TNO-CIVO Toxicology and Nutrition Institute

NYU Medical Center

Institute of Environmental Medicine,
New York University Medical Center

Editorial secretary: Flora de Vrijer

Nasal carcinogenesis in rodents: relevance to human health risk

Proceedings of the TNO-CIVO/NYU Nose Symposium, Veldhoven, Netherlands, 24-28 October 1988

Editing Committee: V.J. Feron & M.C. Bosland

Pudoc Wageningen 1989

CIP data Koninklijke Bibliotheek, Den Haag

ISBN 90-220-0982-3
NUGI 743

Printed in the Netherlands.

ORGANIZING COMMITTEE

V.J. Feron, chairman
M.C. Bosland, vice-chairman and secretary USA
Fl. de Vrijer, general secretary
R.A. Woutersen, vice-secretary
W.J. Hunter
H.A. Milman
K.T. Morgan
P.G.J. Reuzel
H.-B. Richter-Reichhelm
H. Roelfzema
A.C. Upton
A. van der Wielen
J.W.G.M. Wilmer

HOST ORGANIZATIONS

TNO-CIVO Toxicology and Nutrition Institute, Zeist, Netherlands
New York University Medical Center, New York, NY, USA

JOINT ORGANIZATIONS

Bundesgesundheitsamt, Max von Pettenkofer Institut, Berlin, FRG
Chemical Industry Institute of Toxicology, Research Triangle Park, NC, USA
Commission of the European Communities, Luxembourg, Luxembourg
Ministry of Housing, Physical Planning and Environment, Leidschendam, Netherlands
Ministry of Social Affairs and Employment, Voorburg, Netherlands
U.S. Environmental Protection Agency, Washington, DC, USA

Sponsors

Atochem
La Défence 10 Cedex 42, 92091 Paris La Défense, France

BASF AG
Brunekstrasse 80, D-6700 Ludwigshafen, FRG

BP Chemicals Limited
Belgrave House, 76 Buckingham Palace Road, London SW1W 0SU, UK

Bromine Compounds Ltd
Makleff House, P.O. Box 180, Beer Sheva 84101, Israel

Bundesgesundheitsamt
Postfach 330013, D-1000 Berlin 33, FRG

Cassella AG
Hanauer Landstrasse 526, D-6000 Frankfurt am Main 61, FRG

CIBA-GEIGY AG
CH-4002 Basel, Switzerland

Chemical Industry Institute of Toxicology
P.O. Box 12137, Research Tringle Park, NC 27709, USA

Commission of the European Communities
Batiment Jean Monnet, L-2920 Luxembourg, Luxembourg

Dow Chemical Europe SA
Bachtobelstrasse 3, CH-8810 Horgen, Switzerland

DSM
P.O. Box 602, 6160 MH Geleen, Netherlands

Exxon Chemical Holland B.V.
P.O. Box 275, 3100 AG Schiedam, Netherlands

Formaldehyde Institute, Inc.
1330 Connecticut Avenue, N.W., Washington, DC, 20036 USA

Hoechst AG
Postfach 800320, D-6230 Frankfurt am Main 80, FRG

F. Hoffmann-La Roche & Co. AG
Grenzacherstrasse 124, CH-4002 Basel, Switzerland

ICI
Alderley House, Alderley Park, Macclesfield, Cheshire SK10 4TF, UK

MCN
Rijkstraatweg 32, 9752 AE Haren, Netherlands

Sponsors

E. Merck
Frankfurter Strasse, D-6100 Darmstadt 1, FRG

Ministry of Housing, Physical Planning and Environment
P.O. Box 450, 2260 MB Leidschendam, Netherlands

Monsanto Europe N.V.
Tervurenlaan 270-272, P.O. Box 1, B-1150 Brussels, Belgium

National Cancer Institute
Westwood Building, Bethesda, MD 20892, USA

National Institute of Environmetal Health Sciences
P.O. Box 12233, Research Triangle Park, NC 27709, USA

Organon
P.O. Box 20, 5340 BH Oss, Netherlands

E.I. du Pont de Nemours & Comp.
P.O. Box 50, Newark, DE 19714, USA

N.V. Proctor & Gamble European Technical Center S.A.
Temselaan 110, B-1820 Stroombeek Bever, Belgium

The Proctor & Gamble Company, OH 45224, USA
6100 Center Hill Road, Cincinnati, OH 45224, USA

Rhône-Poulenc S.A.
47, Rue de Villiers, 92527 Neuilly/Seine Cedex, France

Sandoz AG
CH-4002 Basel, Switzerland

Shell Nederland B.V.
P.O. Box 1222, 3000 BE Rotterdam, Netherlands

Solvay
Rue de Ransbeek 310, B-1120 Brussels, Belgium

U.S. Environmental Protection Agency
401 M Street, S.W., Washington, DC 20460, USA

CONTENTS

PREFACE

Cancer of the interior of the human nose and accessory sinuses is very rare. This fact is placed in perspective when one considers that the incidence of nasal cancer is about 1% of the incidence of the most common types of cancer in many developed countries, viz. bronchogenic carcinoma in men and breast cancer in women. However, six occupational groups have un unequivocal association with nasal cancer: chromate workers, workers in nickel refineries, makers of isopropyl alcohol, mustard gas manufacturers, wood workers, particularly those making wooden furniture, and boot and shoe workers (Acheson, 1986). In addition, in recent years an increasing number of substances has been found to be capable of inducing nasal tumours in experimental animals upon chronic exposure. Examples are formaldehyde, propylene oxide, p-cresidine, phenylglycidyl ether, 1,2-dibromo-3-chloropropane, acetaldehyde, vinyl chloride, tobacco-specific and other nitrosamines, bis(chloromethyl)ether and hexamethylphosphoramide (Feron et al., 1986).

The primary goal of organizing this symposium was to address issues concerning the relevance of nasal carcinogenesis in rodents to human health risk. Specific aims were:

a. to update the knowledge of nasal tumours induced by chemical and environmental factors such as aldehydes, nickel and wood dust,
b. to discuss in depth the mechanism of action of nasal carcinogens,
c. to compare epidemiological data in man with data on tumours in rodents,
d. to compare regulatory aspects of nasal carcinogens in different countries,
e. to contribute to harmonization of regulations on nasal carcinogens, and
f. to define trends for future research.

This book contains reports and thoughts of a diverse group of investigators and regulators who addressed themselves to the above issues. Part 1 (8 papers) deals with comparative anatomy and physiology of the nasal passages in rodents, primates and man, part 2 consists of 7 papers on nasal tumours in rodents and man, part 3, consisting of 9 papers, covers the important topic of mechanisms of nasal carcinogenesis, and part 4 (4 papers) deals with risk assessment and regulation of nasal carcinogens. Abstracts of 22 posters are included and constitute an integral part of the book. A summary of a panel discussion and some concluding remarks on the state-of-the-art in nasal carcinogenesis research, and needs for future studies concludes the proceedings.

References

1. Acheson, E.D., 1986. Epidemiology of nasal cancer. In: C.S. Barrow (Ed.): Toxicology of the Nasal Passages. Hemisphere Publishing Corporation, Washington, New York, London, p. 135-142.
2. Feron, V.J., R.A. Woutersen & B.J. Spit, 1986. Pathology of chronic nasal toxic responses including cancer. In: C.S. Barrow (Ed.): Toxicology of the Nasal Passages. Hemisphere Publishing Corporation, Washington, New York, London, p. 67-90.

V.J. Feron

Part I

Comparative anatomy and physiology of the nasal passages in rodents, primates and man

COMPARATIVE ASPECTS OF NASAL MORPHOLOGY AND PHYSIOLOGY

Donald F. Proctor

Johns Hopkins Medical Institutions, Baltimore MD 21205, USA

Summary

Because nasal structure in rodents differs significantly from that of man conclusions as to human health risk from toxicological studies in rodents are open to question. The mucociliary apparatus, a major respiratory defense, is basically the same in experimental animals and man. For that reason investigation of injury to and repair of this system in animals deserves consideration as one indication of human health risk.
Keywords: anatomy, physiology, mucociliary, nose, sinus

Introduction

Since the nasal passages of rodents bear little resemblance to ours it is incumbent upon us to emphasize the hazards of extrapolation from rat to man, and to search for similarities which may serve as a useful basis for comparison in toxicological studies. Along the way we should give passing notice to the possibility that some alternative animal might be more suitable than rodents for research in nasal carcinogenesis. A caution against conclusions as to human health should be drawn from the evidence that formaldehyde exposure in rats and mice is a cause of nasal cancer (Albert et al. 1982, Kerns et al. 1983, Swenberg et al. 1980). As yet there is no evidence for such a hazard in man. In this brief paper it is only possible to touch lightly on certain highlights of the problem. The reader is referred elsewhere for more detailed information (Adams & McFarland 1972, Barrow 1986, Hartman & Straus 1933, Hebel & Strandberg 1976, Hoosier & McPherson 1987, Mathew & Sant'Ambrogio 1988, Proctor 1977, Proctor & Andersen 1982, Proctor & Chang 1983, Reznik & Stinson 1983, Schreider 1986).

Nasal Cancer in Man

Nasal cancer is among the rarest of tumors in man (Brinton et al. 1984, Olsen & Jensen 1987). Since it seems likely that this disease is related to exposure to airborne materials (i.e. wood dust, nickel, chromium) (Hadfield 1970, Torjussen 1983, Brinton et al. 1984), and the nasal passage is usually the first part of the respiratory tract to be exposed to inspired air, why is cancer so much more common in the lung, larynx, and pharynx than in the nose? The discrepancy cannot be fully accounted for on the basis of cigarette smoke which bypasses the nasal airway. Workers in a nickel refinery have been found to suffer many more

lung and larynx cancers than nasal. An explanation might be found in either a difference in deposition on respiratory surfaces or a difference in the natural defenses in various respiratory regions. One of our questions should be, are these regional differences in man comparable to those in the rodent? In any event the rarity of nasal tumors should be one subject of inquiry in the study of nasal carcinogenesis.

Another point to be considered in this field of inquiry is the apparent site of origin of nasal cancer. In man nasal cancer is likely to be hidden away in the anterior portion of the middle meatus (a difficult area to explore even for the expert otolaryngologist) and is therefore seldom detected in an early stage. Early symptoms, if any, are readily mistaken for those of infection or allergy. Diagnosis may be clear only after involvement of the ethmoid or maxillary sinuses when the actual point of origin may no longer be evident. In a study of nasal cancer in the boot and shoe industry (Acheson et al. 1970), 61 cases were collected. Of these 22 were said to be in the ethmoid sinuses, 33 in the maxillary antrum, and 6 in the nasal cavity. It is my contention that all of these probably originated in nasal mucosa but were recognized only after involvement of the sinuses most contiguous to the place of maximum pollutant deposition. Sinus cancer rarely if ever originates in the frontals or sphenoids. These facts are compatible with the hypothesis that nasal cancer in man is an airborne disease.

Comparative Anatomy and Physiology

Direct extrapolation from experimental animal toxicological studies to evaluation of human health risk cannot be justified unless we can demonstrate that inspiratory deposition of a carcinogen and its fate after deposition are similar in animal and man, and that the defenses against malignant epithelial change are comparable. Such factors will be affected by the comparative shape of the nasal passages, the nature of airflow through them, the character of secretions in which the offending material is entrapped, the mucociliary currents which move those secretions, and the competence of repair of epithelial cells after injury. We now know a good deal about nasal form and function in normal man (Andersen et al. 1971, Brain et al. 1977, Brain & Valberg 1979, Hounam & Morgan 1977, Proctor et al. 1977, Proctor & Andersen 1982). Similar investigations in experimental animals, especially small rodents, are difficult. For that reason there is a paucity of facts regarding normal animal airway structure and function to use as a basis either for comparison with man or with abnormalities in animals.

If we accept the role of airborne agents in the etiology of nasal cancer the factors involved could be the nature of nasal airflow (in turn influencing the likelihood of deposition of air pollutants on nasal mucosa) and the passage of the offending material through surface secretions and the surface cell layer to reach the basal cells from which all surface cells differentiate. Obviously failure in the mucociliary clearance system will increase the

opportunity of deposited substances to pass through to the basal cell layer. Loss of surface cells (as occurs in man with a simple viral infection) will allow immediate contact of carcinogens with the basal cells. In this connection one author, writing on the epidemiology of nasal cancer in man (Collan 1983), has made the following observation. 'Sinusitis and chronic rhinitis can be considered early symptoms of nasal carcinoma ... many cases had been suffering from these for more than 10 years. Previous nasal trauma appeared to be a risk indicator for nasal cancer...'. This statement suggests that chronic or recurrent damage to the nasal epithelium may facilitate the efective meeting between pathogen and the underlying cells.

We would expect that a meticulous study of the nasal structure and function of various animal species would lead to an easy conclusion as to which species is most appropriate to the search for toxicological evidence reasonably applicable to human health or hazard. The extent of such information is incomplete, but it appears that no single species is ideal for this purpose (Morgan et al. 1984, Morris et al. 1986). There is an interesting example chosen over 30 years ago in the solution of a similar problem (Bang & Bang 1959). In this instance the problem under study was the pathogenesis of respiratory viral infections. The Bangs looked for a naturally occurring and similar animal infection and selected Newcastle virus disease of chickens. In this instance, although the chicken nasal structure differs from that of man even more than does that of the rat, the target organ (the mucus secreting ciliated epithelium) was remarkably alike both in normal form and function and in its reaction to injury and capacity for self repair (Bang & Bang 1969). If there were an animal whose susceptibility to naturally occurring cancer of the nose is similar to that of man its choice for carcinogenesis research would be logical. If no such similarity has been discovered then respiratory epithelial susceptibility to injury and capacity for repair could be an alternative consideration in the choice.

From what is now known I doubt if any animal species is in these respects ideal for nasal carcinogenesis research leading to information relevant to human health. Perhaps we should be more certain that the question has been thoroughly explored prior to accepting the rat (or any other rodent) as the most suitable subject for such investigations.

The biological scientist is frequently tempted by the possibilities of extrapolation from demonstrated data obtained in animal studies to conclusions regarding questions of human health which are not readily susceptible to solutions purely through investigations in man. Such extrapolation is necessary where comparable studies in man are unduly hazardous, and is certainly justifiable where there is a demonstrable similarity between animal and human structure and function. Such similarity is obvious in the larynx and tracheobronchial tree; but the human upper airway differs widely in both structure and function from that of the rodent, and at least partly even from that of primates. Deliberate exposure of human volunteers to suspected

carcinogens is open to criticism. The alternatives are the use of brief supposedly innocuous exposures to hazardous materials, or the use of comparable but harmless substances. An example of the latter would be the study of the effect of an inert dust as one index of what might be the effect of a toxic dust (Andersen et al. 1981). Another possibility is to search for what might be factors in common between animal and man even in this peculiar organ the nose.

The shape of the nasal passage in rodents, and to a large degree in all other animal species, differs from that in man. In man there is an extremely narrow and collapsible entrance, a long turbinated passage leading to the bend at the nasopharynx. Inspiratory airflow thus enters at high linear velocity and, after two to four cm, bends sharply to travel slowly (although not nearly as slowly as in the small bronchi) but with some turbulence along the five to eight cm of the main passage, and then turns a 90^{0} angle to pass downward into the pharynx, larynx, and tracheobronchial tree. The main passage is lined with ciliated mucus secreting epithelium, and the path of flow in mucus generated by the cilia is almost everywhere directed toward the nasopharynx. At the anterior portion of the middle turbinate and meatus there is a narrow region in which mucociliary flow is forward toward squamous epithelium, or forward and then curving backward to join the main stream. In man we know that a large portion of inspired particulate and gaseous matter is deposited in the mucus lining the anterior third of the nasal passage (Andersen et al. 1981, Brain & Valberg 1979, Hounam & Morgan 1977, Pattle 1961). Some of the particles are swept forward to that anterior region from which they can be cleared from the body by nose blowing and cleaning. If the mucociliary system is unimpaired the remainder is carried backward to the nasopharynx to be eventually swallowed.

In contrast, in the rodent the nasal passage offers a more or less straight line for inspired air from ambient to larynx. There is no definite evidence as to airflow linear velocity in rodents, but a suggestion of more turbulence than in man. Sites of deposition are not clearly defined, but mucociliary currents may be more complex and more anteriorly directed than in man, with the possibility of more long term residence at the multiple turning points (Morgan et al. 1984).

Another source of diffciulty in applying findings in any experimental animal to health risk in man is the multifunctional role of the upper airways. Although the double function of deglutition and breathing is in common to all, there are wide differences especially in relation to oronasal breathing and phonation (Proctor 1988).

Over twenty years ago Falk and co-workers published on the effects of irritant gases upon and possible pathogenesis of cancer in a ciliated mucus-secreting epithelium (Kotin et al. 1966). They reviewed the destruction of superficial ciliated epithelium and its repair, a process well known to occur with simple virus infections in man, and suggested that repeated injury by airborne irritants might lead to imperfect repair including squamous metaplasia. In the

recurrent or chronic presence of carcinogen cancer might be the alternative to such metaplasia.

Conclusions

I suggest that those studying nasal carcinogenesis in experimental animals might beware of interpreting their findings in terms of risk to man. I also urge that special efforts be bent on research in the field of injury to and repair of ciliated mucus secreting epithelium, and that nasal defense systems be studied for possible sources of their apparent resistance to malignant change.

References

Acheson, E. D., R. H. Cowdell & B. Jolles, 1970. Nasal cancer in the Northampshire boot and shoe industry. Br. Med. J. 1:385-393.

Adams, D. R. & L. Z. McFarland, 1972. Morphology of the nasal fossae and associated structures of the hamster. J. Morphol. 137:161-179.

Albert, R. E. et al., 1982. Gaseous formaldehyde and hydrogen chloride induction of nasal cancer in the rat. J. N. C. I. 68:597-603.

Andersen, I., Lundqvist & D. F. Proctor, 1971. Human nasal mucosal function in a controlled climate. Arch. Environ. Health 23:408-420.

Andersen, I., L. Mølhave & D. F. Proctor, 1981. Human response to controlled levels of SO_2 and inert dust. Scand. J. Work Environ. Health 7:1-7.

Bang, B. G. & F. B. Bang, 1959. A comparative study of the vertebrate nasal chamber in relation to upper respiratory infections. Bull. Johns Hopkins Hosp. 104:107-125.

Bang, B. G. & F. B. Bang, 1969. Replacement of virus-destroyed epithelium by keratinized squamous cells in vitamin-A-deprived chickens. Proc. Soc. Exp. Biol. Med. 132:50-54.

Barrow, C. S. (Ed.), 1986. Toxicology of the Nasal Passages. McGraw-Hill International Books, Washington.

Brain, J. D., D. F. Proctor & L. M. Reid (Ed.), 1977. Respiratory Defense Mechanisms. M. Dekker, New York.

Brain, J. D. & P. A. Valberg, 1979. Deposition of aerosol in the respiratory tract. Am. Rev. Respir. Dis. 120:1325-1373.

Brinton, L. A. et al., 1984. A case-control study of cancers of the nasal cavity and paranasal sinuses. Am. J. Epidemiol. 119:896-906.

Collan, Y., 1983. Epidemiologic and etiologic aspects and histopathology of nasal carcinoma in Finland. In: G. Reznik & S. F. Stinson (Ed.): Nasal Tumors in Animals and Man. CRC Press, Boca Raton. p. 103-110.

Hadfield, E. H., 1970. A study of adnocarcinoma of the paranasal sinuses in wood workers in the furniture industry. Ann. R. Coll. Surg. (Engl.) 46:301-319.

Hartman, C. G. & W. L. Straus Jr. (Ed.), 1933. The Anatomy of the Rhesus monkey. Williams & Wilkins, Baltimore.

Hebel, R. & M. W. Stromberg, 1976. Anatomy of the Laboratory Rat. Williams & Wilkins. Baltimore.
Hoosier, G. L. van Jr. & C. W. McPherson (Ed.), 1987. Laboratory Hamsters. Academic Press, Orlando.
Hounam, R. F. & A. Morgan, 1977. Particle deposition. In: J. D. Brain, D. F. Proctor & L. M. Reid (Ed.): Respiratory Defense Mechanisms. M. Dekker, New York. p. 125-156.
Kerns, W. D. et al., 1983. Carcinogenicity of formaldehyde in rats and mice after long-term inhalation exposure. Cancer Res. 43:4382-4392.
Mathew, O. P. & G. Sant'Ambrogio (Ed.), 1988. Respiratory Function of the Upper Airway. M. Dekker, New York.
Morgan, K. T. et al., 1984. The nasal mucociliary apparatus, correlation of structure and function in the rat. Am. Rev. Respir. Dis. 130:275-281.
Morris, J. B., R. J. Clay & D. G. Cavanagh, 1986. Species differences in upper respiratory tract deposition of acetone and ethanol vapors. Fund. Appl. Toxicol. 7:671-680.
Olsen, J. H. & O. M. Jensen, 1987. Occupation and risk of cancer in Denmark. Scand. J. Work Environ. Health 13: Suppl. 1:1-90.
Pattle, R. E., 1961. The retention of gases and particles in the human nose. In: C. N. Davies (Ed.): Inhaled Particles and Vapours. Pergamon, Oxford. p. 302-309.
Proctor, D. F., I. Andersen & G. R. Lundqvist, 1977. Human nasal mucosal function at controlled temperatures. Respir. Physiol. 30:109-124.
Proctor, D. F., 1977. The upper airways I. Nasal physiology and defense of the lungs. Am. Rev. Respir. Dis. 115:97-130.
Proctor, D. F. & I. Andersen (Ed.), 1982. The Nose, Upper Airway Physiology and the Atmospheric Environment. Elsevier, Amsterdam.
Proctor, D. F. & J. C. F. Chang, 1983. Comparative anatomy and physiology of the nasal cavity. In: G. Reznik & S. F. Stinson (Ed.): Nasal Tumors in Animals and Man. CRC Press, Boca Raton, Vol. I, p. 1-34.
Proctor, D. F., 1988. Disorders of the extrathoracic airways. In: J. F. Murray & J. A. Nadel (Ed.): Textbook of Respiratory Medicine. W. B. Saunders, Philadelphia. p. 1153-1159.
Reznik, G. & S. F. Stinson (Ed.), 1983. Nasal Tumors in Animals and Man. CRC Press, Boca Raton.
Schreider, J. P., 1986. Comparative anatomy and function of the nasal passages. In: C. S. Barrow (Ed.): Toxicology of the Nasal Passages. McGraw-Hill, Washington. p. 1-25.
Swenberg, J. A. et al., 1980. Induction of squamous carcinoma of the rat nasal cavity by inhalation exposure to formaldehyde-vapor. Cancer Res. 40:3398-3402.
Torjussen, W., 1983. Nasal cancer in nickel workers. In: G. Reznik & S. F. Stinson (Ed.): Nasal Tumors in Animals and Man. CRC Press, Boca Raton. Vol. II. p. 33-54.

THE RESPIRATORY SUBMUCOSAL GLANDS: MORPHOLOGY, EXCRETION PRODUCTS AND INNERVATION

Y.J.B. van Megen and A.B.M. Klaassen

Department of Otorhinolaryngology, St. Radboud Hospital Nijmegen, The Netherlands

Summary

The main part of the respiratory submucosal glandular tissue in the lateral wall of the rat is situated around the ostium of the maxillary sinus and can be divided in a dorsal, simple branched acinious part and in a ventral, very tubulo-acinous glandular part. By using histochemical staining techniques for glycoproteins three different areas can be distinguished. The different areas based on different glycoproteins can also be distinguished by different types of granules in their acinar cells. Histochemical and autoradiographic experiments demonstrated that the glandular acini are mainly innervated by cholinergic fibers and that the excretory ducts are mainly innervated by B-adrenergic fibers. The sensitivity in the glycoprotein secretion to the cholinergic muscarinic agonist metacholine appeared to be higher in the ventral part in comparison with the dorsal part. This higher sensitivity of the ventral part to metacholine stimulation parallels the higher muscarinic receptor density and the lower equilibrium dissociation constant determined by radioligand receptor binding studies.

Introduction

It has been realized already for a long period that the nasal passages are vitally important in the protection of the lower airway system. To that purpose the nasal cavity is equipped with a sophisticated system for filtration, humidification and thermoregulation of the inspired air. The vascularization of the nasal cavity has a crucial function in warming up the inhaled air and regulates the patency of the nasal passages by means of venous erectile tissue (sinusoids). The nasal fluid, mainly a secretory product of the nasal submucosal glands, is involved in the humidification of the inspired air, the protection of the underlying respiratory epithelium and the removal of inhaled pollutants (Phipps, 1981; Widdicombe and Wells, 1982). In particular (glyco)proteins are responsible for the viscosity and gel forming properties of the mucus (Clamp et al., 1978). The acini of the glands produce these (glyco)proteins and it has been suggested that the glandular striated excretory ducts control the ion and water content (Phipps, 1981). In order to perform these functions in an appropriate way, the nasal mucosa is supplied by an intricate network of parasympathetic and sympathetic nerve fibers from the autonomic nerve system (Ishi and Toryama, 1972; Grote et al., 1975). In this article special attention will be paid to the morphology, secretory activity and innervation of the nasal submucosal glands.

Nasal submucosal glands

Morphological aspects

In our studies the rat, especially the nasal lateral wall, was used as

an experimental model for studying the respiratory submucosal glands. In the respiratory area of the rat nasal submucosa most of the glandular tissue in the lateral wall is situated in the region of the maxillary sinus; the boundaries are very irregular. Especially in the floor of the sinus, a large part of the glandular tissue, surrounding the root of the incissor, is situated.

From a morphological point of view no clear cut differences in the structure of this glandular tissue could be observed. However, two parts can be distinguished with regard to the duct system. A dorsal part shows no typical intercalated or striated ducts and drains by some simple excretory ducts into the lumen of the maxillary sinus. On the other hand, a ventral part consists of a very branched tubulo-acinous glandular tissue with many intercalated ducts. From this ventral part one large excretory duct courses towards the vestibule in addition to a number of smaller excretory ducts. No interconnections between these ducts were observed (Klaassen et al., 1981). The dorsal area is comparable with the maxillary gland described by Vidic and Greditzer (1971) and by Bang and Bang (1959). The ventral area can be considered as an anatomical entity, comparable with the lateral nasal gland (Bojsen and Moller, 1964).

Embryogenesis

Developmental data also supoort the observations that two different glands are concerned. In the lateral nasal wall short ducts of the future lateral nasal gland are already present at the level of their vestibular openings on the 16th day of gestation. These ducts grow further in caudoventral direction through the subepithelial mesenchyme of the middle meatus to reach the anterior recess of the maxillary sinus. On the 18th day of gestation one large duct and several smaller ducts can be observed. At that time ramification of the posterior ends and differentiation into acini is observed. Just before birth the anterior part of the subepithelial mesenchyme of the maxillary sinus is completely filled with secretory granules.

A separate glandular system develops in the upper part of the posterior recess of the maxillary sinus. On the 18th day of gestation the first few promordia of this system arise as small invaginations of the posterior end of the maxillary sinus. Shortly thereafter ramification and differentiation into acini is observed. At birth the maxillary sinus is then completely surrounded by glandular tissue of different origin without any distinct anatomical boundary (Klaassen, unpublished observations).

Histochemical aspects

The nasal fluid is mainly a secretory product of the submucosal glands and the epithelial lining but also a mixture of transudate, tears, condensed water of the expired air, loose cells and microorganisms (Mygind, 1978). Biochemical analysis of this fluid revealed the presence of a large variety of chemical substances such as serum proteins, immunoglobulins, electrolytes, enzymes of the intermediate metabolism and some glycoproteins (Mygind, 1978).

By using various histochemical staining techniques for glycoproteins, three different areas can be distinguished in the lateral wall of the rat nasal glands; L_1 and L_3 in the ventral part and L_2 in the dorsal part (Klaassen et al., 1981). Area L_1, a very small part of the glandular tissue, failed to stain with any of the staining procedures for glycoproteins. Area L_2 was PAS (Periodic Acid Shiff)-positive, but did not stain with AB (Alcian Blue, pH 2,6), indicating the presence of neutral

glycoproteins. The affinity for area L_3 for both PAS and AB (pH 2,6) refers to the presence of both neutral and acidic glycoproteins. None of these areas showd an affinity for AB at pH 1.0, suggesting the absence of sulphated glycoproteins. Surprisingly, autoradiographs revealed a heavy incorporation of ^{35}S, an indication for sulphated glycoproteins in the PAS-positive area L_2.

Ultrastructural aspects

Ultrastructural investigations confirmed the complexity of the nasal glandular tissue (Klaassen et al., 1982). The different areas in the lateral wall, based on different glycoproteins, can also be distinguished by different types of granules in their acinar cells. In the areas L_2 (dorsal part; containing neutral glycoproteins) non-confluent, electron-dense granules were present. The area L_3 of the ventral part with neutral and acidic glycoproteins contained confluent, electron-grey and also non-confluent granules with internal structures, possibly representing stages of maturation.

Conclusion

The main part of the glandular tissue in the lateral nasal wall is situated in the ostium of the maxillary sinus and can be divided in a simple branched acinous glandular part and in a ventral very branched tubulo-acinous part. By using various histochemical techniques three different areas can be distinguished; these areas can also be distinguished by different types of granules in their acinous cells.

Secretion

The secretory behaviour of the nasal glands is mainly studied by collections from nasal secretions (Eccles and Wilson, 1973; Burns and Williams, 1977; Malm et al., 1983). Since nasal secretions arise not only from the nasal glands, as mentioned above, previous studies cannot be considered as representative for the secretory activity of the nasal glands. Therefore, the secretory behaviour of the rat nasal glands, under normal conditions and after cholinergic drugs has been studied using morphological and radiobiochemical techniques (Klaassen and Kuijpers, 1986). Autoradiography and electrophoresis provide evidence for the selective incorporation of ^3H-Arginine into all glycoproteins of the nasal submucosal glands. The labelled arginine is rapidly incorporated, into the acinar cells of unstimulated glands, although it takes approximately 4 hours before the labelled protein leaves the cells. The secretion of protein is stimulated by the parasympathetic agonist pilocarpine or metacholine. Histological sections also showed a depletion of secretory granules after pilocarpine treatment. The principal action of this parasympathetic agonist appears to be the stimulation of discharge. The cholinergic antagonist atropine inhibits the secretion; the acinar cells are completely filled with secretory granules following this treatment.

This technique was used to study the secretory behaviour of the histochemically different dorsal (L_2) and ventral (L_3) area of the rat nasal gland. Metacholine causes an increase of 30% in ^{3}glycoprotein secretion between 2 and 5000 µg/kg body weight in the ventral part but only above 2 mg/kg body weight in the dorsal part. Comparable observations with parasympathetic agonists have been made on the discharge of bronchial glands both in vivo and in vitro (Gallaghe et al., 1975; Coles, 1977). In all these experiments, no difference in the amount of radioactivity in

the blood samples could be detected when compared with control animals. A difference in the neural regulation might be an underlying explanation for the higher sensitivity of the ventral part (in comparison with the dorsal glandular part) upon metacholine stimulation.

Innervation

Physiology

Physiological experiments demonstrated already the presence of cholinergic fibers in rat nasal glands. Electrical stimulation of parasympathetic nerves in the nasal glands provokes a secretion which can be inhibited by atropine (Eccles and Wilson, 1973; Anggard, 1977). Although the results are somewhat conflicting, physiological experiments suggested an influence of adrenergic agonists on the secretory activity of the nasal mucosa (Phipps, 1981; Malm, 1983).

Histochemistry

Histochemical and fluoresence techniques have confirmed the presence of cholinergic fibers by the staining acetylcholine esterase in the glandular acini. No cholinergic fibers could be demonstrated in the excretory ducts. In contrast with physiological experiments no adrenergic innervation of the glands could be observed with this method (Grote, 1975; Klaassen et al., 1988).

Differences in parasympathetic innervation of the dorsal and ventral glandular part could be observed with histochemical techniques. The ventral part showed an abundant parasympathetic innervation, whereas hardly any acetylcholine esterase containing fibers could be observed in the dorsal glandular part.

Radioligand receptor binding studies

Radioligand receptor binding studies to homogenates of rat nasal mucosa confirmed the presence of cholinergic muscarinic receptors in rat nasal mucosa (Klaassen et al., 1986). The binding of ^{3}H-1-QNB (Quinuclidinyl-benzilate), a cholinergic muscarinic antagonist, to the nasal mucosa was saturable and the Scatchard plot indicates a homogeneous class of binding sites. Analysis of the data revealed two parameters, an equilibrium dissociation constant (Kd=0.06$\pm$0.02 nM) and a receptor density (Bmax.= 8$\pm$2 pmol/g tissue). The Kd-values are in the same order of magnitude as those found in rat submandibular gland (0.08 nM, Costa and Murphy, 1985; 0.45 nM, Blund et al., 1982).

Inhibition of the ^{3}H-1-QNB binding in rat nasal mucosa by the cholinergic muscarinic agonist Methylfurthretonium showed a monophasic inhibition curve, indicating low affinity agonist binding sites with an inhibition constant (Ki) of 20 µM. This exeptional behaviour distingguished the muscarinic receptors in the rat nasal mucosa from those in rat intestine smooth muscle, which are characterized by a mixture of low and high agonist affinity sites (Rodrigues de Miranda, 1985).

There is a significant difference in Kd-and Bmax.-value in the dorsal (Kd=0.15$\pm$0.01 nM; Bmax.=42$\pm$7 fmol/mg protein) and ventral part (Kd=0.11 $\pm$0.01 nM; Bmax=68$\pm$6 fmol/mg protein) of the rat nasal gland. The agonist inhibition constant of the dorsal part (Ki=18.9 µM) was not significantly higher than the inhibition constant of the ventral part (Ki=11.9 µM).

Autoradiography

In addition to the histochemistry, an in vitro autoradiographic technique was used to demonstrate neuroreceptors in the nasal glands. In general, in vitro autoradiography seems a suitable technique for the localization of neuroreceptors and this technique is more sensitive than the histochemical techniques (Young and Kuhar, 1979; Wamsley, 1983). The autoradiographs of ^{3}H-l-QNB binding showed labelling of the glandular acini. In contrast with the histochemical findings, the autoradiographs showed a slight labelling of the excretory ducts (van Megen et al., 1988). The autoradiographs of ^{125}I-cyanopindolol binding, a B-adrenergic antagonist, showed mainly labelling of the striated excretory ducts, whereas the glandular acini showed hardly any labelling (van Megen et al., 1988).

Conclusion

From histochemical and in vitro autoradiographic experiments it may be concluded that the glycoprotein production by the acini may be mainly under parasympathetic control and the ion and water regulation of the nasal fluid by the excretory ducts are under both sympathetic and parasympathetic control.

The higher sensitivity in glycoprotein of the ventral glandular part to metacholine stimulation parallels the higher receptor density and the lower dissociation constant (i.e. higher affinity).

References

Bang, B.G. & F.B. Bang, 1959. A comparative study of the vertebrate nasal chamber in relations to upper respiratory infections. Part 1: Comparative morphology of the nasal chambers of three commonly used laboratory animals: chicken, rat and ferret. Bull. Johns Hopkins Hosp. 104: 107-149.

Bojsen-Muller, F., 1964. Topography of the nasal glands in rats and some other mammals. Anat. Rec. 150: 11-24.

Burns, J.W. & G.E. Williams, 1977. A simple perfusion method for examination of nasal secretion in the anaesthesized rat. Adv. Exp. Med. Biol. 89: 502-510.

Bylund, D.B., J.R. Martinez & D.L. Pierce, 1982. Regulation of autonomic receptors in rat submandibular gland. Molec. Pharmacol. 21: 27-35.

Clamp, J.R., A. Allen, R.A. Gibbons & G.P. Robberts, 1978. Chemical aspects of mucus. Brit. Med. J. 34: 25-41.

Coles, S., 1977. Regulation of secretory cycles of mucous and serous cells in the human bronchial gland. In M. Elstein & D.V. Parke (Eds.): mucus in health and disease. Plenum Press, New York, pp. 155-168.

Costa, L.G. & S.D. Murphy, 1985. Characterization of muscarinic cholinergic receptors in the submandibular gland of the rat. J. Autonomic Nervous System 13: 287-301.

Eccles, R. & H. Wilson, 1973. The parasympathetic secretory nerves of the nose of the cat. J. Physiol. 230: 213-223.

Gallagher, J.T., P.W. Kent, M. Passatore, R.J. Phipps & P.S. Richardson, 1975. The composition of the tracheal mucus and the nervous control of its secretion in the cat. Proc. R. Soc. Lond. (Biol) 192: 49-76.

Grote, J.J., W. Kuijpers & P.L.M. Huygen, 1975. Selective denervation of the autonomic nerve supply of the nasal mucosa. Acta Otolaryngol. 79: 124-132.

Ishii, T. & M. Toriyama, 1972. Acetylcholinesterase activity in the vasomotor and secretory fibers of the nose. Arch. Klin. Exp. Ohren Nasen Kehlkopfheilkunde 201: 1-10.

Klaassen, A.B.M., W. Kuijpers & J.M. Denucé, 1981. Morphological and histochemical aspects of nasal glands in the rat. Anat. Anz. Jena, 149: 51-63.
Klaassen, A.B.M., P.H.K. Jap & W. Kuijpers, 1982. Ultrastructural aspects of the nasal glands in the rat. Anat. Anz., Jena, 151: 455-466.
Klaassen, A.B.M. & W. Kuijpers, 1986. The secretory activity of rat nasal glands and the effect of cholinergic drugs. Cell tissue Res. 243: 655-659.
Klaassen, A.B.M., W. Kuijpers, H.E. Schres, J.F. Rodrigues de Miranda & A.J. Beld, 1986. Cholinergic receptors in the upper respiratory system of the rat. Arch. Otolaryngol. Head Neck Surg. 112: 428-431.
Klaassen, A.B.M., M.W.S.M. Dooper, J.F. Rodrigues de Miranda & H. Krabbendam, 1987. Differences in innervation and secretory behaviour of the two nasal glandular parts in the rat. Acta Otolaryngol. 104: 545-551.
Klaassen, A.B.M., Y.J.B. van Megen, W. Kuijpers & P. van den Broek, 1988. Autonomic innervation of the nasal mucosa. O.R.L. 50: 32-41.
Malm, L., 1983. Autonomic innervation-pharmacological aspects. In: P. van Cauwenberge & J.J. Grote (Eds.): Nasal hyperreactivity. Belgium, Scientific Soc. Med. Information, Gent, pp. 25-29.
Megen, van, Y.J.B., M.J. Teunissen, A.B.M. Klaassen & J.F. Rodrigues de Miranda, 1988. Autoradiographic analysis of muscarinic receptors in rat nasal glands. Eur. J. Pharmacol. 145: 7-13.
Megen, van, Y.J.B., C.J.B.M. van Ratingen, A.B.M. Klaassen, J.F. Rodrigues de Miranda, C.A.M. van Ginneken & P. van den Broek, 1988. Demonstration of B-adrenergic receptors in rat nasal glands by an in vitro autoradiographic technique. Neurosci. Lett., 90: 75-77.
Mygind, N., 1978. Nasal allergy. Blackwell Scientific Publications, Oxford, London.
Phipps, R.J., 1981. The airway mucociliary system. Int. Rev. Physiol. 23: 213-260.
Rodrigues de Miranda, J.F., H.M.E. Scheres, H.J.M. Salden, A.J. Beld, A.B.M. Klaassen & W. Kuijpers, 1985. Muscarinic receptors in rat nasal mucosa are predominantly of the low affinity agonist type. Eur. J. Pharmacol. 113: 441-445.
Vidic, B. & H.G. Greditzer, 1971. The histochemical and microscopical differentiation of the respiratory glands around the maxillary sinus of the rat. Am. J. Anat., Philadelphia, 132: 491-514.
Widdicombe, J.G. & U.M. Wells, 1982. Airway secretion. In D.F. Proctor & I.B. Andersen (Eds.): The nose, Upper airway physiology and the atmospheric environment. Elsevier Biomedical Press, Amsterdam. pp. 215-244.

Table 1. Morphology, histochemistry, secretion and innervation of the dorsal (L_2) and the ventral (L_3).

	L_2	L_3
Morphology		
tubulo-acinair gland type	simple	branched
acini	large acini small lumen	small acini large lumen
ducts	simple ducts to maxillary sinus	one large intercalated duct to vestibulum
secretoir granules	type 1	type 2
Histochemistry		
glycoproteins		
PAS	+	+
AB (pH 2,6)	-	+
AB (pH 1,0)	-	-
type	neutral sulphated	neutral sialyted
Secretion		
Metacholine stimulation (2 µg/kg body weight)	no degranulation	degranulation
innervation		
Acetylcholineesterase	a few fibers	abundant fibers
Radioligand binding		
Kd (nM)*	0.15±0.01	0.11±0.01
Bmax. (fmol/mg)*	42±7	68±6
Ki_{MeFur} (µM)	18.9	11.9
Autoradiography		
muscarinic receptors	present in acini and excretory ducts	
B-adrenergic receptors	present in excretory ducts	

L= Lateral glandular tissue, type 1= non-confluent, electron dense granulae, type 2= confluent, electron-grey and non-confluent granulae with external structures, * significant difference $p<0.05$.

INTERACTION OF THE NASAL PASSAGES AND THE LOWER AIRWAYS

Thomas V. McCaffrey

Department of Otolaryngology, Mayo Clinic, Rochester, Minnesota, USA

Summary

The upper airway comprising the nasal cavity, pharynx, and larynx represents a high resistance region of the airway. The overall efficiency of respiration is dependent on the regulation of the resistance of this region of the airway. Two major reflex mechanisms regulate the resistance of the upper airway and the nasal airway in particular: reflexes arising from chemoreceptors responsive to hypercapnia and hypoxia and reflexes arising from pulmonary receptors, particularly pulmonary stretch receptors. Reflex regulation of the resistance of the upper airway permits optimum performance of the respiratory system.
Keywords: upper airway, nasal airway, airway resistance, airflow.

Introduction

The airways can be partitioned into two geometrically anatomically and physiologically distinct regions. The upper airway consists of the upper trachea, larynx, pharynx, oral cavity, and nose. Geometrically it represents a nonuniform conduit with constrictions in series at the glottis and the nasal airway. Anatomically this airway is extrathoracic in location, and therefore its caliber is not subject to the effects of intrathoracic pressure changes. Physiologically the upper airway's caliber is controlled by the striated muscles of the larynx and pharynx and the state of congestion of the nasal mucosal blood vessels. The lower airway differs from the upper airway in each of these major aspects. Geometrically it is a dichotomously branching system of tubes anatomically situated within the chest and its caliber is therefore subject to the effects of intrathoracic pressure variations, and finally its diameter is regulated by the smooth muscles of the bronchi and trachea.

The segments of the upper airway which represent the high resistance regions are the glottis and the nasal airway with the nasal airway contributing the greatest proportion of the resistance (Table 1).

Table 1. Contribution of airway regions to total pulmonary resistance in the dog and man.

		Percent of total airway resistance			
		Nasal Airway	Laryngeal Airway	Total Upper Airway	Lower Airway
Dog* (V = .25 L/sec)	Inspiration	79	6	85	15
	Expiration	74	3	77	23
Man** (V = .4 L/sec)	Inspiration	64	5	69	31
	Expiration	58	12	70	30

* Ohnishi & Ogura, 1969
**Ferris, Mead, & Opie, 1964

Although the nasal airway can be bypassed during mouth breathing, resting respiration is preferentially through the nose. The nasal mucosal blood vessels and, therefore, the nasal airway resistance are dynamically regulated by a variety of reflex arcs under physiologic conditions. Most important of these are the chemoreceptor reflexes which respond to changes in blood CO_2 and oxygen concentrations, and the pulmonary reflexes which respond to the state of various pulmonary receptors.

Chemoreceptor reflexes

In man as well as in experimental animals nasal resistance decreases in response to hypercapnia (McCaffrey & Kern, 1979). Figure 1 demonstrates the response of minute ventilation and nasal resistance to increasing inspired CO_2 concentrations. As minute ventilation increases in response to the chemoreceptor stimulation nasal resistance decreases.

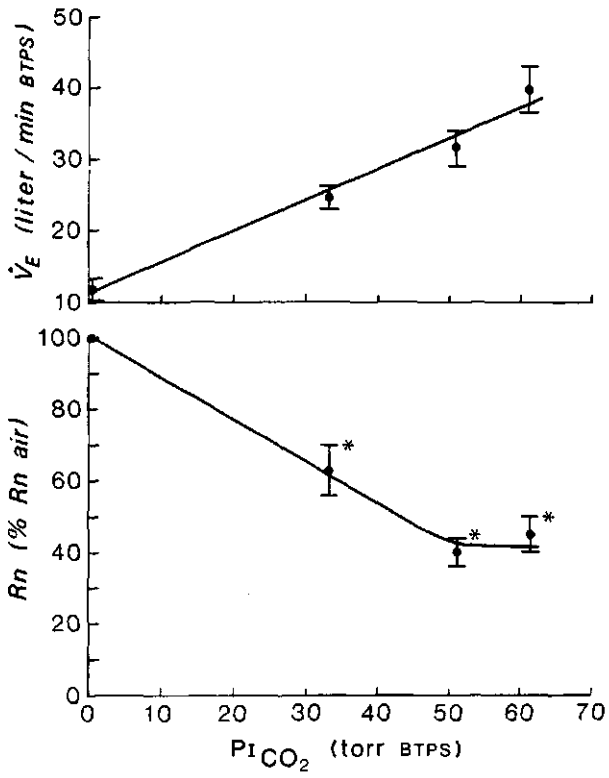


Fig. 1. Response of human nasal airway resistance to hypercapnia.

Stimulation of peripheral chemoreceptors by hypoxia also has an effect on nasal resistance. However, this occurs only at severe levels of an hypoxia and is much less marked than the response to hypercapnia (Fig. 2).

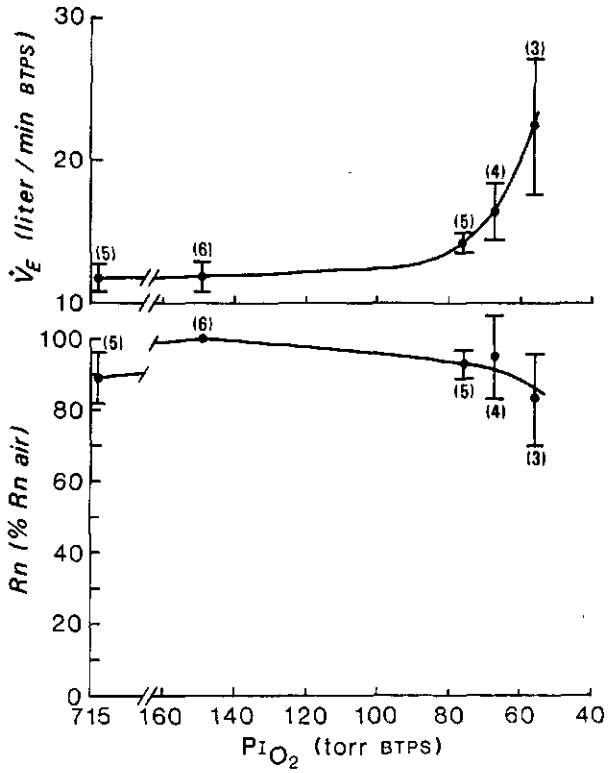


Fig. 2. Response of human nasal airway resistance to hyperoxia and hypoxia.

During both hypoxia and hypercapnia increased minute ventilation is matched by a comparable decrease in nasal resistance. This permits elevated levels of nasal airflow at reduced work levels.

Pulmonary receptor reflexes

There are a variety of receptors within the lung parenchyma with effects on respiration. Several have been examined with respect to their effects on nasal airway resistance (Nishihara & McCaffrey, 1987). Those which have been studied include irritant receptors, pulmonary stretch receptors, and pulmonary J receptors. Pulmonary stretch receptors are situated in major airways and are associated with the smooth muscles of large bronchi. They are stimulated by lung inflation, and their usual respiratory effect is the inhibition of inspiration. In the cat stimulation of stretch receptors, either using lung inflation or by chemical stimulation with veratrum alkaloids, produces an

increase in nasal resistance. Figure 3 shows the effect of stretch receptor stimulation by lung inflation on nasal volume.

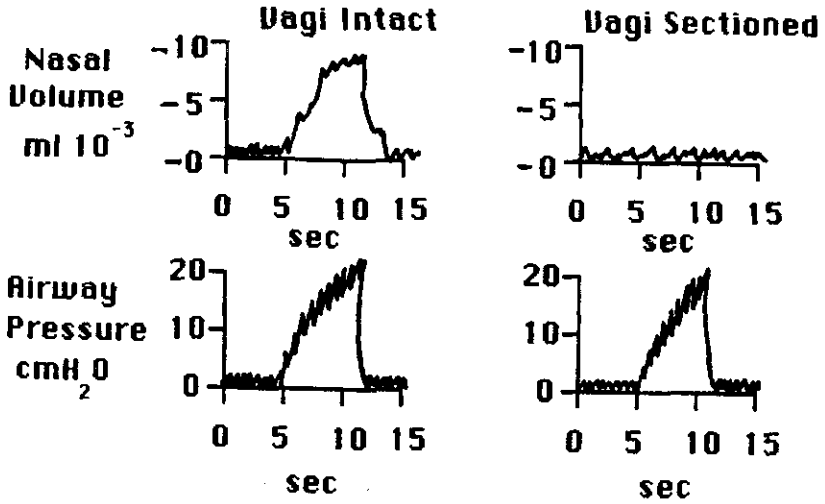


Fig. 3. Effect of lung inflation on nasal volume before and after sectioning the vagus nerves.

Nasal resistance will increase with increases in nasal mucosal volume. It can be seen that nasal volume increases with lung inflation when the vagus nerve is intact; however, this effect is abolished when the vagus nerve is sectioned. Since the pulmonary stretch receptor is the main receptor stimulated by this maneuver it can be assumed that the stretch receptor has a direct influence on nasal resistance in response to lung inflation. Pulmonary irritant receptors are present in the epithelium of medium and small-sized bronchi. Stimulation of these receptors typically produce tachypnea and bronchoconstriction in response to smoke, mechanically irritation, lung hyperinflation, or histamine. No characteristic effect of irritant receptor stimulation has been demonstrated on nasal blood vessels. Pulmonary J receptors are nonmyelenated nerve endings situated near the pulmonary capillaries. Stimulation of these receptors produces apnea in response to pulmonary edema or stimulation by the drug capsicin. Attempts to demonstrate the effects of J reception stimulation on nasal blood vessels have also failed to demonstrate any consistent effect.

These findings suggest that the nasal airway is closely integrated with the function of the respiratory system by several reflex arcs. Immediate and breath-to-breath variation of nasal resistance occurs in response to stretch receptor stimulation. This produces a characteristic fluctuation of nasal resistance in phase with the respiratory cycle. The physiologic significance of this variability, however, is not clear. In addition, both hypoxia and hypercapnia reduce nasal airway resistance in response to chemoreceptor stimulation. This effect would have the obvious value of permitting increased ventilation through the

upper airway at less energy cost. Under conditions of increased respiratory demand nasal and upper airway resistance decrease permitting greater airflow and improved ventilation.

References

Ferris, B., Jr, J. Mean, & L. H. Opie, 1964. Partitioning of respiration flow resistance in man. J. Appl Physiol. 19:653-658.

McCaffrey, Thomas V. & Eugene B. Kern, 1979. Response of nasal airway resistance to hypercapnia and hypoxia in man. Ann. of Otol., Rhinol. and Laryngol. 88:245-252.

Nishihira, S. & T. V. McCaffrey, 1987. Reflex control of nasal blood vessels. Otolaryngol. Head & Neck Surg. 96:273-277.

Ohnishi, T. & J. H. Ogura, 1969. Partitioning of pulmonary resistance in the dog. Laryngoscope, 79:1847-1878.

THE SPECIFIC IMMUNE DEFENCE SYSTEM OF HUMAN NASAL MUCOSA

P. Brandtzaeg

Laboratory for Immunohistochemistry and Immunopathology, Institute of Pathology, University of Oslo, The National Hospital, Rikshospitalet, Oslo, Norway

Summary

The nasal mucosa is protected by a secretory immune system which is under complex and only partly understood immunoregulatory control. B cells of relatively immature memory clones with a potential for J-chain expression, are initially stimulated in mucosa-associated lymphoid tissue (probably including the tonsils) and migrate thereafter through lymph and blood to secretory tissues where they are subjected to terminal differentiation and become immunoglobulin (Ig)-producing immunocytes. Most locally produced Ig is normally dimeric IgA which is selectively transported through the serous type of glandular cells by means of an epithelial receptor protein called the secretory component (SC). IgM is also subjected to SC-mediated transport. Secretory IgA and IgM perform mucosal surface protection by immune exclusion of antigens. In patients with selective IgA deficiency, secretory IgA is lacking but it may be satisfactorily replaced by secretory IgM. In other IgA-deficient patients, however, immunoregulatory compensation gives rise to a large number of IgD-producing cells in the nasal mucosa. IgD cannot act as a secretory antibody and these patients are prone to have recurrent infections. These observations show that there are large individual variations in the secretory immune system.
Keywords: nasal mucosa, tonsils, secretory immunoglobulins, secretory component, mucosal immunity, B cells, T cells, IgA deficiency.

Introduction

The nasal cavity and nasopharyngeal region are mainly covered by a thin, specialized epithelium that constitutes a weak mechanical barrier. Adequate surface protection, therefore, depends on intimate cooperation between natural nonspecific defence mechanisms and acquired specific immunity, mediated mainly by secretory antibodies. The term "immune exclusion" has been coined for this function (Fig. 1).

Immune exclusion

The importance of locally produced antibodies for mucosal defence was not fully realized until after the identification and characterization of secretory immunoglobulin A (SIgA) in the early 1960s. However, Walsh & Cannon (1938) had in the 1930s shown convincingly in experimental animals that intranasal immunization resulted in local formation of antibodies. Numerous subsequent studies verified this observation and demonstrated antibody activity in IgA of nasopharyngeal secretions (for review, see Brandtzaeg, 1984).

The pioneering work of Rossen et al. (1966) showed that IgA was a prominent protein component of nasal secretions and that most of it could be characterized as SIgA which is mainly a dimer of about 390 kD (Fig. 2).

In addition to the light and heavy chains, SIgA contains a disulphide-linked polypeptide of about 15 kD called the J ("joining") chain. J chain is also found in pentameric IgM (Mestecky, Zikan & Butler, 1971). SIgA differs from serum-type dimeric IgA by the additional content of an epithelial glycoprotein now called the secretory component (SC). It has a molecular mass of about 80 kD. Secretory IgM (SIgM) is likewise associated with SC, although only 60-70% of the polymers retain SC after purification (Brandtzaeg, 1975). SC serves as an epithelial receptor for both dimeric IgA (Fig. 2) and pentameric IgM and is thus responsible for their external transport (Brandtzaeg, 1985).

Although antibodies in external secretions are chiefly carried by actively transported SIgA (and SIgM), nasopharyngeal fluids contain considerable amounts of IgG antibodies as a result of passive leakage through the surface epithelium (Fig. 1). Observations in patients and in experimental animals have indicated that such IgG, whether locally produced or serum-derived, participates in immune exclusion in the upper respiratory tract. Traces of IgD and IgE are externally translocated in a way similar to IgG, or IgE may be carried by mucosal mast cells (Brandtzaeg, 1984).

Immune regulation

Secretory immunity depends on complex and only partly understood regulatory mechanisms for the generation and differentiation of B cells which mainly end up as IgA-producing immunocytes adjacent to glandular structures (Fig. 1). The primary responses seem to take place chiefly in organized mucosa-associated lymphoepithelial tissue (MALT) where the B cells proliferate under the influence of antigen and T-cell regulation. MALT includes Peyer's patches and other gut-associated lymphoid tissue (GALT), bronchus-associated lymphoid tissue (BALT), and probably also the tonsils. After migration to secretory tissues, the B cells are in addition subjected to "second signals" causing both local proliferation and terminal differentiation to plasma cells (Fig. 1).

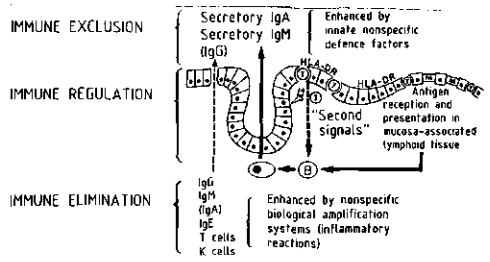


Fig. 1. Schematic representation of the three main components of mucosal defence: immune exclusion, immune regulation, and immune elimination. Antigen stimulation in mucosa-associated lymphoid tissue provides "first signals" to B cells which migrate to secretory sites via lymph and peripheral blood. "Second signals" (dashed bold arrow) induce local proliferation and terminal differentiation of the extravasated B cells. Most plasma cells generated in this way produce J chain-containing dimeric IgA which is translocated to the lumen as stabilized secretory IgA. Some IgM is also actively transported by the epithelial cells, whereas IgG reaches the surface only by passive diffusion (dashed thin arrow).

It is generally accepted that molecules encoded by the class II region of the human major histocompatibility complex (MHC) are critical as restricting elements in immune regulation (Janeway et al., 1984). These gene products are called Ia antigens in animal - the best known human counterparts being the HLA-DR molecules. Helper T cells (Th) can only be stimulated by antigens presented to them by accessory cells in the context of class II surface determinants. DR and similar molecules thus exert a genetically determined "guidance" function in the immune response to foreign material. Such class II determinants are expressed not only by macrophages and dendritic histiocytes, but also by certain epithelial cells (Fig. 1) in tonsils and nasal mucosa (Brandtzaeg, 1984). Lympho-epithelial interactions may therefore be important in mucosal immune regulation (Brandtzaeg et al., 1988).

Immune elimination

Foreign material that is able to penetrate the mucosal barrier will normally be subjected to immune elimination from the body interior (Fig. 1). This process may be initiated by acquired (specific) humoral immunity involving antibodies of various Ig classes, probably often in combination with cell-mediated immunity that depends on different functions of activated T cells.

Humoral and cell-mediated immunity is enhanced by a variety of nonspecific biological amplification systems such as activated complement, phagocytosis, release of histamine from mast cells, release of various lymphokines from activated T cells, etc. All these factors aim at efficient neutralization and elimination of foreign material.

Inflammation and tissue damage induced by these amplification systems may be sufficiently persistent to cause overt clinical disease if immune elimination is not successful. These nonspecific mechanisms may thus in various ways evolve into immunopathology in mucous membranes.

Ig production in nasal mucosa

The mucosa on the inferior and middle turbinates contains two principally different populations of immunocytes: one dominated by IgA cells present in the glandular areas and another dominated by IgG cells in the stroma beneath the surface epithelium (Brandtzaeg, 1984). Our observations indicate that the gland-associated immunocytes are mainly responsible for local secretory immunity whereas those beneath the surface are involved in immune elimination.

Except for an increased proportion of IgG-producing cells, the composition of the nasal gland-associated immunocyte population is quite similar to that of lacrimal glands (Brandtzaeg, 1983). Both secretory sites contain on an average a substantial proportion of IgD-producing cells, in contrast to the gastrointestinal mucosa. IgE-producing cells are normally not found but occasional immunocytes of this class may appear in allergic individuals. Mucosal mast cells with IgE are often seen in such patients, even when local IgE immunocytes are absent (Brandtzaeg, 1984).

In subjects with selective IgA deficiency, the proportion of nasal IgG-producing cells is relatively high in nasal mucosa. A more striking feature, however, is a remarkably high proportion of IgD-producing cells (24-82%) in most such individuals (Brandtzaeg et al., 1979). Others lack nasal IgD cells but have a relatively high number of local IgM immunocytes; these patients suffer from fewer upper respiratory tract infections than those with marked local IgD production (Brandtzaeg et al., 1987).

Most nasal IgM and IgD immunocytes are J chain-positive and the same is

true for 45-70% of the IgG cells. Such J chain-positive immunocytes probably belong to the secretory immune system along with the IgA-producing cells which show more than 98% J chain expression associated with extensive dimer production (Brandtzaeg & Korsrud, 1984). In the extrafollicular compartment of normal tonsils only 35-50% of the IgA-producing cells are J chain-positive (Brandtzaeg & Korsrud, 1984; Korsrud & Brandtzaeg, 1981).

Most tonsillar IgA immunocytes are of the IgA1 subclass (median proportion, 95%), but there are large individual variations (Kett et al., 1986). It is remarkable that also nasal mucosa usually contains mainly (90-95%) IgA1 immunocytes, which is in contrast to the dominance of IgA2 immunocytes seen in the distal gut (Kett et al., 1986).

Dichotomy of secretory immunity

The striking discrepancy between intestinal mucosa and secretory sites of the upper respiratory tract regarding the occurrence of IgD immunocytes and subclass distribution of IgA immunocytes, has led us to propose that the former tissue receives most of its B cells from GALT, whereas the latter sites are seeded mainly by B cells from BALT and tonsils (Brandtzaeg et al., 1986). Similar dichotomy of the secretory immune system has been suggested in the rat by recent homing studies on B cells (Parmely, 1985, van der Brugge-Gammelkoorn, Claassen & Sminia, 1986). In man, precursors from GALT may develop preferentially into IgA2-producing cells via direct switching from IgM expression, whereas secretory immunity of the upper respiratory tract may depend more on differentiation to IgA1 immunocytes through sequential switching of B cells according to the order of the C_H genes (Brandtzaeg et al., 1986).

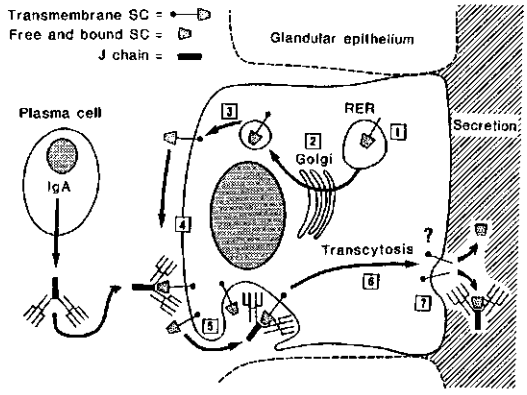


Fig. 2. Model for SC-mediated epithelial transport of dimeric IgA. **[1]** Synthesis and core glycosylation of transmembrane SC in rough endoplasmic reticulum (RER) of epithelial cell. **[2]** Terminal glycosylation (•) in Golgi complex. **[3]** Expression of SC at basolateral cell surfaces. **[4]** Complexing of SC with J chain-containing dimeric IgA. **[5]** Endocytosis of ligand-receptor complexes and excess SC. **[6]** Transcytosis of vesicles. **[7]** Cleavage and release of SIgA and free SC. The cleavage mechanism and the fate of the cytoplasmic tail of transmembrane SC are unknown (?). During the external translocation, covalent stabilization of the IgA-SC complexes regularly occurs (two disulphide bridges indicated in SIgA between SC and one of the IgA subunits).

The J chain-positive IgD and IgG immunocytes found in nasal mucosa and lacrimal glands normally - and especially in IgA deficiency (Brandtzaeg & Korsrud, 1984) - may hence be considered as "spin-off" from the vectorial differentiation process apparently dominating in this region. Salivary and mammary glands probably receive precursor cells in similar numbers from GALT and tonsils/BALT and are hence in an intermediate position, both with regard to frequency of IgD immunocytes (Brandtzaeg, 1983) and accumulation of IgA1- and IgA2-producing cells (Kett et al., 1986).

The notion that B cells of BALT and tonsils have appreciably more IgD-producing potential than those from GALT is supported by the fact that tonsils normally contain far more IgD-producing immunocytes than GALT (Brandtzaeg, Surjan & Berdal, 1978; Bjerke & Brandtzaeg, 1986). This difference may depend on environmental rather than intrinsic factors. Many bacterial species that are common in the upper respiratory tract have Fc receptors for IgD and may thus exert mitogenic effects on IgD-expressing B lymphocytes (Forsgren & Grubb, 1979). By contrast, Escherichia coli does not bind to IgD (Forsgren & Grubb, 1979) and lipopolysaccharides inhibit the expression of IgD on B cells in vitro (Parkhouse & Cooper, 1977).

Relation between nasal immunity and clinical state

The concept of a "common mucosal immune system" implies that GALT supplies primed B cells to secretory sites of the upper respiratory tract. However, recent observations in IgA deficiency have suggested that the nasal mucosa of only some patients is populated by B cells that have originated in GALT, or at least show properties like GALT-derived cells in that terminal differentiation leads to prominent local production of IgM (Brandtzaeg et al., 1987). These patients have fewer upper airway infections than those whose IgA immunocytes are replaced largely by IgD-producing cells in nasal mucosa - either because IgD acts as a disease-promoting antibody by blocking complement-activating IgG and IgM antibodies, or because B cells derived from tonsils/BALT are less likely to terminate with IgM production (which is the basis for generation of compensatory SIgM). Patients who are unable to compensate satisfactorily their IgA deficiency with any class of local Ig production are even more susceptible to infections (Brandtzaeg et al., 1987).

As a consequence of such individual immunoregulatory variations in the mucosal defence of the upper airways, the putative role of lymphoepithelial structures of Waldeyer's ring in reinforcing mucosal immunity of the whole upper respiratory tract, needs reconsideration.

References

Bjerke, K. & P. Brandtzaeg, 1986. Immunoglobulin- and J chain-producing cells associated with lymphoid follicles in the human appendix, colon and ileum, including Peyer's patches. Clin. Exp. Immunol. 64:432-441.

Brandtzaeg, P., 1975. Human secretory immunoglobulin M. An immunochemical and immunohistochemical study. Immunology 29:559-570.

Brandtzaeg, P., 1983. The secretory immune system of lactating human mammary glands compared with other exocrine organs. Ann. N.Y. Acad. Sci. 409:353-381.

Brandtzaeg, P., 1984. Immune functions of human nasal mucosa and tonsils in health and disease. In: J. Bienenstock (Ed.): Immunology of the Lung and Upper Respiratory Tract. New York: McGraw-Hill. pp. 28-95.

Brandtzaeg, P., 1985. Role of J chain and secretory component in receptor-mediated glandular and hepatic transport of immunoglobulins in man. Scand. J. Immunol. 22:111-146.

Brandtzaeg, P. & F.R. Korsrud, 1984. Significance of different J chain profiles in human tissues: generation of IgA and IgM with binding site for secretory component is related to the J chain expressing capacity of the total local immunocyte population, including IgG and IgD producing cells, and depends on the clinical state of the tissue. Clin. Exp. Immunol. 58:709-718.

Brandtzaeg, P., L. Surjan & P. Berdal, 1978. Immunoglobulin systems of human tonsils. I. Control subjects of various ages: quantification of Ig-producing cells, tonsillar morphometry and serum Ig concentrations. Clin. Exp. Immunol. 31:367-381.

Brandtzaeg, P., S.T. Gjeruldsen, F. Korsrud, K. Baklien, P. Berdal & J. Ek, 1979. The human secretory immune system shows striking heterogeneity with regard to involvement of J chain-positive IgD immunocytes. J. Immunol. 122:503-510.

Brandtzaeg, P., K. Kett, T.O. Rognum, R. Söderström, J. Björkander, T. Söderström, B. Petruson & L.Å. Hanson, 1986. The distribution of mucosal IgA and IgG subclass-producing immunocytes and alterations in various disorders. Monogr. Allergy 20:179-194.

Brandtzaeg, P., G. Karlsson, G. Hansson, B. Petruson, J. Björkander & L.Å. Hanson, 1987. The clinacal condition of IgA-deficient patients is related to the proportion of IgD- and IgM-producing cells in their nasal mucosa. Clin. Exp. Immunol. 67:626-636.

Brandtzaeg, P., L.M. Sollid, P.S. Thrane, D. Kvale, K. Bjerke, H. Scott, K. Kett & T.O. Rognum, 1988. Lymphoepithelial interactions in the mucosal immune system. Gut 29:1116-1130.

Forsgren, A. & A.O. Grubb, 1979. Many bacterial species bind human IgD. J. Immunol. 122:1468-1472.

Janeway, C.A., K. Bottomly, J. Babich, P. Conrad, S. Conzen, B. Jones, J. Kaye, M. Katz, L. McVay, D.B. Murphy & J. Tite, 1984. Quantitative variations in Ia antigen expression plays a central role in immune regulation. Immunol. Today 5:99-105.

Kett, K., P. Brandtzaeg, J. Radl & J.F. Haaijman, Different subclass distribution of IgA-producing cells in human lymphoid organs and various secretory tissues. J. Immunol. 136:3631-3635.

Korsrud, F.R. & P. Brandtzaeg, 1981. Immunohistochemical evaluation of J-chain expression by intra- and extra-follicular immunoglobulin-producing human tonsillar cells. Scand. J. Immunol. 13:271-280.

Mestecky, J., J. Zikan & W. Butler, 1971. Immunoglobulin M and secretory immunoglobulin A: evidence for a common polypeptide chain different from light chains. Science 171:1163-1165.

Parkhouse, R.M.E. & M.D. Cooper, 1977. A model for the differentiation of B lymphocytes with implications for the biological role of IgD. Immunol. Rev. 37:105-126.

Parmely, M.J., 1985. Kinetics of mammary and intestinal IgA-producing cells in the lactating rat. J. Reprod. Immunol. 8:89-93.

Rossen, R.D., W.T. Butler, W.E. Vannier, R.G. Douglas & A.G. Steinberg, 1966. The sedimentation and antigenic properties of proteins in nasal and other external secretions. J. Immunol. 97:925-938.

van der Brugge-Gammelkoorn, G.J., E. Claassen & T. Sminia, 1986. Anti-TPN-forming cells in bronchus-associated lymphoid tissue (BALT) and paratracheal lymph node (PTLN) of the rat after intratracheal priming and boosting with TNP-KLH. Immunology 57:405-409.

Walsh, T.E. & P.R. Cannon, 1938. Immunization of the respiratory tract. A comparative study of the antibody content of the respiratory and other tissues following active, passive and regional immunization. J. Immunol. 35:31-46.

NASAL LYMPHOID TISSUE, ITS SPECIALIZED POSITION IN THE UPPER RESPIRATORY TRACT

C.F. Kuper[1], B.J. Spit[1], J.P. Bruijntjes[1], E.G.J. Hendriksen[1] and D.M.H. Hameleers[2]

[1] TNO-CIVO Toxicology and Nutrition Institute, Zeist, Netherlands
[2] Department of Histology, Medical Faculty, Vrije Universiteit, Amsterdam, Netherlands

Summary

The morphology of paired lymphoid tissue in the rat nasal mucosa, at the entrance of the nasopharyngeal duct, was investigated with conventional and immunocytochemical methods, by means of light and electron microscopy. This lymphoid tissue was denoted nasal lymphoid tissue or NALT. The morphological characteristics indicate that NALT plays an important role in the mucosal immune system of the respiratory tract.

Introduction

Mucosal tissue in the respiratory tract comprises epithelium that is exposed directly to infectious and antigenic agents. This tissue is the site of an extensive immunologic system to protect the body against the entry and damaging activities of noxious agents. Associated with the mucosa of the lower and upper respiratory tract are lymphoid accumulations. The lymphoid tissue in the lower respiratory tract is known as bronchus-associated lymphoid tissue (BALT), and already extensively investigated (Gregson et al., 1979a,b; Bienenstock, 1985; Van der Brugge-Gamelkoorn et al., 1985a, b; Plesch, 1982). In rats, only limited information is available on nasal lymphoid tissue or NALT. Already some decennia ago, massive lymphoid cell accumulations associated with the mucosa of the nasopharyngeal duct of the rat were described (Kelemen, 1947; Giddens et al., 1971), but it was shown only recently that these lymphoid aggregates form organized lymphoid structures that are part of a common mucosal immune system (Spit et al., 1988, in press; Koornstra et al., 1989, in press).

Materials and Methods

Young adult male albino Wistar rats (Cpb:WU Wistar random; Central Institute for the Breeding of Laboratory Animals, Zeist, The Netherlands) were used. NALT was collected and frozen at -159°C. Cryostat sections of NALT were fixed in pure acetone and stained via the indirect immunohistochemical method. The following antibodies were used: W3/13 (T cell), W3/25 (T helper cell), OX8 (T non-helper or T suppressor/cytotoxic/large granular cell), IgG clone OX12, IgM, IgA, IgE and Mas 029c (Ia), all from Seralab, England) and ED1, ED2, ED3, kindly provided by C.D. Dijkstra, Vrije Universiteit, The Netherlands.

Results and Discussion

Nasal lymphoid tissue (NALT) is located at both sides of the entrance of the nasopharyngeal duct, at the level of the second palatal ridge (Fig. 1). Although not investigated systematically, these lymphoid aggregates are probably not the only organized lymphoid tissues in the

mucosa of the nasal passages, but they are the largest and possibly the only aggregates with a fixed localization.

NALT comprises discrete and complementary T and B cell compartments, and specialized epithelium (Figs. 2a and b).

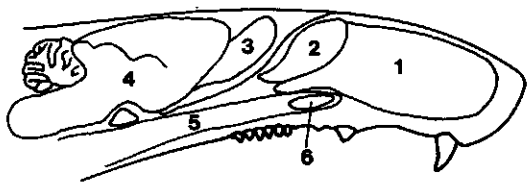
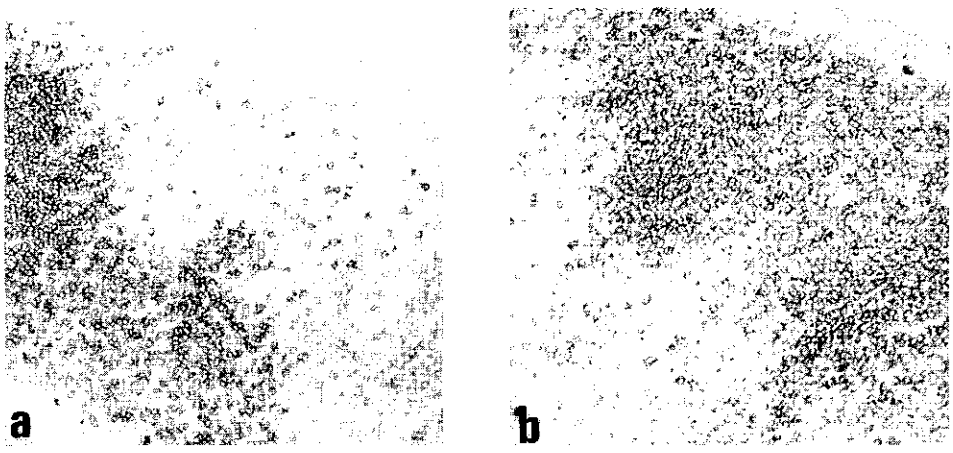


Fig. 1. Schematic presentation of a longitudinal section through the head of a rat. The lower jar is removed. 1. respiratory epithelium, 2. olfactory epithelium, 3. bulbus olfactorius, 4. brain, 5. nasopharyngeal duct, 6. nasal lymphoid tissue or NALT.



Figs. 2a and b. Serial cryostat sections of rat nasal lymphoid tissue stained for T lymphocytes (W3/13 surface marker; Fig. 2a), or B lymphocytes (surface IgM; Fig. 2b).

The following morphological characteristics apply to conventionally housed, unstimulated Wistar rats, which means that they were mildly exposed to antigens. A schematic presentation of the cellular composition is given in Fig. 3.

The T cell compartment was populated by T helper (W3/25) and T non-helper (T suppressor/cytotoxic/large granular; OX8) cells in almost equal numbers. In addition, a considerable number of macrophages were found, which were positive for ED1, a marker that is found in macrophages and monocytes (Dijkstra et al., 1985). The ED1 positive cells were round as well as dendritic-like cells. Most B cells in the B cell compartment contained surface IgM (sIgM) or IgG (sIgG). Only a few B cells were positive for surface IgA or IgE. Some macrophages with the ED1 marker were found dispersed in the B cell compartment. Ample Ia expression was found in the

follicles. This is suggestive for cells with immune-initiating and immune-regulating properties (Sertl et al. 1986). The border between NALT and the surrounding connective tissue was populated by ED1 positive macrophages and, in addition, by macrophages carrying the ED2 or ED3 surface markers. ED2 is a marker for tissue macrophages and ED3 staining is characteristic for macrophages in lymphoid organs (Dijkstra et al. 1985; Beelen et al., 1987). Both the ED2 as the ED3 positive cells had a dendritic-like appearance.

The epithelium overlying the nasal lymphoid tissues contained few goblet cells. The ciliated cells were slightly modified with shorter and fewer cilia, and rather cuboidal than columnar. The most characteristic epithelial cells were the non-ciliated or M cells (Spit et al., 1988, in press). These M cells appear to be an important feature of mucosa-associated lymphoid tissues (Madara et al., 1984; Bye et al., 1984; Bienenstock, 1985). Different types of the non-ciliated cells could be observed in NALT, possibly expressions of the state of maturity of these cells. They had microvilli or merely an irregular outline of the apical plasma membrane. They contained electron-lucent cytoplasm which is rich in organelles or electron-dense, organelle-poor cytoplasm. The antigen uptake property of the M cells was clearly demonstrated in nasal application experiments with horse radish peroxidase (HRP) alone or coupled to 5 nm gold complex (HRP-gold). HRP in NALT sections was visualized by diaminobenzidine-tetrahydrochloride and examined by transmission electron microscopy. After exposure to HRP for 45 min, electron-dense material was found in the apical cytoplasm and in connection with the lateral and basal margins of these cells (Figs. 4a and b). The distribution pattern of HRP-gold was also restricted to the M cells. Five-nm gold particles were found

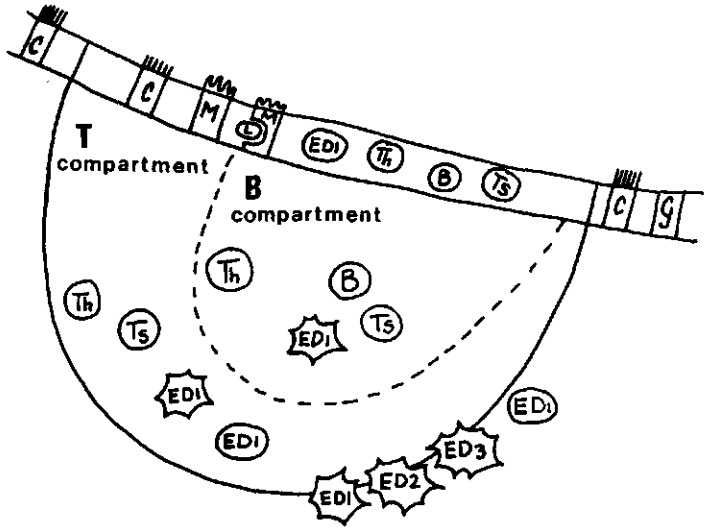
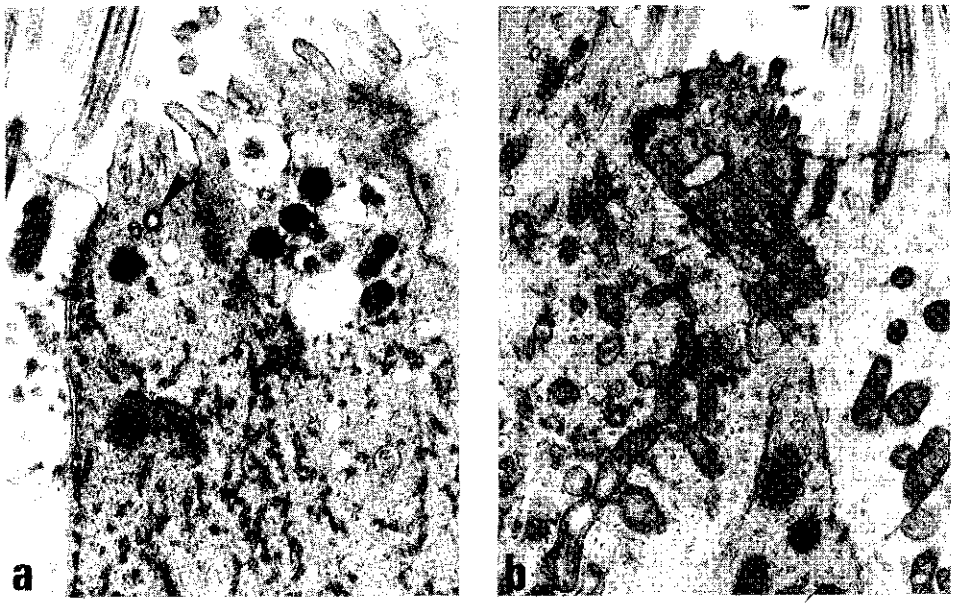


Fig. 3. Schematic presentation of the cellular composition of rat NALT. C = ciliated epithelial cell, G = goblet cell, M = M cell, L = lymphocyte, Th = T helper lymphocyte, Ts = T non-helper (suppressor/cytotoxic/large granular) lymphocyte, B = B lymphocyte, ED1 = ED1+ macrophage, ED2 = ED2+ macrophage, ED3 = ED3+ macrophage.



Figs. 4a and b. Lymphoepithelium of rat NALT, after exposure to horse radish peroxidase (HRP) for 45 min. One of the two M cells contain cytoplasmic electron dense material, associated with HRP exposure (arrowhead; Fig. 4a). Electron dense material is also found in connection with the upper part of the lateral margins of M cells (Fig. 4b).

in the vacuoles in the cytoplasm of the M cells. No gold particles were observed in the lateral and basal margins, probably partly due to the particle size. These results indicate that the non-ciliated cells are a major site of entry for antigens and particles. This is supported by the finding that the M cells stained positively for Ia. Many lymphocytes infiltrated the epithelium (Fig. 5). Both B and T lymphocytes were observed between the epithelial cells. B cells were positive for sIgM. The T cells expressed a marker for the T helper or T non-helper.

The functions of NALT in the mucosal immune system are still largely unknown. The high number of so-called M cells and the presence of numerous lymphocytes in NALT epithelium of rats with a low antigenic load indicate that NALT is an important part of the mucosal immune system in the respiratory tract. The role of NALT in the induction of tolerance for aeroantigens, in analogy to the role of GALT in induction of food tolerance, is presently investigated.

Acknowledgements

The study was supported by a grant of the Ministry of Welfare, Health and Cultural affairs, The Netherlands

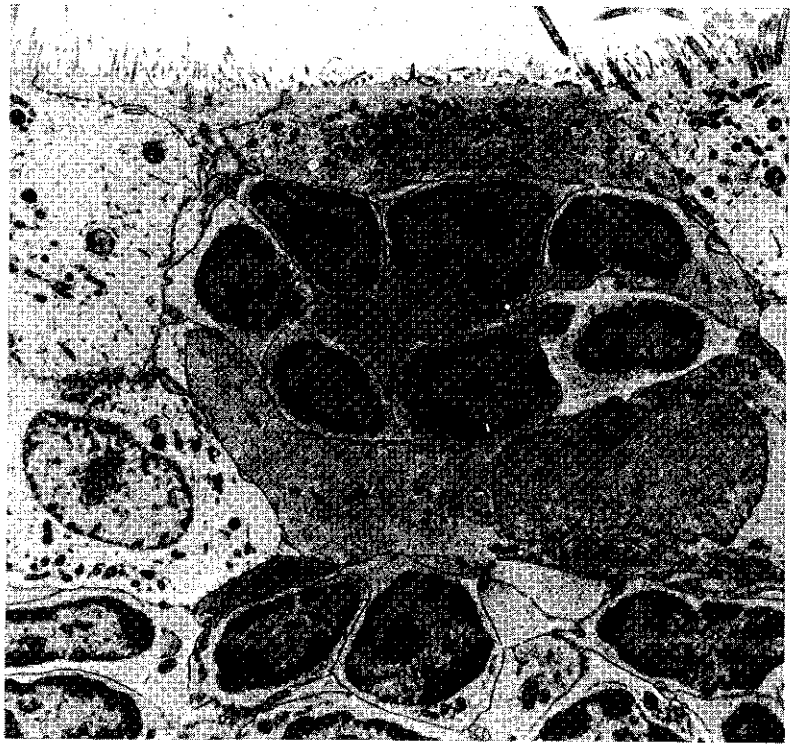


Fig. 5. Lymphoepithelium of rat NALT. Lymphocytes are present in the epithelium in close association with a non-ciliated epithelial cell.

References

Beelen R.H.J., I.L. Eestermans, E.A. Döpp & C.D. Dijkstra, 1987. Monoclonal antibodies ED1, ED2, and ED3 against rat macrophages: Expression of recognized antigens in different stages of differentiation. Transplant. Proc. 19:3166-3170.

Bienenstock J., 1985. Bronchus-associated lymphoid tissue. Int. Archs. Allergy Appl. Immun. 76, Suppl. I:62-69.

Bye W.A., C.H. Allan & J.S. Trier, 1984. Structure, distribution, and origin of M-cells in Peyer's patches of mouse ileum. Gastroenterology 86:789-801.

Dijkstra C.D., E.A. Döpp, P. Joling & G. Kraal, 1985. The heterogeneity of mononuclear phagocytes in lymphoid organs: distinct macrophage subpopulations in the rat recognized by monoclonal antibodies ED1, ED2, ED3. Immunology 54:589-599.

Giddens W.E., C.K. Whitehair & G.R. Carter, 1971. Morphologic and Microbiologic features of nasal cavity and middle ear in germfree, defined-flora, conventional and chronic respiratory disease-affected rats. Am. J. Vet. Res. 32:99-114.

Gregson R.L., M.J. Davey & D.E. Prentice, 1979a. Postnatal development of bronchus-associated lymphoid tissue (BALT) in the rat Rattus norvegicus. Lab. Animals 13:231-238.

Gregson R.L., M.J. Davey & D.E. Prentice, 1979b. The response of rat bronchus-associated lymphoid tissue to local antigenic challlenge. Br. J. Exp. Path. 60:471-482.
Kelemen G., 1947. The junction of the nasal cavity and the pharyngeal tube in the rat. Arch. Otolaryngol. 45:159-168.
Koornstra P.J., A.M. Duijvesteijn, S.R. Joosten-Achjanij, F.I.C.R.S. de Jong, L.F.M. Vlek & J.H.M. van de Beek, 1989. Waldeyer's ring equivalent (WRE) in the rat: FACS analysis and immunohistology of WRE cells during aging. Eur. J. Celbiology. In press.
Madara J.L., W.A. Bye & J.S. Trier, 1984. Structural features of and cholesterol distribution in M-cell membranes in guinea pig, rat, and mouse Peyer's patches. Gastroenterology 87:1091-1103.
Plesch B.E.C., 1982. Histology and immunohistochemistry of bronchus associated lymphoid tissue (BALT) in the rat. Adv. Exp. Med. Biol. 149:491-497.
Sertl K., T. Takemura, E. Tschachler, V.J. Ferrans, M.A. Kaliner, E.M. Shevach, 1986. Dendritic cells with antigen-presenting capability reside in airway epithelium, lung parenchyma and visceral pleura. J. Exp. Med. 163:436-451.
Spit B.J., E.G.J. Hendriksen, J.P. Bruijntjes & C.F. Kuper, 1988. Nasal lymphoid tissue in the rat. Cell. Tissue Res. In press.
Van der Brugge-Gamelkoorn G.J., B.E.C. Plesch, T. Sminia & H.L. Langevoort, 1985a. Histological changes in rat bronchus-associated lymphoid tissue after administration of five different antigens. Respiration 48:29-35.
Van der Brugge-Gamelkoorn G.J., M.B. Van de Ende & T. Sminia, 1985b. Non-lymphoid cells of bronchus-associated lymphoid tissue of the rat in situ and in suspension. With special reference to interdigitating and follicular dendriitic cells. Cell Tissue Res 239:177-182.

AIRFLOW, MUCOCILIARY CLEARANCE, AND LESION DISTRIBUTION IN THE NASAL PASSAGES OF EXPERIMENTAL ANIMALS

K.T. Morgan and T.M. Monticello

Chemical Industry Institute of Toxicology, Research Triangle Park, North Carolina, USA

Summary

Neoplasms and non-neoplastic responses induced in the nasal passages of experimental animals by inhaled air pollutants vary considerably in location and nature. The distribution of nasal lesions is a consequence of the combined effects of regional deposition of the inhaled material and local tissue susceptibility. For certain particulate matter, and highly water soluble gases such as formaldehyde, airflow patterns appear to play a major role in regional deposition and consequent lesion distribution in the nose. For these materials, airflow patterns may also contribute to species specific differences in the distribution of lesions in the respiratory tract. Gases with a low water solubility, such as methyl bromide, are probably deposited more uniformly over the nasal mucosa, and the site-specificity of nasal lesions is attributable to local tissue susceptibility as a consequence of regional metabolism. In addition to metabolism, other airway defenses may influence the distribution of nasal lesions. The mucociliary apparatus provides an effective epithelial defense against deposited particulate matter. Similar protection by the mucociliary apparatus has been postulated for inhaled toxic gases, but this remains to be demonstrated experimentally. Inhibition of mucociliary function can provide a sensitive indicator of regional nasal toxicity of inhaled air pollutants. Identification of the principal factors that influence the distribution and nature of nasal lesions is important when attempting to determine potential human risks using data derived from laboratory animals.

Introduction

Rats and mice are used extensively for inhalation toxicology studies designed to assess risks, or understand mechanisms, of diseases in humans. The toxicologic pathologist can play an important role in this research process by adequately reporting the nature and location of lesions in the nasal passages (Jiang, 1986). Much remains to be learned about the structure and function of the nose, and only limited information exists concerning the mechanisms responsible for neoplastic and pre-neoplastic responses in this complex organ. During the interpretation of nasal lesions, pathologists can benefit from knowledge of the factors that influence lesion distribution in the nose.

Site specificity of nasal lesions

Until recently the nasal passages were inadequately examined in inhalation toxicology studies. Publications on nasal lesions in laboratory animals often contain an incomplete description of the

distribution of lesions, even when the nature of the responses is clearly reported. However, due to the complexity of the nose, precise localization of responses requires detailed mapping (Jiang et al, 1986), which is not always justified. An alternative approach is to generate an appropriate narrative. In rats exposed to irritant gases there is clear site-specificity for nasal lesions. Figure 1 indicates examples of sites of nasal lesions induced in rats by exposure to a range of chemicals.

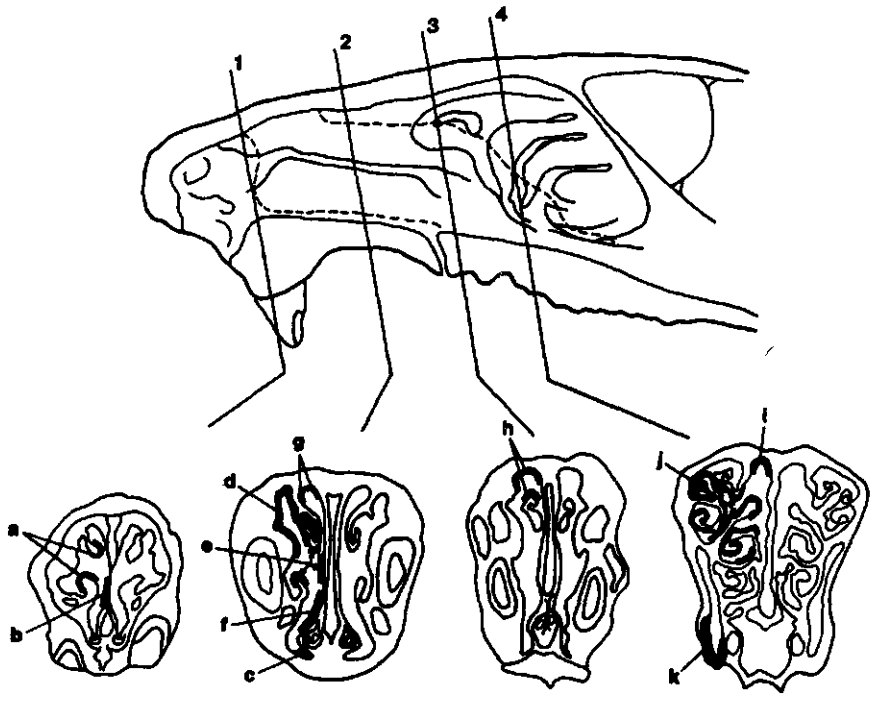


Figure 1. Diagram of rat nasal passages opened along the mid-line with the septum removed to reveal the turbinates (upper diagram). Lines numbered 1-4 indicate section levels used routinely in this laboratory, which are shown in lower portion of the figure. Some important sites to look for nasal lesions are indicated on the cross sections: (a) tips of atrioturbinates, (b) septum in nasal vestibule, (c) squamous epithelium in ventral meatus, (d) lining of lateral meatus, (e) margins of middle meatus: ventral margin of nasoturbinate, dorsal margin of maxillo-turbinate, and adjacent septum, (f) ventral septum, (g, h, i) olfactory epithelium in dorsal meatus, (j) olfactory mucosa on ethmoid scrolls, (k) Steno's gland.

Awareness of these sites is important for pathologists and histotechnologists working in the field of inhalation toxicology. Such awareness will result in optimal selection and preparation of nasal sections and identification of treatment-induced lesions.

Factors that Influence Nasal Lesion Distribution

Two major factors influence the distribution of nasal lesions induced by chemical gases or vapors, (a) regional deposition, and (b) local tissue susceptibility. Nasal lesions induced by formaldehyde and methyl bromide, which are shown diagrammatically in Figure 2, provide useful examples of each of these mechanisms.

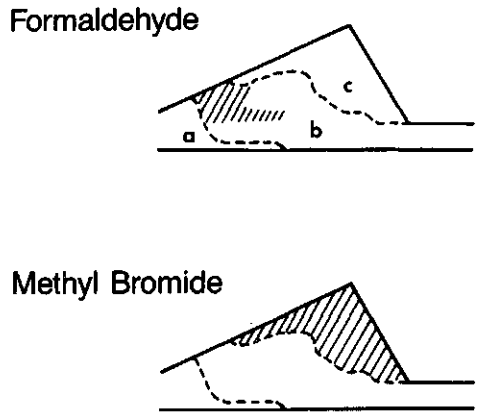


Figure 2. Diagrammatic representation of the nasal passages of the rat showing location of the three major epithelial types (a = squamous, b = respiratory, c = olfactory) and sites of nasal lesions (cross-hatching) induced by formaldehyde and methyl bromide.

The distribution of formaldehyde-induced lesions is largely attributable to regional deposition as a result of its high water solubility (55 gm/100 ml) and nasal airflow patterns (Morgan et al, 1988). Lesions induced in the olfactory epithelium by methyl bromide, which has a low water solubility (0.09 gm/100 ml) and leaves the respiratory epithelium unaffected, are probably a consequence of tissue susceptibility as a result of regional metabolism of this compound (Hurtt et al, 1988).

Deposition. Numerous studies have been carried out on the deposition of airborne chemicals by the nose. These studies can be divided into two main types: Uptake studies, that investigate the ability of the nose or the upper airways to remove materials from inspired air, and regional deposition studies, designed to determine which areas of the nose or upper airways receive the highest 'dose' of the deposited material.

Uptake studies have demonstrated that the nose removes a number of chemicals from inspired air, with uptake efficiencies ranging from almost 100% for formaldehyde in the dog and hydrogen fluoride in the rat, down to less than 1% for carbon monoxide in humans and dogs with

the other gases lying between these two extremes. Very few attempts have been made to characterize regional deposition of inhaled materials in the nose. Whole body autoradiography demonstrated nasal deposition of ^{14}Carbon derived from radio-labelled formaldehyde. However, attempts to use this approach to localize regional deposition patterns are confounded by the labile nature of reactive materials, such as formaldehyde, which are rapidly metabolized or translocated from the deposition site.

Schreider (1983) examined particle deposition in casts of guinea pigs and rats, using monodisperse aerosols of methylene blue, ranging in size from 1 to 7 μm diameter. All the deposited material was found in the front third of the nose, with the highest concentration being just posterior to the nares. It was concluded that impaction, presumably as a consequence of nasal airflow patterns, was the principal process of deposition in the nasal passages for larger particles. Torjussen (1983) implicated airflow patterns in the deposition of particles on the anterior curvature of the middle turbinate of humans, and stated that this site-specific deposition probably accounts for the frequency of lesions in this site in nickel workers. Studies of particle deposition in the human nose using a radio-aerosol revealed two major deposition sites which were correlated with airflow characteristics: (1) close to the ostium internum where turbulent eddies are well developed, and (2) the anterior region of the middle turbinate, where the direction of airflow changes from upward to horizontal (Itoh et al, 1985). Recent work in this laboratory, in rats and monkeys, demonstrated a good correlation between nasal airflow patterns and the distribution of formaldehyde-induced lesions. These findings are consistent with the proposal made by Proetz (1951) that airflow patterns play an important role in the regional deposition of inhaled materials in the nose, with the consequent potential to influence lesion location and severity. Therefore, species differences in regional gas deposition as a result of nasal airflow characteristics may represent an important issue for consideration during human risk assessments based on animal studies.

Tissue Susceptibility. The nasal passages are lined by several different types of mucosa, each possessing characteristic morphologic, biochemical, and physiologic propertes which may influence their susceptibility to toxic chemicals. The olfactory epithelial toxicity of methyl bromide provides a good example of site specific lesions that are attributable to tissue susceptibility as a result of regional metabolism, and are unrelated to nasal airflow patterns (Hurtt et al, 1988). This gas is specifically toxic to olfactory epithelium, which is extensively destroyed in rats by a single 6 hr exposure to 200 ppm, while the other nasal epithelia remain morphologically unaffected. Methyl bromide has a very low water solubility, which would be expected to result in slower uptake by the watery nasal secretions, and greater access of this chemical to the recesses of the ethmoid region, resulting in a similar 'dose' to all regions of the nose. It has been proposed that methyl bromide-induced degeneration of the olfactory epithelium in rats is the result of a primary biochemical lesion in olfactory sustentacular cells and mature sensory cells (Hurtt et al, 1988). Such a biochemical lesion would account for the site specificity of the epithelial damage seen at the light microscopic level.

Mucociliary apparatus. Airway defenses play a major role in influencing the outcome of exposure to airborne irritants. In addition to regional metabolism, referred to above, one of the major defenses of the nasal airway is the mucociliary apparatus. Detection of inhibition of nasal mucociliary function provides a sensitive indicator of toxicity in rats exposed to gaseous irritants. It has been established that the mucociliary apparatus provides protection of the airway epithelium against inhaled particulate matter. This system may also contribute to the removal of inhaled, water-soluble reactive gases. However, for non-reactive gases the mucociliary apparatus probably provides no significant protection (Morris et al, 1986). If the nasal mucociliary apparatus does protect the epithelium from exposure to certain highly reactive gases, it may influence the distribution and severity of nasal lesions.

Conclusion

Identification and characterization of the factors that influence the distribution and nature of nasal lesions is important when attempting to determine potential human risks using data derived from laboratory animals.

References

Hurtt, M.E., Thomas, D.A., Working, P.K., Monticello, T.M., and Morgan, K.T. (1988). Degeneration and regeneration of the olfactory epithelium following inhalation exposure to methyl bromide: pathology, cell kinetics, and olfactory function. Toxicol. Appl. Pharmacol., 94, 311-328.

Itoh, H., Smaldone, G.c., Swift, D.L. and Wagner, H.N. (1985). Mechanisms of aerosol deposition in a nasal model. J. Aerosol Sci., 16, 529-534.

Jiang, X.Z., Morgan, K.T., and Beauchamp, R.W.Jr. Histopathology of acute and subacute nasal toxicity. In: Toxicology of the Nasal Passages (Craig S. Barrow, Ed.), Hemisphere, New York, 1986, pp. 51-66.

Morgan, K.T., Monticello, T.M., Fleishman, A., and Patra, A.L. (1988). Preparation of rat nasal airway casts and their application to studies of nasal airflow. Proceedings of a Symposium on Lung Dosimetry, Extrapolation Modeling of Inhaled Particles and Gases. Duke University, 1987 (In Press).

Morris, J.B., Clay, R.J., and Cavanagh, D.G. (1986). Species differences in upper respiratory tract deposition of acetone and ethanol vapors. Fund. Appl. Toxicol., 7, 671-680.

Proetz, A.W. (1951). Air currents in the upper respiratory tract and their clinical importance. Ann. Otol. Rhinol. Laryngol., 60, 439-467.

Schreider, J.P. (1983). Nasal airway anatomy and inhalation deposition in experimental animals and people. In: Nasal Tumors in Animals and Man. (Gerd Reznik and Sherman F. Stinson, Eds.) Vol. III, pp 1-26. CRC Press, Boca Raton, Florida.

Torjussen, W. (1983). Nasal cancer in nickel workers. Histopathological findings and nickel concentrations in the nasal mucosa of nickel workers, and a short review of chromium and arsenic. Vol. 2, pp. 33-53, In: Nasal Tumors in Animals and Man (Reznik, G., and Stinson, S.F., Eds.). CRC Press Inc., Boca Raton.

INHALED TOXICANT-INDUCED PROLIFERATIVE RESPONSES IN NASAL EPITHELIUM OF LABORATORY ANIMALS

Jack R. Harkema[1], Thomas M. Monticello[2], and Jon A. Hotchkiss[1]

[1]Lovelace Inhalation Toxicology Research Institute
P. O. Box 5890, Albuquerque, NM, 87185, USA
[2]Chemical Industry Institute of Toxicology
P. O. Box 12137, Research Triangle Park, NC, 27709, USA

Introduction

Proliferation of epithelial cells lining respiratory airways is an important response to injury following inhalation of irritants. This response is necessary to repair damaged epithelium and to protect the airway mucosa from further insult by thickening the epithelial barrier (epithelial hyperplasia). There is little quantitative information in the literature, however, characterizing the proliferative responses of the nasal surface epithelium to inhaled irritants. Site specific association of necrosis, hyperplasia, and malignancy in the airway epithelium suggests that enhanced cell proliferation may be important in the carcinogenic process. Enhanced cell proliferation is generally thought to be involved in both the early and late phases of cancer formation. This paper will focus on alterations in cell proliferation of nasal airway epithelium in rats and monkeys after single or multiple exposures to ozone, a common oxidant pollutant in photochemical smog, or to formaldehyde gas, an important industrial chemical. Formaldehyde gas is a known nasal carcinogen in rats. The respiratory tract carcinogenicity of ozone is open to question.

Ozone-induced alterations in nasal epithelial cell proliferation

The responses of the nasal epithelium of bonnet monkeys to exposures of 0.15 ppm ozone (8 hr/day for 6 or 90 days) and 0.30 ppm (8 hr/day for 90 days) have been studied at the University of California-Davis and the California Primate Center (Harkema et al., 1987a). Morphometric changes were evident only in the transitional and respiratory epithelia in the anterior, nonturbinate area of the nasal cavity. Transitional epithelium (TE) is a nonciliated, cuboidal/columnar, stratified epithelium, which lines the luminal surface of the anterior nasal cavity between the nasal vestibule and the nasal turbinate region (Harkema et al., 1987b). Respiratory epithelium (RE), which is a ciliated, pseudostratified epithelium with goblet cells, lines the remainder of the main chamber of the nasal cavity, except for a relatively small area in the dorsoposterior aspect of the airway covered by olfactory epithelium. One of the most significant changes observed in this study was secretory cell metaplasia in these two surface epithelia of the anterior nasal cavity after both 6-day and 90-day ozone exposures. After 6 days of exposure to 0.15 ppm ozone, total cell numbers in both epithelial populations were significantly greater than those in animals exposed only to filtered air. After 90 days of 0.15 ppm ozone, the total epithelial cells/mm of basal lamina had decreased to control levels. Ninety-day exposure to 0.30 ppm ozone did not produce a significantly greater number of epithelial cells/mm of basal lamina than

did the exposure to 0.15 ppm. Dramatic shifts in epithelial cell populations of RE and TE were noted after short and long term exposures to ozone. In the TE, luminal nonciliated cells without secretory granules were significantly decreased after 6-day and 90-day exposures, while the number of nonciliated cells with secretory granules were significantly increased. Small mucous granule cells increased after 6 and 90 days of ozone exposure. This latter cell type is prominent in the TE and RE of bonnet monkeys and is located in the midepithelial layers between the apical and basal cells (Harkema et al., 1987b). No such cell type in the nasal epithelium of rodents has been described. In contrast to the small mucous granule cells in the ozone-exposed monkeys, the population densities of basal cells after 6- and 90-day exposure to 0.15 ppm ozone were significantly less than those of controls. Quantitative changes in epithelial cell populations in RE of the ozone-exposed monkeys were also evident. There were increases in small mucous granular cells, intermediate cells, and luminal secretory cells, decreases in the relative numbers of ciliated cells, and no changes in the number of basal cells.

Concurrent with the changes in epithelial cell numbers, there was luminal epithelial cell necrosis in the TE and RE of ozone-exposed monkeys. An inflammatory cell influx was only present in the hyperplastic nasal epithelia after 6 days of ozone exposure.

The results of this study suggested that nasal epithelial cell proliferation increased early (before 6 days) after the initiation of the ozone exposure, with new cell production being greater than cell loss by necrosis. This resulted in thickened, hyperplastic epithelium in the anterior nasal airway after 6 days of irritant exposure. In addition, it appeared that the "burst" of cell proliferation ceased sometime between 6 and 90 days of exposure, because the TE and RE were no longer hyperplastic after 90 days of exposure. At this later time point, these epithelia did exhibit secretory cell metaplasia, which suggested an adaptive/protective response to the continued, long-term exposure. This study, however, was not designed to characterize cell turnover or to determine which epithelial cells were rapidly dividing and were responsible for the early hyperplastic and subsequent metaplastic changes in the nasal epithelium.

Recent studies at the Lovelace Inhalation Toxicology Research Institute have characterized the proliferative response of rat nasal epithelium to short-term ozone exposure (Harkema et al., 1988; Johnson et al., 1988). In these studies, one group of Fischer 344/N rats was exposed to 0.12 or 0.80 ppm ozone for 3 or 7 days (6 hr/day) and immediately sacrificed, while another group was exposed for 7 days to ozone and then to filtered air (0.0 ppm ozone) for 3 or 7 days before sacrifice. Animals exposed to 0.12 ppm had no microscopic lesions in the nasal mucosa. Hyperplasia of TE covering the lateral wall and nasal and maxilloturbinates of the anterior nasal cavity was evident in the rats exposed to 0.80 ppm ozone and sacrificed at 7 days. The epithelium in rats also had a concomitant increase in intraepithelial mucosubstances. Rats exposed to the same concentration of ozone, but sacrificed after 3 days of exposure, had no evidence of hyperplasia. However, using bromodeoxyuridine (BrdU, a thymidine analog) and immunohistochemistry, to detect BrdU-incorporated DNA, a tremendous increase in S-phase cells (i.e., cells undergoing DNA replication) in the TE was detected. In addition, there were increased numbers of mitotic figures and intraepithelial neutrophils in the TE of the rats exposed for 3 days to 0.8 ppm. There was a marked decrease in S-phase cells in the 7 day, 0.8 ppm-exposed rats compared to the 3 day-exposed

animals. By 3 and 7 days post-exposure, there was no evidence of epithelial hyperplasia or increased cell proliferation (cells in the S-phase of the cell cycle) in the TE. Rats sacrificed 3 and 7 days after the end of the 7 day exposure to 0.8 ppm ozone had secretory cell metaplasia and increased amounts of intraepithelial mucosubstances in the TE.

Results from these studies demonstrated that the nasal TE of the rat, like that of the monkey, responds to short-term ozone exposure by increasing the number of epithelial cells/mm of basal lamina (hyperplasia), resulting in a thicker surface epithelial barrier. The hyperplastic response was reversed by 3 days post-exposure, but the TE exhibited secretory metaplasia with increased amounts of intraepithelial mucosubstances through 7 days after the end of the exposure. The increase in cell proliferation in response to ozone occurs early after the initiation of the exposure (3 days) when there was no histologic evidence of epithelial cell damage and prior to epithelial hyperplasia.

The differences in exposure concentrations and times make it difficult to compare the exposure-response relationships in the two species. However, the limited data suggest that in comparison to the nasal mucosa of the monkey, the nasal epithelium of the rat appeared to be less sensitive to ozone (lesions induced by high, but not by low concentrations). In addition, nasal epithelial damage to high concentrations of ozone was restricted to TE of the lateral wall and turbinates in rats (not along the septum), but was present in both TE and RE on the lateral wall and septum of the monkey.

Formaldehyde-induced alterations in nasal epithelial cell proliferation

Researchers at the Chemical Industry Institute of Toxicology have investigated the role of cell proliferation in formaldehyde-induced toxicity (Chang et al., 1983; Swenberg et al., 1986; Monticello et al., in press). Alterations in cell proliferation were shown to be a sensitive indicator of formaldehyde-induced toxic insult in the rodent, with the amount of cell proliferation following exposure to between 2 and 15 ppm formaldehyde varying greatly (Swenberg et al., 1987). This dose-responsive change in cell proliferation should be factored into the dose response for chemically-induced carcinogenesis studies (Swenberg et al., 1987), since cell proliferation is considered essential in chemical carcinogenesis.

Formaldehyde-induced cancer in the rat nasal epithelium may be directly related to these increases in cell proliferation, that presumably result from formaldehyde-induced cytotoxicity (Swenberg et al., 1983). Since formaldehyde is mutagenic and reacts preferentially with single-stranded DNA (von Hippel and Wong, 1971; Auerbach, 1977), studies of cell proliferation may have special relevance to the initiation of formaldehyde-induced nasal carcinogenesis. Increases in cell proliferation not only could enhance the likelihood of interaction of formaldehyde with DNA and the fixation of adducts before DNA repair could occur (Swenberg et al., 1983), but could also alter the clonal expansion of initiated cells to a large enough population that the chance of a second or third critical mutational event occurs (Swenberg et al., 1987; Farber and Sarma, 1987). Additional studies have been conducted or are currently in progress, in both the rat and nonhuman primate, to further investigate the role of cell proliferation in formaldehyde-induced toxicity. The results of these studies may be used for interspecies comparisons and improved risk estimation.

Rats exposed to 6 or 15 ppm formaldehyde, 6 hr/day, (Swenberg et al., 1986), or 5 ppm formaldehyde, 8 hr/day, (Wilmer et al., 1987), for 3 days, had a marked increase in cell proliferation, which is confined to the anterior portion of the nasal cavity. Monkeys exposed to 6 ppm formaldehyde for 5 days (6 hr/day), also had significant increases in cell proliferation (Monticello et al., in press), the greatest of which occurred in the anterior nasal cavity, similar to the rodent. Unlike the rodent, however, increases in cell proliferation were also present in the posterior regions of the nasal passages and in the nasopharynx and distal airways (i.e., trachea and carina). Another difference between these two species, was that cell proliferation rates in the monkey nasal mucosa remained elevated, immediately following six weeks of exposure, while in the rat cell proliferation rates returned to near control levels following 9 days of exposure to 6 ppm (Swenberg et al., 1986). Finally, no increase in cell proliferation was detected in the maxillary sinuses of the monkey, consistent with the absence of any histological lesions in this site (Monticello et al., in press).

Formaldehyde-induced nasal lesions were similar in the rat and monkey, characterized by epithelial degeneration, hyperplasia and varying degrees of squamous metaplasia. However, the distribution of the nasal lesions differed. Formaldehyde-induced lesions in the monkey nasal passages were more widespread, and extended to the nasopharynx (Monticello et al., in press); in the rodent, following a similar exposure time and concentration, lesions were confined to the anterior portion of the nasal cavity (Monticello and Morgan, unpublished observations). A good correlation did exist between the distribution of these lesions and areas of increased cell proliferation.

Discussion and summary

The nasal epithelium in the anterior aspect of the nasal cavities of rats and monkeys is sensitive to irritant exposure and responds to the insult by increasing the proliferative rate of surface epithelial cells along the airway lumen. Both irritants induce microscopic lesions and increases in cell proliferation. Lesions and proliferation increases are most severe in the anterior aspect of the nasal cavity, although less severe alterations are found in the more distal airways (i.e., nasopharynx, trachea, and/or bronchi). The nasal responses of the two toxicants differ, however, in the type of surface epithelial metaplasia they induce in the nasal airway. Ozone induces a predominantly secretory cell metaplasia, while formaldehyde induces squamous metaplasia.

It is interesting that significant species-specific differences in nasal epithelial responses have been identified for both ozone and formaldehyde. The monkey appears to be more sensitive for induction of proliferative lesions than the rat from exposures to either ozone or formaldehyde, based on severity or extent of lesions, and/or alterations in epithelial cell proliferation in the nasal airways. The reasons for the species differences are unknown, but may be due to differences in epithelial cell sensitivity, intranasal airflow patterns, thickness or biochemical nature of luminal mucus, and/or xenobiotic detoxification capabilities of the nasal epithelia. The latter three factors may be of significance in influencing the actual dose of toxicant reaching the various target cells and/or molecules.

Cell proliferation is an important mechanism of the nasal epithelium for repairing toxicant-induced injury and for altering the surface epithelium (i.e., increasing the epithelial thickness) to protect the

nasal mucosa from subsequent exposures. Unfortunately, the reparative response could potentially increase both the initiating and promoting events of nasal carcinogenesis. Therefore, cell proliferation could play a key role in the development of nasal tumors induced by inhaled irritants that are also carcinogens.

References

Auerbach, C., M. Moutschen-Dahmen, and J. Moutschen, 1977. Genetic and Cytogenetical Effects of Formaldehyde and Related Compounds. Mutation Research 39: 317-362.

Chang, J. C. F., E. A. Gross, J. A. Swenberg, and C. S. Barrow, 1983. Nasal Cavity Deposition, Histopathology and Cell Proliferation After Single or Repeated Formaldehyde Exposure in B6C3F$_1$ Mice and F344 Rats. Toxicology and Applied Pharmacology 68: 161-176.

Farber, E. and D. R. S. Sarma, 1987. Hepatocarcinogenesis: A Dynamic Cellular Perspective. Laboratory Investigation 56: 4-22.

Harkema, J. R., C. G. Plopper, D. M. Hyde, J. A. St. George, D. W. Wilson, and D. L. Dungworth, 1987a. Response of the Macaque Nasal Epithelium to Ambient Levels of Ozone: A Morphologic and Morphometric Study of the Transitional and Respiratory Epithelium. American Journal of Pathology 128: 29-44.

Harkema, J. R., C. G. Plopper, D. M. Hyde, D. W. Wilson, J. A. St. George, and V. J. Wong, 1987b. Nonolfactory Surface Epithelium of the Nasal Cavity of the Bonnet Monkey: A Morphologic and Morphometric Study of the Transitional and Respiratory Epithelium. American Journal of Anatomy 180: 266-279.

Harkema, J. R., J. A. Hotchkiss, and R. F. Henderson, submitted, 1988. Effects of 0.12 and 0.80 ppm Ozone on Rat Nasal and Nasopharyngeal Epithelial Mucosubstances: Quantitative Histochemistry. Toxicology and Applied Pharmacology.

Johnson, N. F., J. A. Hotchkiss, J. R. Harkema, and R. F. Henderson, submitted, 1988. Regional Differences in Cell Proliferation in Rat Nasal Epithelium Following Ozone Exposure. Toxicology and Applied Pharmacology.

Kerns, W. D., K. L. Pavkov, D. J. Donofrio, E. J. Gralla, and J. A. Swenberg, 1983. Carcinogenicity of Formaldehyde in Rats and Mice After Long-Term Inhalation Exposure. Cancer Research 43: 4382-4392.

Monticello, T. M., K. T. Morgan, J. I. Everitt, and J. A. Popp, in press. Effects of Formaldehyde Gas on the Respiratory Tract of Rhesus Monkeys: Pathology and Cell Proliferation. American Journal of Pathology.

Swenberg, J. A., E. A. Gross, J. Martin, and J. A. Popp, 1983. Mechanisms of Formaldehyde Toxicity. In: J. E. Gibson, ed., Formaldehyde Toxicity. Hemisphere Publishing Corporation, NY, pp. 137-147.

Swenberg, J. A., E. A. Gross, and H. W. Randall, 1986. Localization and Quantitation of Cell Proliferation Following Exposure to Nasal Irritants. In: C. S. Barrow, ed., Toxicology of the Nasal Passages. Hemisphere Publishing Corporation, NY, pp. 291-298.

Swenberg, J. A., F. C. Richardson, and Boucheron, J. A. et al., 1987. High- to Low-Dose Extrapolation: Critical Determinations Involved in the Dose Response of Carcinogenic Substances. Environmental Health Perspectives 76: 57-63.

von Hippel, P. H. and K. Y. Wong, 1971. Dynamic Aspects of Native DNA Structure: Kinetics of Formaldehyde Reaction with Calf Thymus DNA. Journal of Molecular Biology 61: 587-613.

Wilmer, J. W., R. A. Woutersen, L. M. Appelman, W. R. Leeman, and V. J. Feron, 1987. Subacute (4-Week) Inhalation Toxicity Study of Formaldehyde in Male Rats: 8-Hour Intermittent Versus 8-Hour Continuous Exposures. Journal of Applied Toxicology 7(1): 15-16.

Partially supported by the United States Department of Energy Office of Health and Environmental Research under Contract DE-AC04-76EV01013 and by National Institute of Health Grant ES04282.

HISTOCHEMISTRY AND BIOCHEMISTRY OF THE RAT NASAL MUCOSA

Matthew S. Bogdanffy

Haskell Laboratory for Toxicology and Industrial Medicine,
E. I. du Pont de Nemours and Co., Newark, Delaware, 19714, U.S.A.

Summary

The nasal mucosa is a highly metabolically active tissue. The distribution of enzyme activity between cells of the respiratory and olfactory mucosae has been shown, by enzyme histochemistry and immunohistochemistry, to be cell-specific. A variety of enzymes have been identified which may be useful as cellular markers and/or markers of cellular differentiation. In addition, several enzymes involved in xenobiotic biotransformation have been localized in specific cell types. The localization of these enzymes has aided the identification of cellular targets for metabolically activated chemicals, has shed light on their mechanisms of toxicity, and has highlighted the important role of the nasal mucosa as an extrahepatic site of biotransformation.

Introduction

A basic tenet of biology is that structure supports function. This is true particularly in the nasal cavity where, as described in the previous chapters, the anatomy of the nasal cavity is exquisitely suited for the functions of humidification, purification, and olfaction of inspired air. Physiological and biochemical functions taking place in the nasal mucosa contribute significantly to these processes. Mucus production and ciliary activity within the nasal mucosa are energy-demanding processes and as a result the nasal mucosa is a highly metabolically active and enzyme-rich tissue.

The cellular composition of the mucosa has been extensively studied (see previous chapters). Over twelve different types of cells can be found in the nasal epithelium and a variety of glands can be found in the lamina propria. Each of these have distinct metabolic functions and play different roles in xenobiotic biotransformation and chemical-induced nasal toxicity. Many enzymes have been demonstrated histochemically. Some of these have important roles in the mechanism of action of several nasal toxicants. Others have no known function as enzymes of metabolic activation or detoxication but may serve as useful markers of cell type and enzyme viability. This review of histochemically identified enzymes in nasal mucosa is divided into those that have value as enzyme/cell markers and those that also have implications for the mechanism of action of several xenobiotic nasal toxicants.

Cellular Markers

Recent application of methods for histochemical demonstration of common markers of cellular enzyme activity to nasal mucosa was made possible by the use of cold glycol methacrylate embedding technique (Randall et al., 1987). A summary of the distribution of these and other enzymes is presented in Table 1.

Ciliary movement is driven by energy released during hydrolysis of adenosine triphosphate. The enzyme responsible for catalyzing the hydrolysis is the magnesium-dependent adenosine triphosphatase (ATPase), a major protein in the sturctural component of cilia. The primary location of ATPase in nasal epithelium is the luminal plasma membrane and of cilia respiratory epithelial cells, sustentacular cells, and the ciliary processes of sensory cells. Neither the cell body of sensory cells nor nerve bundles in the lamina propria of olfactory mucosa demonstrate any appreciable reaction.

Acid phosphatase (AcP) and alkaline phosphatase (AlkP), nonspecific hydrolases of phosphate esters, are located in lysosomes and plasma membranes, respectively. Within the respiratory mucosa, AlkP and AcP are, in general, evenly distributed however AlkP is particularly evident in ciliated and goblet respiratory epithelial cells. Within olfactory mucosa, AcP reaction product appears strong in the cytoplasm of mature olfactory sensory cells. Acid phosphatase is weak in less mature sensory cells and basal cells. In contrast, AlkP is active in basal cells and clusters of mature sensory cells.

Glucose-6-phosphatase (G6P) is responsible for dephosphorylation of glucose-6-phosphate and liberation of free glucose into the blood or extracellular space. Glucose-6-phosphatase stains weakly throughout the nasal mucosa although strong in bones supporting the tissue.

γGlutamyl transpeptidase (γGT) is a membrane-bound enzyme involved in translocation of amino acids into the cell with concurrent transfer of the γglutamyl moiety of glutathione. γGlutamyl transpeptidase has more commonly been used as a marker of hepatocyte transformation in initiation-promotion studies of chemical carcinogenesis. In the nasal cavity γGT activity appears in most cell types of respiratory and olfactory mucosa with the exception of seromucous glands and olfactory neuronal cells. γGlutamyl transpeptidase activity is prominent in the luminal membrane surface of ducts of Bowman's glands and also in acinar cells of these glands which is interesting because it suggests a role of the cells in protein production, specifically glycoprotein biosynthesis.

Carbonic anhydrase (CA) has also been localized in nasal mucosa by histochemical techniques (Brown et al., 1984). Carbonic anhydrase, which catalyzes the hydration of carbon dioxide to form cabonic acid, stains positively in sensory cells of olfactory epithelium. Interestingly, two populations of sensory cells were revealed by this technique; one with intense cytoplasmic staining, and the other with a more prominent nuclear staining.

Xenobiotic Metabolizing Enzymes

Aldehyde dehydrogenase (AldH) is an important detoxication enzyme for many aldehydes. Acetaldehyde, a nasal carcinogen, is metabolized by nasal mucosal AldH (Casanova-Schmitz et al., 1984) approximately five times more readily in respiratory mucosa than olfactory mucosa suggesting that the former is better equipped to detoxify inhaled aldehydes. Interestingly, subchronic exposure to 400 ppm acetaldehyde has little to no effect on respiratory epithelium while causing severe degeneration of the olfactory neuronal cell layer. Biochemical studies alone cannot answer the question of which respiratory epithelial cell type(s) possess aldehyde dehydrogenase, however histochemical studies can provide the answer.

Histochemical studies have shown that acetaldehyde dehydrogenase is localized almost exclusively in epithelial cells of the respiratory mucosa with very little activity detectable in olfactory mucosa (Bogdanffy et al., 1986)(Figure 1a). Reaction product was polarized towards the luminal aspect of ciliated respiratory epithelial cells. The subcellular distribution of AldH activity in respiratory mucosa suggests the enzyme being detected is located in mitochondria which are known to be numerous within the luminal aspect of ciliated nasal cells (Monteiro-Riviere and Popp, 1984).

In the studies of AldH, acetaldehyde was used as the substrate. A related dehydrogenase, formaldehyde dehydrogenase (FdH), is distinct from AldH in its specificity for formaldehyde as a substrate and glutathione as a cofactor. Chronic exposure of rats to formaldehyde, in contrast to acetaldehyde, results in nasal tumors of the anterior nasal cavity. Recently, the histochemical distribution of FdH was studied in rat nasal mucosa (Keller et al., 1988). In contrast to AldH, FdH was visualized in both mucosae and particularly within neuronal cells of olfactory epithelium showing that AldH and FdH, two toxicologically-related but biochemically separate enzymes, have distinctly different distributions within nasal mucosa. Further, while the primary site of nasal injury due to acetaldehyde inhalation is olfactory mucosa, respiratory mucosa is the primary site of injury following formaldehyde exposure. Thus, the histochemical distribution of these detoxication enzymes correlates with regional resistance to inhaled aldehydes and may offer partial explanation for the distinct regional specificity of aldehyde-induced nasal lesions.

Nonspecific carboxylesterase (αnaphthylbutyrate esterase, NBE) also shows large differences in both distribution and quantitative activity between respiratory and olfactory mucosa (Bogdanffy et al., 1987). Within respiratory mucosa, the majority of activity appears in the epithelium with only moderate or weak activity in underlying seromucous glands (Figure 1b). Carboxylesterase activity is somewhat variable in cell types of the respiratory epithelium. A moderate amount of activity can be found in ciliated and cuboidal cells and less in basal cells. Only weak to negligable activity was detected in goblet cells and seromucous glands. In olfactory mucosa, sustentacular cells and acini of Bowman's glands constitute the majority of activity (Figure 1C). It is interesting to note that basal cells of the olfactory mucosa do not possess NBE activity suggesting that this enzyme does not appear until differentiation along the path of becoming sustentacular cells or ducts of Bowman's glands. Nonspecific esterase applied in the early phases of olfactory epithelial regeneration may provide some insight into the cellular origin of the regenerating tissue. The cell-specific staining may allow differentiated neuronal cells in regenerating olfactory epithelium to be distinguished from sustentactular cells and ducts of Bowman's glands based on the lack of NBE stain. Both basal cells and ducts of Bowman's glands have been suggested as progenitor cells for sensorsy cells and sustentacular cells (Matulionis, 1976; Uraih, et al., 1987; Hurtt et al., 1988).

Nasal mucosal esterases hydrolyze a wide variety of substrates (Stott & McKenna, 1985; Bogdanffy et al., 1987; Dahl et al., 1987; Patteson et al., 1988). Inhalation studies show that glycol ether acetates (Miller et al., 1984;), acrylate esters (Miller et al., 1985), and dibasic esters (Keenan et al., 1988) cause degeneration of the olfactory sensory cell layer. Since sensory cells do not possess NBE activity, the neighboring sustenctacular cells, which do hydrolyze esters, may be the site of hydrolysis of the inhaled esters. Accumulation of toxic acid metabolites

within sustentacular cells may lead to their death and the consequent death of the dependent sensory cells. A similar mechanism has been proposed for methyl bromide-induced lesions of olfactory epithelium (Thomas & Morgan, 1988). Alternatively, acid metabolites may be transported across tight junctions from sustenacular cells to neuonal cells.

Over the last five years nasal cytochrome P-450 has been shown to be involved in the biotransformation of many xenobiotic and endogenous substrates. Interest in this area has come primarily from experimental carcinogenesis studies with nitrosamines and other classes of chemicals such as ethylene dibromide and hexamethyl phosphoramide which produce nasal tumors following chronic oral or inhalation exposure.

The phenobarbital-inducible isozyme of cytochrome P-450 and NADPH cytochrome P-450 reductase has been localized by immunohistochemistry (Voigt et al., 1985; Baron et al., 1988). The β-naphthoflavone-inducible form of P-450, cytochrome P-450c, has been localized by immunohistochemistry in olfactory mucosa of rats by Foster et al. (1986). The pattern of enzyme distribution for both P-450 isozymes is similar to that for NBE and parallels the distribution of covalently bound radiolabelled compounds known to be metabolized to reactive products by cytochrome P-450.

In respiratory mucosa, most cell types in the epithelium and cells of the seromucous glands of the lamina propria stain positive for cytochrome P-450. In olfactory mucosa, the sustentacular cells and cells of Bowman's glands are also positive for cytochrome P-450. Similar to NBE, basal cells of the olfactory epithelium do not stain positive for P-450 which is interesting in that P-450 expression is often considered a marker of cellular differentiation. The distribution of benzo(α)pyrene hydroxylase (BαPOH), a cytochrome P-450-dependent monooxygenase catalyzing the hydroxylation of the polycyclic aromatic carcinogen benzo(α)pyrene, corresponds with that of P-450.

Two other enzymes related to metabolic activation and detoxication of chemicals, epoxide hydrolase (EH) and glutathione S-transferase (GSHt), have also been localized immunocytochemically (Baron et al., 1988). Epoxide hydrolase catalyzes the hydration of electrophilic epoxide moieties to vicinal diols in alkene and arene molecules. In many cases, the epoxide has been formed through cytochrome P-450-mediated monooxygenases. Hydrolysis of the epoxide by EH is, in most cases, a detoxication reaction, but in some cases may lead to substrates for further metabolic activation. Similarly, GSHt, which catalyzes the conjugation of glutathione with electrophilic centers facilitating water solubility and excretion may also form more toxic end products. Both EH and GSHt have patterns of distribution similar to the cytochrome P-450 enzymes. Therefore in the case of EH- or GSHt-mediated detoxication of chemicals activated by P-450, the nasal mucosa, with the exception of sensory cells, is afforded some degree of protection. In the case where EH and GSHt result in more biologically active metabolites, the nasal mucosa may be a target site for chemical-induced toxicity.

Conclusions

Histochemical study of nasal mucosa has revealed enzymes with the capacity for metabolic activation and detoxication of inhaled chemicals or chemicals reaching the nasal mucosa through the systemic circulation.

Techniques such as enzyme histochemistry and immunohistochemistry show that these biotransformation enzymes are not evenly distributed through the heterogeity of the mucosal cell population while quantitative biochemical studies have demonstrated high enzymatic activities in the mucosa. Biochemical and histochemical studies together can identify target cell populations and aid in the extrapolation of risk to the human nasal mucosa of chemical exposure. Future studies must include evaluation of human nasal tissue before adequate extrapolation can be made from the rodent model to humans.

References

Baron, J., J.P. Burke, F.P. Guengerich, W.B. Jakoby, & J.M. Voigt, 1988. Sites for xenobiotic activation and detoxication within the respiratory tract : Implications for chemically induced toxicity. Toxicol. Appl. Pharmacol. 93: 493-505.

Bogdanffy, M.S., H.W. Randall & K.T. Morgan, 1986. Histochemical Localization of aldehyde dehydrogenase in the respiratory tract of the Fischer-344 rat. Toxicol. Appl. Pharmacol. 82:560-567.

Bogdanffy, M.S., H.W. Randall, & K.T. Morgan, 1987. Biochemical quantitation and histochemical localization of carboxylesterase in the nasal passages of the Fischer-344 rat and B6C3F1 mouse. Toxicol. Appl. Pharmacol. 88:183-194.

Brown, D., L.-M. Garcia-Segura, & L. Orci, 1984. Carbonic anhydrase is present in olfactory receptor cells. Histochemistry 80:307-309.

Casanova-Schmitz, M., R.M. David, & H.d'A. Heck, 1984. Oxidation of formaldehyde and acetaldehyde by NAD^+-dependent dehydrogenases in rat nasal mucosal homogenates. Biochem. Pharmacol. 33:1137-1142.

Dahl, A.R., S.C. Miller, & J. Petridou-Fischer, 1987. Carboxylesterases in the respiratory tract of rabbits, rats, and syrian hamsters. Toxicol. Lett. 36:129-136.

Foster, J.R., C.R. Elcombe, A.R. Boobis, D.S. Davies, D. Sesardic, J. McQuade, R.T. Robson, C. Hayward, & E.A. Lock, 1986. Immunocytochemical localization of cytochrome P-450 in hepatic and extra-hepatic tissues of the rat with a monoclonal antibody against cytochrome P-450c. Biochem. Pharmacol. 35:4543-4554.

Hurtt, M.E., D.A. Thomas, P.K. Working, T.M. Monticello, & K.T. Morgan, 1988. Degeneration and regeneration of the olfactory epithelium following inhalation exposure to methyl bromide: Pathology, cell kinetics, and olfactory function. Toxicol. Appl. Pharmacol. 94:311-328.

Keenan, C.M., M.S. Bogdanffy, & D.P. Kelly, 1988. Subchronic inhalation study with dibasic esters (DBE) : Recovery of nasal lesions. Toxicologist 8:23.

Keller, D.A., H.d'A. Heck, H.W. Randall, & K.T. Morgan, 1988. Histochemical localization of formaldehyde dehydrogenase (FDH) activity in the rat. Toxicologist 8:20.

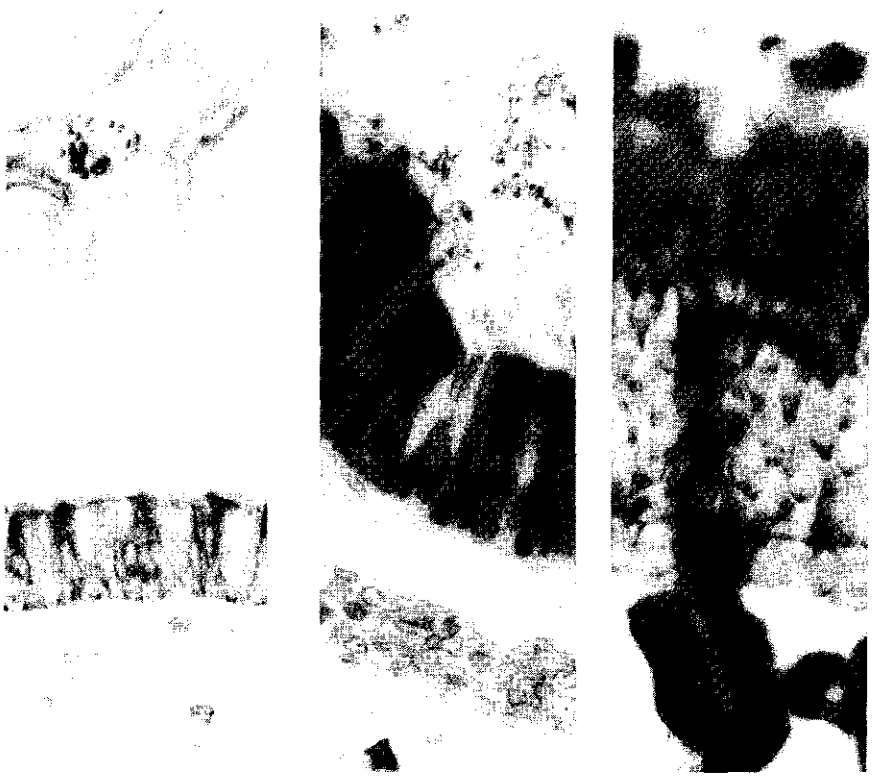
Matulionis, D.H., 1976. Ultrastructural study of the mouse olfactory epithelium following destruction by $ZnSO_4$ and its subsequent regeneration. Am. J. Anat. 142:67-90.

Miller, R.R., E.A. Hermann, J.T. Young, L.L. Calhoun, & P.E. Kartl, 1984. Propylene glycol monomethyl ether acetate (PGMEA) metabolism, disposition, and short-term vapor inhalation toxicity studies. Toxicol. Appl. Pharmacol. 75:521-530.

Miller, R.R., J.T. Young, R.J. Kociba, D.G. Keyes, K.M. Bodner, L.L. Calhoun, & J.A. Ayres, 1985. Chronic toxicity and oncogenicity bioassay of inhaled ethyl acrylate in Fischer-344 rats and B6C3F1 mice. Drug Chem. Toxicol. 8:1-42.

Patterson, C.A., C.R. Kee, & M.S. Bogdanffy, 1988. Kinetics of nasal

mucosal carboxylesterase-mediated hydrolysis of dibasic esters. Toxicologist 8:22.
Randall, H.W., M.S. Bogdanffy, & K.T. Morgan, 1987. Enzyme histochemistry of the rat nasal mucosa embedded in cold glycol methacrylate. Am. J. Anat. 179:10-17.
Stott, W.T. & M.J. McKenna (1985). Hydrolysis of several glycol ether acetates and acrylate esters by nasal mucosal carboxylesterase in vitro. Fundam. Appl. Toxicol. 5:399-404.
Thomas, D.A., & K.T. Morgan, 1988. Olfactory toxicity : Studies of methylbromide. CIIT Activities 8:1-7.
Uraih, L.C., F.A. Talley, K. Mitsumori, B.N. Gupta, J.R. Bucher, & G.A. Boorman, 1987. Ultrastructural changes in the nasal muscosa of Fischer-344 rats and B6C3F1 mice following an acute exposure to methylisocyanate. Environ. Health Persp. 72:77-88.
Voigt, J.M., F.P. Guengerich & J. Baron, 1985. Localization of a cytochrome P-450 isozyme (cytochrome P-450 PB-B) and NADPH-cytochrome P-450 reductase in rat nasal mucosa. Cancer Lett. 27:241-247.



(A) (B) (C)

Figure 1. Light micrograph of nasal mucosa stained for (A) aldehyde dehydrogenase, (B) and (C) carboxylesterase. (A) respiratory and olfactory mucosa, (B) respiratory mucosa, (C) olfactory mucosa. Magnifications are (A) 450X, (b) and (C) 570X.

TABLE 1
Qualitative Assessment of Enzyme Histochemical Stain Intensity in Regions of the Rat Nose[a]

Cell Type	ATP	AcP	AlkP	G6P	γGT	CA	AldH	FdH	NBE	P450 Red	P-450	EH	GSHt	BαPOH
Squamous epithelium	ND	+[b]	++	+	+	ND	+	ND	+	ND	ND	ND	ND	ND
Respiratory epithelium	+++	+	+	+	++	-	+++	√[b]	++	√	√	√	√	√
Seromucous glands	ND	ND	ND	ND	ND	ND	ND	√	+	√	√	√	√	√
Olfactory sustentacular cells	++	+	++	+	++	-	-	√	++	√	√	√	√	√
Olfactory Nerves	+	+++	++	+	-	√	-	√	-	-	-	-	-	-
Bowman's Glands	++	+	++	+	+++	√	+	√	+++	√	√	√	√	√

[a]Abrreviations are: ATP, adenosine triphosphatase; AcP, acid phosphatase; AlkP, alkaline phosphatase; G6P, glucose-6-phosphatase; γGT, γglutamyl transpeptidase; CA, carbonic anhydrase; AldH, acetaldehyde dehydrogenase; FdH, formaldehyde dehydrogenase; NBE, αnaphthyl butyrate esterase; P450 Red, NADPH cytochrome P-450 reductase; P-450, several inducible forms of cytochrome P-450 (see Voigt et al., 1985; Baron et al., 1988; Foster et al., 1986); EH, epoxide hydrolase; GSHt, several inducible forms of glutathione S-transferases (see Baron et al., 1988); BαPOH, benzo(α)pyrene hydroxlase.

[b]For studies in which qualitative evaluations were made of stain intensity, the scores are: - = no reaction; + = weak reaction; ++ = moderately strong reaction; +++ = strong reaction. For studies in which no qualitative evaluation was made, √ and - indicate enzyme present or absent, respectively.

Part 2

Neoplastic nasal changes after chronic exposure of rodents, primates and man to carcinogens

RODENT NASAL CARCINOGENS AND CLASSIFICATION OF NASAL TUMORS IN RODENTS

G. A. Boorman, R. H. Brown, L. C. Uraih

National Toxicology Program,(Boorman,Uraih) and Experimental Pathology Laboratories (Brown), Research Triangle Park, NC, USA

Summary

Spontaneous tumors of the nasal cavity are uncommon in rodents and occur in less than 0.5% of the F344 rats and B6C3F1 mice in two-year studies. Only 10 of nearly 350 chemicals tested in two-year studies in rodents by the National Cancer Institute/National Toxicology Program (NCI/NTP) were positive for tumors of the nasal passage. Spontaneous and induced tumors have been observed in squamous, and respiratory epithelium, while only induced tumors were found in the olfactory epithelium. Mesenchymal tumors while rare may also occur in the nasal passage and may be chemically induced.
Keywords: rat, mouse, nasal passage, cancer, carcinogens

Introduction

Recent emphasis on air pollutants and use of inhalation studies to identify potential carcinogens has resulted in increased interest in tumors of the nasal passages. With increasing use of inhalation as a route of exposure, the nasal passage is more commonly a target site in toxicity and carcinogenicity studies. The association of formaldehyde with squamous cell carcinomas in the nasal passage of rats generated further interest in nasal carcinogenesis, in addition to prompting research on the anatomy and function of the nasal passage in rodents.

Although earlier procedures only required that gross lesions of the nasal passage be trimmed in and evaluated, current standard procedures include examination of multiple levels of the nasal passage. In addition, characterization and classification and an understanding of the biology of the proliferative lesions in the nasal cavity is crucial to the interpretation of these studies.

The National Toxicology Program (NTP) has a data base of nearly 350 completed long-term toxicity and carcinogenicity studies, primarily using the B6C3F1 mouse and the F344 rat. We have recently reviewed the spontaneous and induced tumors of the nasal passage from the NTP data base. Some chemicals given systemically caused tumors at multiple sites including nasal passage; others caused liver or stomach tumors when given systemically but nasal tumors when chemical exposure was by inhalation.

Results and discussion

Spontaneous tumors of the nasal passage occur at less than 0.5% incidence in B6C3F1 mice and F344 rats (Haseman, et al., 1984). In a recent review of spontaneous tumors in rats from studies completed since 1976, there were six tumors (two adenomas of respiratory epithelium, 1 squamous cell papilloma, 3 squamous cell carcinomas) of the nasal cavity in 3,885 control males and no nasal cavity tumors in 3,933 control female F344 rats. There were no recorded spontaneous tumors of olfactory epithelium in either rats or mice in our data base.

Since the inception of the NTP/NCI testing program, over 350 long-term evaluations for the potential carcinogenicity of chemicals has been conducted in rats and mice. During this period only 10 chemicals caused tumors of the nasal passage (Table 1). All ten studies were positive in rats but only three chemicals caused tumors of the nasal passage in mice. In the early NCI studies, pathology was limited to selected organs and gross lesions and did not include a routine examination of the nasal passage. However, four of the first 203 chemicals were found to cause tumors of the nasal cavity of rats (Stinson, 1983). With procarbazine, the tumors were recognized by invasion of the olfactory bulb of the brain since the nasal cavity was not saved. Of the first four NCI positive studies (Table 1), all chemicals were given orally or parenterally and three (procarbazine, Tris (Aziridinyl) phosphine sulfide, and p-cresidine) caused tumors of the olfactory epithelium. The other chemical (1,4 Dioxane) caused squamous cell carcinomas and a few adenocarcinomas of the respiratory epithelium. All four chemicals were positive in other sites in rats and mice indicating multispecie and multisite carcinogenic potential. Three chemicals appear to have a predilection for the olfactory epithelium. Specific localization to the olfactory epithelium following systemic administration has been shown for 4-(Methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) (Belinsky, et al., 1987). When NNK is given intraperitoneally the promutagenic O^6-methylguanine concentration is highest in the nasal mucosa followed by the liver and the lung. By 4 days after treatment, necrosis of the olfactory epithelium is seen (Belinsky, et al., 1987). Others have shown that the related nitrosamine, N-nitrosodimethylamine, causes DNA methylation of the nasal mucosa at similar levels as NNK but produces fewer tumors, suggesting that DNA methylation alone may not account for the strong NNK tumorigenicity in the nasal mucosa (Hecht et al., 1986).

A second group of chemicals (Table 1) were given systemically but caused tumors of both the respiratory and olfactory epithelium. Additional information has lead to the conclusion, that in most cases, some chemical exposure to the nasal passage occurred by inhalation. This group includes 1,4-dioxane, 2,6 xylidine and dimethylvinyl chloride which were given in the drinking water, in the feed and by gavage respectively. The chemical 1,4-dioxane is volatile and a part of the chemical may have reached the nasal cavity by

exhalation. In the case of 2, 6 xylidine, the chemical sublimated off the feed and collected in the bottom of the polycarbonate shoe box style cage. The exposure route was judged to be in part by inhalation (NTP, In Press). Although dimethylvinyl chloride (1-chloro-2-methylpropene) was given by gavage, metabolism studies showed that 25-35% of the gavaged compound was exhaled as parent material thus effectively exposing the nasal mucosa via inhalation (NTP, 1986). These three chemicals caused a high incidence of tumors of the nasal cavity, 1,4-dioxane causing squamous cell carcinomas, dimethylvinyl chloride caused papillomas, adenocarcinomas, squamous cell carcinomas, undifferentiated carcinomas and rhabdomyosarcomas while 2,6 xylidine caused multiple tumor types in the nasal cavity including rhabdomyosarcomas. Two of these chemicals were tested in mice causing tumors at sites other than the nasal cavity. A test of 2, 6 xylidine in mice was not conducted.

The chemicals 1.2-dibromo-3-chloropropane and 1,2-dibromoethane given by inhalation were also carcinogenic for the nasal cavity and other sites in both rats and mice. However, the incidence of tumors was highest in the nasal passage generally 80% or higher for rats.

Table 1. Chemicals positive for nasal cavity tumors in NCI/NTP studies.

Chemical	Route	Rats/Mice	Tumor cell type
A. Systemic carcinogens			
Procarbazine	ip	R/M	Neuroepithelium
Tris (Aziridinyl)Phosphine sulfide	ip	R	Neuroepithelium
P-Cresidine	feed	R	Neuroepithelium
B. Systemic carcinogens with some inhalation exposure			
1,4 Dioxane	Water	R	Squamous Cell
2,6 Xylidine	feed	R	Multiple
Dimethylvinyl chloride	Gavage	R	Multiple
C. Inhalation multisite carcinogens			
1,2-Dibromo-3-chloropropane	inhal	R/M	Multiple
1,2-dibromo-ethane (Ethylene Dibromide)	inhal	R/M	Multiple (Rats) Resp. (Female mice)

D. Carcinogens acting primarily on the nasal cavity

Propylene Oxide inhal	R/M	Respiratory (Rats) Vascular (Mice)
1,2 Epoxybutane inhal	R	Respiratory

Multiple = tumors of respiratory and olfactory epithelium

The chemicals in the last group appear to act primarily on the nasal cavity. Both propylene oxide and 1,2 epoxybutane. had their effect limited to the nasal cavity and induced a very low incidence of tumors. In the case of propylene oxide only two of 50 male and three of 50 female rats exposed to the highest concentration had papillary adenomas of the nasal cavity. There was an increased incidence of hyperplasia and marked inflammation of the nasal cavity. In mice an increased incidence of vascular tumors of the nasal cavity was seen. With 1,2 -epoxybutane 7 of 50 male and 2 of 50 female rats exposed to the highest concentration had papillary adenomas of the nasal cavity. Nonneoplastic changes in the nose included severe inflammation, epithelial hyperplasia, erosions and squamous metaplasia (NTP, 1988). Five of the male rats from the high concentration dose group had neoplasms of the lung but there were no other tumor sites in the females and no carcinogenic effect in mice. These chemicals appear to be weak carcinogens with an effect seen only in the high dose group and limited generally to the respiratory epithelium.

In the NTP data base, it appears that chemicals causing tumors of the nasal cavity fall into four catagories; 1) chemicals given systemically that can localize in the nasal mucosa, often olfactory epithelium and cause neoplasms, 2) chemicals given systemically but where some inhalation exposure may have occurred and with tumors at multiple sites including nasal cavity 3) carcinogens given by inhalation that cause tumors of both olfactory and respiratory epithelium and 4) chemicals that are not carcinogenic at other sites but when given by inhalation will cause a low incidence of benign tumors of the respiratory epithelium. The first three groups of chemicals appear to be clearly carcinogenic since they usually cause tumors at multiple sites, usually both benign and malignant tumors of the nasal cavity are seen. It is interesting to note that when tumors are found in the nasal passage the incidence is quite high (may exceed 80%), higher than at other positive tumor sites. This would suggest that for some chemicals the nasal passage is a sensitive target tissue. On the other hand, nearly half of the 350 chemicals tested to date were considered positive and in only 10 cases was the nasal cavity a tumor target site. This indicates that for most studies and routes the nasal cavity is less sensitive to carcinogens than the liver which is the most frequent positive target site.

The potential hazard of the last two chemicals listed in Table 1 is more difficult to determine. In all cases the

number of tumors of the nasal passage was small, the respiratory epithelial tumors were benign, marked inflammatory and hyperplastic changes were present in the nasal passage and increased incidence of tumors was not found outside the respiratory system. These chemicals may be considered weakly carcinogenic but certainly pose less potential hazard than other chemicals listed in the table.

Evaluation of tumors of the nasal passage require accurate and uniform diagnostic terminology. Multiple levels of the nasal passage need to be examined to include all epithelial cell types. Tumors need to be characterized as to tissue or epithelial cell of origin, including squamous epithelium, respiratory epithelium, olfactory epithelium, (more recently a transitional epithelium has been recognized) or mesenchymal tissue. Other than tumors of the olfactory epithelium, tumor classification is similar to terminology and diagnostic criteria used for these epithelial and mesenchymal tissues in other sites. The classification also needs to be simple based on the differentiation of the tumor and the cell of origin. While multiple patterns exist for tumors arising in the olfactory epithelium, we prefer to use olfactory neuroblastoma for all tumors of the olfactory epithelium with or without the characteristic rosettes. A proposed classification for proliferative lesions of the nasal passage follows in Table 2.

Table 2. Proliferative Lesions of the Nasal Passage.

I. Squamous Epithelium
A. Squamous Hyperplasia
B. Squamous Cell Papilloma
C. Squamous Cell Carcinoma
II. Respiratory Epithelium
A. Hyperplasia
B. Adenoma
C. Adenocarcinoma
D. Keratinizing Squamous Metaplasia
E. Squamous Cell Carcinoma
III. Olfactory Epithelium
A. Hyperplasia
B. Olfactory neuroblastoma
IV. Mesenchymal Tissue
A. Fibroma, Fibrosarcoma
B. Schwannoma, Neurofibrosarcoma
C. Hemangioma, Hemangiosarcoma
D. Chondroma, Chondrosarcoma
E. Osteoma, Osteosarcoma
F. Rhabdomyosarcoma
G. Anaplastic Sarcoma
V. Nasal Lacrimal Duct
A. Squamous Cell Papilloma
B. Squamous Cell Carcinoma
VI. Glands (Steno's Glands, Bowman's Glands,etc)
A. Adenoma
B. Adenocarcinoma

References

Belinsky, S. A., M. E. Doolan, C. M. White, R. R. Maronpot, A. E. Pegg & M. W. Anderson, 1987. Molecular dosimetry of DNA adducts formation and cell toxicity in rat nasal mucosa following exposure to the tobacco specific nitrosamine 4-(Methylnitrosamino)-1-(3-pyridyl)-1-butanone and their relationship to induction of neoplasia. Cancer Res. 47:6058-6065.

Bioassay of 1,4-dioxane for possible carcinogenicity, NCI carcinogenesis Tech. Rep. Ser. No. 80, DHEW publ. No. (NIH) 78-1330, 1978; Bioassay of procarbazine for possible carcinogenicity, NCI carcinogenesis Tech. Rep. Ser. No. 19, DHEW publ. No. (NIH) 79-819, 1979; Bioassay of Thio-TEPA for possible carcinogenicity, NCI carcinogenesis Tech. Rep. Ser. No. 58, DHEW publ. No. (NIH) 78-1308, 1978; Bioassay of p-cresidine for possible carcinogenicity, NCI carcinogenesis Tech. Rep. Ser. No. 142, DHEW publ. No. (NIH) 79-1397, U.S. Government Printing Office, Washington, D. C., 1979

Haseman, J. K., D. D. Crawford, J. E. Huff, G. A. Boorman, & E. E. McConnell, 1984. Results from 86 two-year carcinogenicity studies conducted by the National Toxicology Program. J. Toxicol. Environ. Health 14:621-639.

Hecht, S. S., N. Trushin, A. Castonguay, & A. Rivenson, 1986. Comparative tumorigenicity and DNA methylation in F344 rats by 4-(Methylnitrosamino)-1-(3-pyridyl)-1-butanone and N-Nitrosodimethylamine. Cancer Res. 46:498-502.

National Toxicology Program (NTP),1982. Toxicology and Carcinogenesis studies of 1,2-dibromoethane in F344/N rats and B6C3F1 mice. NTP TR 210; NTP, 1982. Toxicology and Carcinogenesis studies of 1,2-dibromo-3-chloropropane in F344/N rats and B6C3F1 mice. NTP TR 206; NTP, 1985. Toxicology and Carcinogenesis studies of propylene oxide in F344/N rats and B6C3F1 mice. NTP TR 267; NTP, 1986. Toxicology and Carcinogenesis studies of dimethylvinyl chloride (1-chloro-2-methylpropene) in F344/N rats and B6C3F1 mice. NTP TR 316; NTP, 1988. Toxicology and Carcinogenesis studies of 1,2-epoxybutane in F344/N rats and B6C3F1 mice. NTP TR 329; NTP (in press). Toxicology and Carcinogenesis studies of 2,6 xylidine in F344/N rats and B6C3F1 mice. NTP TR 278, U. S. Department of Health and Human Services, Public Health Service, National Institutes of Health, Research Triangle Park, NC

Stinson, S. F., 1983. Nasal cavity cancer in laboratory animal bioassays of environmental compounds. In: G. Reznik & S. F. Stinson (Ed.): Nasal tumors in animals and man, Vol III, CRC Press, Boca Raton, Florida, p. 157-170.

NITROSAMINE-INDUCED NASAL CAVITY CARCINOGENESIS IN RODENTS

Hildegard M. Schüller

Experimental Oncology Laboratory, Department of Pathobiology, College of Veterinary Medicine, University of Tennessee, Knoxville, TN, U.S.A.

Summary

Numerous N-nitrosamines induce cancer in the nasal cavities of rodents. Most of such tumors arise in the respiratory and olfactory portion of the nose. Because of diagnostic difficulties, the histological classification and pathogenesis of these neoplasms is still a matter of debate. Serial sacrifice experiments in rats have provided evidence that the tumors in the respiratory portion originate from submucous glands while the neoplasms in the olfactory region arise from proliferating basal cells. In both areas, differentiation into neuroendocrine tumor cells was observed. Moreover, autoradiographic and biochemical studies in rats and hamsters injected with radiolabelled nitrosamines have shown that the nasal epithelia (mucous cells and sustentacular cells)) as well as cells in the submucous glands metabolize these compounds. However, the mechanisms responsible for the pronounced organotrophy of many nitrosamines for the nasal cavity are far from understood and will require extensive research in the future.

Introduction

Tumors of the nasal cavity are not among the most prominent cancers in man. Except for a few occupational and environmental hazards, epidemiology (Collan, 1983; Prasad, 1983) has generally failed to link cancers at this site to any particular class of chemical carcinogens. However, inhaled hazardous materials which would easily travel down into the lower respiratory organs of man are likely to be trapped and absorbed in the more complex and narrow nasal passages of rodents. Moreover, a large proportion of the rodent's nasal passages is coated by a respiratory epithelium identical to the one found in the large bronchi of man. With respect to aerodynamic considerations of inhaled materials as well as considerations on cell type specific responses to systemically administered chemical carcinogens, many chemicals which are typical lung carcinogens in man are therefore likely to induce nasal cavity cancer in rodents. N-nitrosamines constitute a class of chemical carcinogens believed to require metabolic activation in the host organism (Lijinsky, 1980; Dipple, 1988). While epidemiological evidence for their carcinogenic potential in man is sparse, there are numerous reports of their strong carcinogenic effects in a variety of rodent organ systems, including the nasal

cavity (Reznik-Schüller, 1983). One of the most prominent but least understood characteristics of nitrosamines is their pronounced organotrophy (Lijinsky, 1988). Although there are pronounced interspecies differences with respect to the organ affected and induced cancer type, a given nitrosamine usually induces cancer in the same organ of one species regardless of its route of administration. Accordingly, most nitrosamines which induce nasal cavity cancer in rodents have this effect after systemic administration and do not require direct deposition in the nasal passages by inhalation. Experiments conducted in hamsters (Reznik-Schüller, 1978, 1982), mice (Brittebo & Tjälve, 1983) and rats (Reznik-Schüller, 1983; Brittebo & Tjälve, 1983) have provided evidence that nitrosamines which are nasal cavity carcinogens are metabolized by epithelial cells of the nasal passages and submucous glands. Moreover, recent research conducted in this author's laboratory indicates that selective uptake of nitrosamines via specific cellular receptors may further add to the organ and cell type specific carcinogenicity of such compounds.

This review will focus on research data suited to add to our understanding of the mechanisms of nitrosamine-induced nasal cavity carcinogenesis in rodents. The reader interested in comprehensive information on rodent nasal cavity cancer induced by chemicals, including nitrosamines, is referred to a recent series of excellent reviews (Reznik & Stinson, 1983).

Morphology and Pathogenesis of Tumors

The pathological classification of rodent nasal cavity tumors is complicated by difficulties in diagnosing by light microscopy the cell types constituting nasal passage epithelia. Many of these cell types can only be diagnosed with certainty by electron microscopic and/or immunocytochemical techniques. Such diagnostic difficulties are reflected in the lack of an internationally applied tumor classification for this organ system as well as vague descriptions rather than precise tumor classifications in many published articles. This is an important factor to consider, when analyzing and comparing pathology data on rodent nasal cavity generated in different laboratories. Generally, the induced nasal cavity tumors can be subdivided into three categories: tumors arising in the atrioturbinates (coated by squamous epithelium), tumors arising in the respiratory portion (coated by respiratory epithelium), and tumors originating in the olfactory portion (coated by olfactory epithelium of the nose. The vast majority of nitrosamine-induced nasal cavity tumors are localized in the respiratory and olfactory portion of the nose while tumors arising from squamous epithelium in the apex of the nose are less frequent. This may well reflect the distribution of metabolically competent cells within the nasal epithelia with squamous cells in the nasal apex being poor in cytoplasmic enzyme systems while cells in

other parts of the nose (mucous cells, sustentacular cells, neuroendocrine cells, and cells of the submucous glands) have abundant enzyme systems involved in xenobiotic metabolism. Most nitrosamines induce tumors at several sites of the nose, although data in published articles are often lacking detailed anatomical information on the localization of the neoplasms. The tumor types inducible by nitrosamines are summarized in Table 1.

Table 1: Histological tumor types induced by nitrosamines in the rodent nasal cavity.

Anatomical site	Tumor types
Atrioturbinals (squamous epithelium)	Papillomas Squamous cell carcinomas
Respiratory portion (respiratory epithelium, submucous glands)	Adenomas Adenocarcinomas Papillary polyps Squamous cell carcinomas Neuroendocrine carcinomas
Olfactory portion (olfactory epithelium, submucous glands)	Adenomas Adenocarcinomas Papillary polyps Squamous cell carcinomas Neuroendocrine carcinomas Neuroblastomas Esthesioneuroepitheliomas (olfactory neuroepithelioma)

The pathogenesis of nitrosamine-induced nasal cavity tumors has been a matter of debate for many years (Herrold, 1969; Pour, et al., 1974; Reznik-Schüller, 1983). However, in only one of these studies was the sequential development of tumors actually investigated in a serial sacrifice type experiment (Reznik-Schüller, 1983). In that experiment, F344 rats were given N-nitrosomethylpiperazine (NMP) which induces a high incidence of adenocarcinomas and squamous cell carcinomas in the respiratory portion of the nose as well as a high incidence of olfactory neuroepitheliomas in the olfactory region (Lijinsky & Reuber, 1982). In the respiratory portion, the earliest detectable lesions were focal toxic degenera-

tions in acinar cells of the submucous glands. Subsequently, foci of atypical hyperplastic acinar cells developed while respiratory epithelia close to excretory ducts of submucous glands also showed focal hyperplasias. The lesions in the submucous glands progressed further into adenomas and adenocarcinomas while those within the respiratory epithelium itself did not. A substantial proportion of the tumor cells demonstrated ultrastructural features of neuroendocrine cells, some of which later underwent squamous metaplasia. These data suggest that NMP-induced adenocarcinomas and squamous cell carcinomas in the respiratory portion of the rat nasal cavity are derived from acinar cells in the submucous glands. On the other hand, the first detectable lesions in the olfactory region were hypertrophy of smooth endoplasmic reticulum in sustentacular cells of the olfactory epithelium along with toxic degeneration of acinar cells in submucous glands (Bowman's glands). Unlike the tumor development in the respiratory region, neoplasms in this part of he nose did not arise from submucous glands. In contrast, basal cells of the olfactory epithelium proliferated and differentiated into neuroendocrine cells which then gave rise to neuroendocrine carcinomas as assessed by electron microscopy. These tumors demonstrated formation of rosettes and pseudorosettes, one of the principal diagnostic criteria for histopathology diagnosis as olfactory neuroepithelioma. Unfortunately, the skulls had been decalcified with Decal, so that immunocytochemical stains for neuroendocrine markers could not be conducted. The classification of the NMP-induced tumors in the olfactory region as neuroendocrine neoplasms is therefore somewhat speculative and still awaits immunocytochemical verification.

Biochemistry

N-nitrosamines are believed to require metabolic activation in the host organism (Lijinsky, 1980). Such metabolism is believed to involve α-oxidation to form α-hydroxy nitrosamines as an initial step (Preussmann & Wiessler, 1987). This step in turn, is believed to be mediated by cytochrome P-450-dependent enzymes (Preussmann & Wiessler, 1987). However, experiments conducted in systems other than nasal cavity have provided evidence that monoamine oxidases (Lake et al., 1983) or prostaglandin synthetase enzymes (Schüller et al., 1987; Hegedus et al., 1987) may also participate in the initial phase of nitrosamine metabolism. Autoradiographic studies in Syrian golden hamsters injected with tritiated diethylnitrosamine (Reznik-Schüller, 1982) provided evidence that the compound may be metabolized by epithelial cells in the nasal passages. Interestingly, the cells with the heaviest radioactive labelling (submucous glands, mucous cells, sustentacular cells) were the same cell types which in the rat NMP study showed the earliest morphological lesions in the nasal cavity (Reznik-Schüller, 1983). Moreover, histochemical studies in the lungs have shown that mucous

cytochrome P-450 (Azzopardi & Thurlbeck, 1968). It hence appears, that the presence of enzyme systems capable of metabolizing nitrosamines is one important factor in determining which cell type(s) will develop early lesions after nitrosamine treatment. Epithelial cells of the nasal passages were confirmed as sites of nitrosamine metabolism by studies which used whole body autoradiography to monitor the tissue distribution of various nitrosamines in mice and rats (Brittebo & Tjälve, 1983). Moreover, in vitro experiments using $^{14}CO_2$-production or the formation of tissue-bound metabolites as endpoints further supported the interpretation that nasal epithelial cells metabolize nitrosamines (Brittebo & Tjälve, 1983). Most recently, it has been shown that the rat nasal mucosa demonstrates a pronounced accumulation and persistence of O^6 and 7-methylguanine after administration of N-methyl-N-benzylnitrosamine (Benthem et al., 1988). Although these data provide further evidence for the nasal mucosa as a site of nitrosamine metabolism they do not add in an understanding of the mechanisms of nitrosamine carcinogenesis because the nitrosamine under study is not a nasal carcinogen in the rat.

Conclusions

Although numerous articles have reported a carcinogenic effect of nitrosamines in the rodent nasal cavity, extremely little is known on the mechanisms of nitrosamine carcinogenesis in this organ. Only just a handful of experiments have made an effort to elucidate the mechanisms which lead to the development of nasal cavity tumors in rodents treated with nitrosamines. Pathogenesis and autoradiographic studies have identified mucous cells, cells in the submucous glands and possibly neuroendocrine cells as important targets and sites of nitrosamine metabolism in the rodent nasal cavity. However, what significance - if any - such metabolism and/or the formation and persistence of DNA-adducts may have for tumor initiation by nitrosamines will require extensive research in the future.

References

Azzopardi, A., Thurlbeck, W.M. 1968: Oxidative enzyme pattern of the bronchial glands. Am. Rev. Resp. Dis. 97:1038-1045.

Brittebo, E.B., Tjälve, H. 1983: Metabolism of N-nitrosamines by the nasal mucosa. In: Reznik, G., Stinson, S.F. (eds.): Nasal tumors in animals and man. CRC Press, Boca Raton, Vol. III, pp. 233-250.

Collin, Y. 1983: Epidemiologic and etiologic aspects of nasal carcinoma in Finland. In: Reznik, G., Stinson, S.F., (eds.): Nasal tumors in animals and man. CRC Press, Boca Raton, Vol. 1, pp. 103-110.

Dipple, A. 1988: Reactive metabolites of carcinogens and their interactions with DNA. In: Politzer, P., Martin, Jr., F.J. (eds.): Chemical carcinogens. Bioactive

molecules, Vol. 5, Elsevier (publ.) Amsterdam, pp. 32-62.
Hegedus, T.J., Falzon, M., Margaretten, N., Gazdar, A.F., Schüller, H.M. 1987: Inhibition of N-diethylnitrosamine metabolism in human lung cancer cell lines with features of well differentiated pulmonary endocrine cells. Biochem. Pharmacol. 36:3339-3343.
Herrold, K.M. 1967: Induction of olfactory neuroepithelium tumors in Syrian hamsters by diethylnitrosamine. Cancer 17:114-126.
Lake, B.G., Rowland, I.R., Harris, R.A., Collins, M.A., Phillips, J.C., Cottrell, R.C., Gangolli, S.D. 1983: The effects of substrates and inhibitors of monoamine oxidase on hepatic dimethylnitrosamine metabolism and mutagenicity. Adv. Exp. Med. Biol. 136:1143-1147.
Lijinsky, W. 1980: Significance of in vivo formation of N-nitroso compounds. Oncology 37:223-225.
Lijinsky, W. 1988: Nucleic acid alkylation by N-nitroso compounds related to organ specific carcinogenesis. In: Politzer, P. & Martin, F J., Jr. (eds.): Bioactive molecules Vol. 5: Chemical carcinogens. Elsevier, New York: 242-263.
Lijinsky, W., Reuber, M.D. 1982: Transnitrosation by nitrosamines in vivo. In: Bartsch, H., O'Neill, I.K., Castegnaro, M., Okuda, M. (eds.): Analysis and formation of N-nitroso compounds. IARC Sci. Publ. No. 41:625-631.
Pour, P., Cardesa, A., Althoff, J., Mohr, U. 1974: Tumorigenesis in the nasal olfactory region of Syrian golden hamsters as a result of Di-n-propylnitrosamine and related compounds. Cancer Res. 34:16-28.
Prasad, U. 1983: Nasopharyngeal carcinoma in man. In: Reznik, G., Stinson, S.F. (eds.): Nasal tumors in animals and man. CRC Press, Boca Raton, Vol. I, pp. 151-186,
Preussmann, R., Wiessler, M. 1987: The enigma of the organ specificity of carcinogenic nitrosamines. TIPS 8:185-189.
Reznik, G., Stinson, S.F. 1983: Nasal tumors in animals and man. Vols. 1-3, CRC Press, Boca Raton.
Reznik-Schüller, H. 1978: Ultrastructure of N-diethyl nitrosamine-induced tumours in the nasal olfactory region of the Syrian golden hamster. J. Pathol. 124:161-171.
Reznik-Schüller, H.M. 1982: Labelling in cells of the nasal cavity of hamsters treated with triticated N-nitrosodiethylamine. Cancer Lett. 16:109-114.
Reznik-Schüller, H.M. 1983: Pathogenesis of tumors induced with N-nitrosomethylpiperazine in the olfactory region of the rat nasal cavity. J. Natl. Cancer Inst. 71:165-172.
Reznik-Schüller, H.M. 1983: Nitrosamine-induced nasal cavity carcinogenesis. In: Reznik, G., Stinson, S.F. (eds.): Nasal tumors in animals and man. CRC Press, Boca Raton, Vol. III, pp. 47-78.
Schüller, H.M., Falzon, M., Gazdar, A.F., Hegedus, T.J. 1987: Cell type specific differences in metabolic

activation of N-nitrosodiethylamine by human lung cancer cell lines. In: Relevance of N-nitroso compounds for human cancer. Bartsch, H., O'Neill, I.K., Schulte-Hermann, R. (eds.): IARC Sci. Publ. No. 84; Oxford University Press (London), pp. 138-140.

vanBenthem, J., Vermeulen, E., den Engelse, L., Wilmer, J.W.G.M., Scherer, E. 1988: Organ-specific carcinogenesis by N-methyl-N-benzylnitrosamine does not correlate with formation, persistence or accumulation of O^6-methylguanine or 7-methylguanine in target and non-target tissues of the rat: A quantitative immunocytochemical study. In: Proc.: Conference on the advances in the biology and chemistry of N-nitroso and related compounds. Omaha, Nebraska, p. 24.

LOCALIZATION OF NASAL TUMOURS IN RATS EXPOSED TO ACETALDEHYDE OR FORMALDEHYDE

R.A. Woutersen and V.J. Feron

TNO-CIVO Toxicology and Nutrition Institute, Zeist, Netherlands

Abstract

Chronic exposure to acetaldehyde may lead to nasal squamous cell carcinomas and adenocarcinomas in rats (Woutersen et al., 1986). To determine a more precise localization of these tumours all slides of the nasal passages of the rats used in the carcinogenicity study with acetaldehyde were reexamined. The majority of the adenocarcinomas (77%) occurred in the endo- and ecto-turbinates region and the dorsomedial area of the nose at cross section V and VI (see Fig. 1). Most squamous cell carcinomas were seen to originate from the respiratory epithelium lining the naso- and maxilloturbinates and adjacent lateral wall, and the mid-ventral septum at cross section II, III and IV. Acetaldehyde-induced squamous cell carcinomas may originate more posterior in the nose than those induced by formaldehyde. The present findings demonstrate that the nasal tumours invariably originated from epithelium which had been severely damaged by the aldehydes tested.

Introduction

Short- and long-term inhalation studies in hamsters and rats demonstrated that acetaldehyde at concentrations of 750 ppm and higher is capable of inducing (a) inflammatory changes and severe hyper- and metaplasia of the epithelium in the upper parts of the respiratory tract, and (b) tumours of the laryngeal and nasal epithelium (Feron et al., 1982; Woutersen et al., 1986, 1987). Formaldehyde has been found to be a nasal carcinogen in Fischer-344 rats (Swenberg et al., 1980; Kerns et al., 1983) and in Cpb:WU Wistar rats (Feron et al., 1988; Woutersen et al., 1989), inducing squamous cell carcinomas and possibly polypoid adenomas. The more precise localization of nasal tumours associated with chronic exposure of F-344 rats to formaldehyde gas has recently been described by Morgan et al. (1986). In our studies, we found that low effective concentrations of acetaldehyde (400-1000 ppm) only affect the olfactory epithelium while low effective concentrations of formaldehyde (2-3 ppm) only damage the respiratory epithelium. However, the exact localization of the acetaldehyde-induced tumours was not further delineated. The present study was undertaken to determine more precisely the sites of origin of the neoplasms observed in the studies with acetaldehyde and to compare them with those seen in the formaldehyde studies.

Materials and methods

The material used for this study was derived from the same tissue sections and paraffin blocks of the previously reported chronic inhalation toxicity and carcinogenicity study of acetaldehyde in Cpb:WU Wistar rats (Woutersen et al., 1986). The findings with acetaldehyde have been compared with those seen in studies performed with formaldehyde (Morgan et al., 1986; Feron et al., 1988). Sections of the nasal passages (6 standard cross sections; Fig. 1) were examined for all rats reported to have a

nasal tumour. For each animal, the locations of the tumour(s) were recorded with the cross section and the most presumable site of origin in that cross level. The results of each case were assigned an accuracy rating as described by Morgan et al. (1986). It was difficult to determine the site of origin of large tumours which had extensively invaded the nose and in case of adenocarcinomas also frequently the brain. Such tumours received a low accuracy rating. For small neoplasms confined to a single section it was relatively easy te define the site of origin and such tumours received a high accuracy rating.

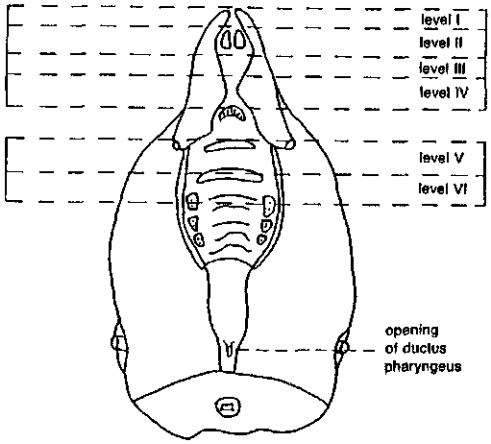


Fig. 1. Diagram of cross sections through the nose used for sectioning.

Results and discussion

Acetaldehyde

Adenocarcinomas

In the 3000/1000 ppm exposure group 40/101 (19/48 males and 21/53 females) had developed an adenocarcinoma. For the mid- and low-concentration groups these figures are: 55/105 (28/52 males and 27/53 females) and 20/96 (14/48 males and 6/48 females). For 66/115 (57%) adenocarcinomas, it was possible to establish the cross section where the tumour originated with a high accuracy rate (Table 1). The majority of the adenocarcinomas had developed from olfactory epithelium lining the ecto- and endoturbinates at standard cross section VI (48%, Table 1), whereas 18% most probably originated from olfactory epithelium at cross section V. Six adenocarcinomas most probably originated from cross section III and 13 from cross section IV. The adenocarcinomas found at cross section III seem to originate from glandular epithelium present in the submucosa beneath the respiratory epithelium. These tumours frequently grew endophytically along the structures of the nasal passages and invaded nerve bundles without exhibiting infiltrative growth into the lumen of the nasal cavity. Incidentally, it was clear that the origin of such a tumour was not the epithelium of the Bowman's glands, but that of the glands beneath the respiratory epithelium.

Table 1. Apparent sites of origin of adenocarcinomas induced in the rat nasal cavity by chronic inhalation exposure to acetaldehyde vapour.

Sex	Accuracy rating	Total	Total adenocarcinomas (%)[1] at cross section[2]						Unable to determine
			I	II	III	IV	V	VI	
Male	High	32	0	0	9	13	22	56	0
	Low	29	0	0	0	10	28	24	38
Female	High	34	0	0	9	9	12	70	0
	Low	20	0	0	0	15	10	30	45
Total		115	0	0	5	11	18	48	17

[1] rounded to nearest whole number
[2] see Figure 1

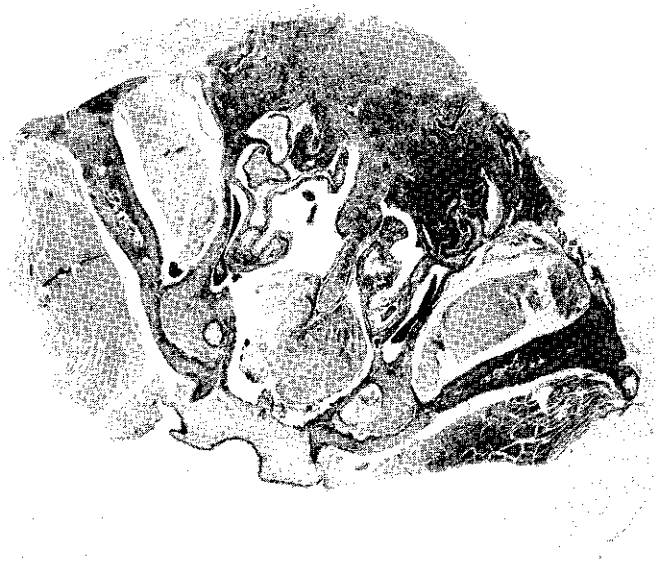


Fig. 2. Large nasal adenocarcinoma growing outside the nose and a small squamous cell carcinoma of the nasal septum; female rat exposed to 1500/3000 ppm acetaldehyde for 84 weeks. H&E, x10.

The adenocarcinomas consisted of compact sheets and cords of cells separated by strands of fibrous tissue widely varying in thickness. The tumour cells were pleomorphic and bizarre mitotic figures were frequently observed. The tumours often contained dark and light cells with big hyperchromatic round to oval nuclei. The dark cells were small with scanty cytoplasm, sharp nuclear membranes, fine nuclear chromatin pattern and small, distinct nucleoli. Several tumours exhibited rosettes, pseudorosettes, pallisading and glandular formations indicative of a neurogenic

origin. The neurogenic origin, however, should appear from unequivocal presence of neurotubules, neurosecretory granules, or neuritic processes. From electron microscopical studies performed on acetaldehyde-induced adenocarcinomas, it appeared that the cytoplasm of the tumour cells contained small mitochondria, a few strands of rough endoplasmic reticulum, and many free polyribosomes but no characteristic structures. Neurofibrils could not be identified. In view of their localization, prevailing histologic pattern and cytology, the nasal adenocarcinomas are considered to be derived from the olfactory stem or sustentacular cells (including the basal cells of the glands of Bowman) and might be classified as nasal neuroepitheliomas subdivided in several subtypes based on the prevailing type of tumour cells (Feron et al., 1989).

Squamous cell carcinomas

In the 3000/1000 ppm group, 40/101 (17/48 males and 23/53 females) had developed a squamous cell carcinoma. For the mid- and low-concentration groups these figures are 18/105 (10/52 males and 8/53 females) and 2/96 (2/48 males and 0/48 females). It was possible to establish the origin of 47/60 of these tumours with a high accuracy rate (Table 2).

Table 2. Apparent sites of origin of squamous cell carcinomas in the rat nasal cavity by chronic inhalation exposure to acetaldehyde vapour.

Sex	Accuracy rating	Total	Total adenocarcinomas (%)[1] at cross section[2]						Unable to determine
			I	II	III	IV	V	VI	
Male	High	20	0	10	45	35	5	5	0
	Low	9	0	22	0	45	11	0	22
Female	High	27	7	22	33	26	7	4	0
	Low	4	0	25	0	0	0	0	75
Total		60	3	15	35	32	7	3	8

[1] rounded to nearest whole number
[2] see Figure 1

Most of the squamous cell carcinomas were seen to originate from metaplastic keratinized stratified squamous respiratory epithelium lining the naso- and maxilloturbinates with the adjacent lateral wall or the mid-ventral part of the septum at cross sections III (35%) or IV (32%). About 10% of the squamous cell carcinomas appeared to originate from the epithelium at cross sections V or VI. One of these tumours developed from the squamous epithelium lining the most ventral part of the nose at cross section V (Fig. 2). All other squamous cell carcinomas located posterior in the nose appeared to be derived from metaplastic keratinized squamous olfactory epithelium.
Eight carcinomas-in-situ have been included in the total number of squamous cell carcinomas. These early carcinomas were only seen in female rats (3 in the mid- and 5 in the top-concentration group). Four of these CIS were located at cross section II; 3 at cross section III and 1 at cross section IV.
One small papilloma was seen in a female low-concentration rat. The tumour originated from the respiratory epithelium lining the maxillary turbinate at cross section III.

Fifteen out of 302 rats developed both an adenocarcinoma as well as a squamous cell carcinoma (Fig. 2). This was seen in 13 top-concentration rats (7 males and 6 females) and in 2 animals (one male and one female) of the mid-concentration group.

Formaldehyde

The squamous cell carcinomas seen in our studies with formaldehyde (Feron et al., 1988 and Woutersen et al., 1989) were seen to originate from the same region in the nasal cavity as previously described by Morgan et al. (1986). They reported that most squamous cell neoplasms occurred on the lateral side of the nasoturbinate and adjacent lateral wall at cross section II. Other tumours were located on the mid-ventral nasal septum (cross section II and III) and on the dorsal septum and the roof of the dorsal meatus (cross level II and III). Only a small number was found on the maxilloturbinate at cross section II and III.
A total of 3 compound-related polypoid adenomas has been observed in our studies with formaldehyde viz. 2 in male rats exposed to 20 ppm formaldehyde for 4 or 8 weeks, respectively, followed by an observation period of over 2 years (Feron et al., 1987, 1988), and one in a male rat exposed to 10 ppm formaldehyde for 3 months followed by an observation period of 25 months (Feron et al., 1988). One of the formaldehyde-related tumours had derived from the epithelium lining the maxilloturbinate and the adjacent lateral wall; it was an exophytic sessile tumour protruding into the nasal cavity and remaining free in the lumen. The other formaldehyde-induced tumour was a small pedunculated adenoma originating from the lateral wall at the level of the nasoturbinate (cross section III).
In addition to solid sheets of epithelial cells and microcysts it contained one large cystic structure partially lined by keratinized stratified squamous epithelium. The third polypoid adenoma was a small pedunculated adenoma derived from the ventral margin of the nasoturbinate (Fig. 3). In view of their localization, histology and cytology, there seems to be little doubt that polypoid adenomas originate from respiratory epithelium.

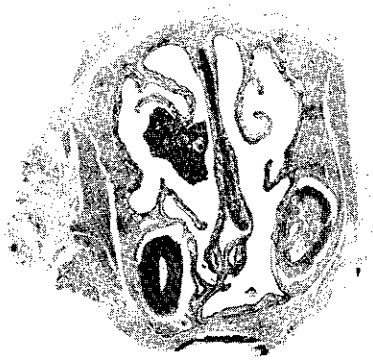


Fig. 3. Polypoid adenoma originating from the ventral margin of the nasoturbinate at cross section III; male animal exposed to 10 ppm formaldehyde for 13 weeks and killed after a recovery period of over 2 years. H&E, x10.

Concluding remarks

- Localization of the formaldehyde-induced squamous cell carcinomas and polypoid adenomas was comparable in the studies evaluated.
- Acetaldehyde-induced squamous cell carcinomas may originate more posterior in the nose than those induced by formaldehyde.
- Acetaldehyde-induced squamous cell carcinomas may be derived from metaplastic keratinized squamous olfactory epithelium.
- Acetaldehyde as well as formaldehyde have been found to induce nasal carcinomas at exposure concentrations which also cause much damage to the nasal mucosa followed by regenerative hyperplasia and metaplasia.
- The cytotoxic effects of these aldehydes may be very important for the induction of nasal carcinomas.

References

Feron, V.J., A. Kruysse & R.A. Woutersen, 1982. Respiratory tract tumours in hamsters exposed to acetaldehyde vapour alone or simultaneously to benzo(α)pyrene or diethylnitrosamine. Eur. J. Cancer Clin. Oncol. 18:13-31.

Feron, V.J., H.R. Immel, J.W.G.M. Wilmer, R.A. Woutersen & A. Zwart, 1987. Nasal tumours in rats after severe injury to the nasal mucosa and exposure to formaldehyde vapour: preliminary results. In: The role of Formaldehyde in Biological Systems (E. Tyiak and G. Gullner, Eds.), Sote Press, Budapest, p. 73-77.

Feron, V.J., J.P. Bruijntjes, R.A. Woutersen, H.R. Immel & L.M. Appelman, 1988. Nasal tumours in rats after short-term exposure to a cytotoxic concentration of formaldehyde. Cancer Lett. 39:101-111.

Feron, V.J., R.A. Woutersen, A. van Garderen-Hoetmer & H.C. Dreef-van der Meulen. Upper Respiratory Tract Tumours in Cpb:WU (Wistar random) rats. Environm. Health Perspectives, In press.

Kerns, W.D., K.L. Pavkov, D.J. Donofrio, E.J. Gralla & J.A. Swenberg, 1983. Carcinogenicity of formaldehyde in rats and mice after long-term inhalation exposure. Cancer Res. 43:4382-4392.

Morgan, K.T., X-Z Jiang, J.B. Starr & W.D. Kerns, 1986. More precise localization of nasal tumours associated with chronic exposure of F-344 rats to formaldehyde gas. Toxicol. Appl. Pharmacol. 82:264-271.

Swenberg, J.A., W.D. Kerns, R.I. Mitchell, E.J. Gralla & K.L. Pavkov, 1980. Induction of squamous cell carcinomas of the rat nasal cavity by inhalation exposure to formaldehyde vapour. Cancer Res. 40:3398-3402.

Woutersen, R.A., L.M. Appelman, V.J. Feron & C.A. van der Heijden, 1984. Inhalation toxicity of acetaldehyde in rats. II. Carcinogenicity study: interim results after 15 months. Toxicology 31:123-133.

Woutersen, R.A., L.M. Appelman, A. van Garderen-Hoetmer & V.J. Feron, 1986. Inhalation toxicity of acetaldehyde in rats. III. Carcinogenicity study. Toxicology 41:213-231.

Woutersen, R.A. & V.J. Feron, 1987. Inhalation toxicity of acetaldehyde in rats. IV. Progression and regression of nasal lesions after discontinuation of exposure. Toxicology 47:295-305.

Woutersen, R.A., A. van Garderen-Hoetmer, J.P. Bruijntjes, A. Zwart & V.J. Feron, 1989. Nasal tumours in rats after severe injury to the nasal mucosa and prolonged exposure to 10 ppm formaldehyde. J. Appl. Toxicol., In press.

ROLE OF TISSUE DAMAGE IN NASAL CARCINOGENESIS

V.J. Feron and R.A. Woutersen

TNO-CIVO Toxicology and Nutrition Institute, Zeist, The Netherlands

Summary

Examples are given to substantiate that cancer often develops in chronically injured tissue both in man and experimental animals. Cytotoxic effects and epithelial hyperproliferation appear to play an almost essential role in the development of nasal tumours in rats exposed to formaldehyde. Consequently, exposure of humans to non-cytotoxic levels of formaldehyde is considered to represent a negligible cancer risk. In rats nasal squamous cell carcinoma may be part of the malocclusion syndrome. It was hypothesized that wood dust concentrations not leading to irritation of the nasal mucosa are low enough to avoid substantial risk of nasal cancer. Tissue- and compound-specific factors are discussed to determine the role of tissue injury and hyperproliferation in (nasal) carcinogenesis.
Keywords: nose, tissue damage, hyperproliferation, malocclusion syndrome, formaldehyde, wood dust, ethyl acrylate.

Introduction

There is substantial evidence that cancer often develops in chronically injured tissue: e.g. colon cancer is frequently seen in patients with chronic colitis (Laroye, 1974); skin cancer may occur in burn scars (Berenblum, 1944); and many lung tumours grow in areas of scarring (Bennett, Sasser & Ferguson, 1969). Known promoting factors are poor dental hygiene for mouth cancer, vocal abuse for laryngeal cancer, and chronic sinusitis for cancer of the maxillary sinus (Sato, 1987). A large body of animal data suggests that chronic tissue injury induced by chemical or physical agents could be a major factor in tumour development in connective and epithelial tissues (Grasso, 1987): formation of subcutaneous sarcomas at the site of repeated injection of substances such as common salt, distilled water or glucose, "solid state" carcinogenesis, and bladder cancer in rodents following foreign body implants.

Malignant tumours induced in the nasal epithelium by irritating carcinogens such as acetaldehyde (Woutersen et al., 1986) and formaldehyde (Kerns et al., 1983) have been found to arise only from epithelium which is severely damaged. Moreover, irritation of the nasal mucosa leading to epithelial hyperplasia, metaplasia and dysplasia are considered etiological factors in the development of nasal cancer in workers exposed to wood dust (Mohtashamipur & Norpoth, 1983; Boysen, 1985). Therefore, it was deemed desirable to analyze data on occurrence and induction of nasal tumours in experimental animals with the aim to improve the insight into the role of tissue damage, followed by regenerative hyperproliferation, in nasal carcinogenesis. This analysis is described in the present paper.

Irritating nasal carcinogens in rodents

Formaldehyde

Formaldehyde is a highly irritating, genotoxic carcinogen capable of inducing malignant tumours in the nose of rats after long-term inhalation exposure (Kerns et al., 1983). The nasal toxicity of formaldehyde is characterized by inhibition of mucociliary function (Morgan, Gross & Patterson, 1986), reaction with albumin and other small proteins present in nasal mucus (Bogdanffy et al., 1987), reaction with glutathione followed by detoxification by formaldehyde dehydrogenase (Heck & Casanova-Schmitz, 1984), and, when biotransformation is overwhelmed or even inactivated, rhinitis, degeneration and necrosis followed by regenerative hyperplasia and metaplasia of the respiratory epithelium (Swenberg et al., 1983). These nasal effects have been found in rats exposed to concentrations ranging from 2 to 20 ppm, the "no-cytotoxic-effect-level" being 1 ppm (Woutersen et al., 1987; Appelman et al., 1988).

As far as the genotoxicity is concerned, formaldehyde appears to be a direct-acting mutagen which induces DNA-protein cross-links in nasal respiratory epithelium following inhalation exposure ("local genotoxicity") (Ma & Harris, 1988). A series of studies has clearly demonstrated a strong deviation from linearity of the formation of DNA-protein cross-links in the nasal epithelium of rats (Heck & Casanova, 1987; Casanova & Heck, 1987). One of the reasons for this non-linearity is inactivation of formaldehyde by glutathione, which apparently is much more effective at low than at high formaldehyde concentrations.

A high incidence of nasal carcinomas in rats (about 40%) occurred at exposure concentrations of about 15 ppm (Kerns et al., 1983). In a recent 28-month inhalation study in Wistar rats, no formaldehyde related nasal tumours were observed at 10 ppm (Feron et al., 1987; Woutersen et al., 1989). These tumour data and the aforementioned toxicity data clearly demonstrate that exposure levels causing nasal tumours also cause rhinitis, necrosis and hyper- and metaplasia of the nasal mucosa. Moreover, in a study on the more precise localization of the formaldehyde-induced nasal tumours in rats, Morgan et al (1986) showed that humans invariably occurred at locations of mucociliary inhibition, hyperplasia and metaplasia. The dose-response curve for nasal tumours is very steep and extremely non-linear, while its shape appears to correspond with that of the dose-response curves for DNA-protein cross-links, inhibition of the mucociliary function, increased cell proliferation, and hyper- and metaplasia of the nasal epithelium. Obviously, an association exists between the cytotoxic and carcinogenic effects. In other words, the steep, non-linear dose-response curve for nasal tumours - indicating a disproportionate decrease in cancer risk at low concentrations - is most probably due to the fact that defence mechanisms of the nose (mucociliary clearance, detoxification by dehydrogenase, DNA-repair) are very effective at low concentrations, but can be overwhelmed and inactivated at high concentrations; consequently, cell and tissue damage and finally tumours occur at high concentrations.

These data and considerations suggest that the induction of nasal carcinomas in rats by formaldehyde requires repeated exposure to levels that cause considerable damage to the nasal epithelium followed by regenerative hyperplasia and metaplasia. This increased cell replication and subsequent cycles of DNA-damage, provoked by long-term repeated exposure to formaldehyde, may strongly enhance the likelihood of relevant

DNA-damage, and, moreover, may strongly enhance the progression of initiated/preneoplastic cells to cancer. This also means that formaldehyde in concentrations not leading to cell damage most probably cannot act as a complete carcinogen (causing initiation, promotion and progression), and as a result is very unlikely to induce cancer by itself. Therefore, it is concluded that cytotoxic effects of formaldehyde play a highly significant role, perhaps a role close to an essential one, in the formation of nasal tumours by formaldehyde.

This conclusion is strongly supported by the results of a recently completed long-term inhalation study, in which male Wistar rats with a severely damaged or an undamaged nasal mucosa were exposed to 0, 0.1, 1.0 or 10 ppm formaldehyde vapour for 6 hours/day, 5 days/week, during either 28 months or 3 months followed by a non-exposure, observation period of 25 months (Woutersen et al., 1989). The damage of the nasal mucosa was induced by bilateral intranasal electrocoagulation.

Table 1. Incidence of nasal tumours in rats with a damaged or undamaged nasal mucosa exposed to formaldehyde vapour for 3 or 28 months.

Formaldehyde concentration (ppm)	Exposure period and no. of rats with a damaged or undamaged nose bearing a nasal tumour/no. of rats examined			
	3 months *		28 months	
	Damaged	Undamaged	Damaged	Undamaged
0	0/57	0/26	1/54	0/26
0.1	2/57	0/30	1/58	1/26
1.0	2/53	0/29	0/56	1/28
10	2/54	2/26	17/58	1/26

* Followed by a 25-month non-exposure, observation period

The incidence of nasal tumours in the various groups is presented in Table 1, which shows that treatment-related nasal tumours only occurred in the 10 ppm group of rats with a damaged nose and exposed to formaldehyde for the full period of 28 months. Obviously, severe damage to the nasal mucosa in combination with prolonged exposure to a relatively high cytotoxic concentration of formaldehyde leads to tumour formation. Ten ppm induced extensive and severe hyperplasia and metaplasia in the intact nasal mucosa, but no tumours. Clearly, for tumour formation "drastic" conditions are required: severe damage plus a relatively high concentration (dose).

Acetaldehyde

Acetaldehyde is, just like formaldehyde, a highly irritating genotoxic nasal carcinogen in rats. At very high concentrations (1500 to 3000 ppm) acetaldehyde induced malignant tumours of both the respiratory and the olfactory nasal epithelium, while at lower concentrations (e.g. 750 ppm) only carcinomas of the olfactory epithelium were found (Woutersen et al., 1986). Comparable to the occurrence of nasal tumours after inhalation of formaldehyde, also with acetaldehyde nasal tumours were seen to arise from epithelium which had been damaged by acetaldehyde indicating a significant

role of the cytotoxicity of acetaldehyde in its carcinogenicity. However, the lowest exposure concentration of acetaldehyde tested in a long-term rat study (750 ppm) induced both cytotoxic and neoplastic changes, which means that it cannot be excluded that non-cytotoxic levels are capable of inducing nasal tumours in rats. Consequently, for the time being, the role of tissue damage in acetaldehyde carcinogenesis is less convincing than in formaldehyde carcinogenesis.

Cytotoxic nasal non-carcinogens in rodents

Acrolein, a very irritating unsaturated aldehyde, induced rhinitis, and hyperplasia of the nasal respiratory and olfactory epithelium in Syrian hamsters (Feron & Kruysse, 1977); after discontinuation of the exposure, the nasal mucosa partially recovered in most animals. Chronic exposure of Syrian hamsters to furfural, a heterocyclic aldehyde, led to highly characteristic alterations of the olfactory epithelium viz. atrophy, cystic, glandlike structures and accumulations of sensory cells in the lamina propria, and karyo- and cytomegaly of Bowman's glands (Feron & Kruysse, 1978). Dimethylamine is very irritant to the skin, eyes, and the respiratory tract. At concentrations ranging from 175 to 500 ppm, it induces ulcerative rhinitis and metaplasia of the nasal epithelium in rats. Exposure of rats to dimethylamine at concentrations of 0, 10, 50 and 175 ppm for 12 months resulted in concentration-dependent lesions of the nasal mucosa. The most severe lesions occurred in the olfactory mucosa with destruction of sensory cells and nerve bundles. No nasal tumours occurred (Buckley et al., 1985). Ethyl acrylate has been found to cause ulceration, hyperplasia and hyperkeratosis and also papillomas and squamous cell carcinomas in the forestomach of rats and mice that were given doses of 100 and 200 mg/kg body weight/day by gavage on 5 days/week for two years (Maronpot, 1983). In a 2 year inhalation study with ethyl acrylate in rats, using exposure levels ranging from 25 to 225 ppm, ethyl acrylate was not carcinogenic (Miller et al., 1985). However, treatment-related effects were found in the nasal olfactory mucosa; the changes consisted of atrophy of sensory cells, basal cell hyperplasia and an increase in intra-epithelial glandular elements. There is limited evidence for genotoxic potential of ethyl acrylate, but its toxicological relevance in mammals is very doubtful (Ashby, 1986). Ethyl acrylate is very rapidly hydrolized by carboxylesterase to acrylic acid and ethanol at the site of original contact (Miller et al., 1981). Ethyl acrylate that is not hydrolized is detoxified by binding to non-protein sulphydryls (DeBethizy et al., 1987). The results of biochemical and histochemical carboxylesterase activity and glutathione binding studies in the forestomach and nasal epithelium (Miller et al., 1981; Ghanayem, Maronpot & Matthews, 1985; Bogdanffy, Randall & Morgan, 1987), and inhalation toxicity studies with acrylic acid in rats (Miller et al., 1981), seem to allow the conclusion that acrylic acid or acrylic acid plus ethyl acrylate are responsible for the nasal effects seen after exposure to ethyl acrylate, while ethyl acrylate itself is the major ultimate toxic agent for the forestomach. Apparently, differences in response between the forestomach and the nose may be ascribed to variations in enzyme activities and specificities, to differences in the nature and binding capacities of thiols, or to other factors which may contribute to tissue-specific sensitivities (J.J. Clary, I.C. Munro and J. Orr, personal communication, 1988). Also the presence of tumours in the forestomach and the absence of nasal tumours may be attributed to tissue-specific differences, despite the presence of hyperproliferative changes in both organs.

Wood dust

Epidemiological studies have shown a clear association between carcinoma of the nasal passages and paranasal structures and occupational exposure to wood dust (Scheidt, Erhardt & Bartsch, 1987). Reduction in mucociliary transport, and metaplasia with dysplasia of the nasal epithelium have been reported in wood workers (Wilhelmsson et al., 1985; Boyson, Voss & Solberg, 1986). Epithelial metaplasia has been regarded as a possible precursor to nasal carcinoma (Boysen, 1985). Chemicals introduced to wood or wood's natural constituents, and also mechanical irritation of the nasal mucosa by wood dust particles have been suggested as causative or enhancing agents (Mohtashamipur & Norpoth, 1983; Boysen, 1985). Maybe tissue damage followed by hyperproliferative changes is also a key factor in wood dust carcinogenesis.

Nasal squamous cell carcinoma in rats part of the malocclusion syndrome?

Seven out of 661 untreated male control rats (Cpb:WU; Wistar random strain) have been found to bear a nasal tumour, which in all cases appeared to be a squamous cell carcinoma (Feron et al., 1989). In about 10% of the rats of this strain "overgrowth" of an incisor tooth and malocclusion occurs; frequently this condition is accompanied by odontitis, periodontitis, maxillary sinusitis and rhinitis. In three of the seven untreated controls with a nasal squamous cell carcinoma, the tumour was seen to be associated with severe necrotizing (peri)odontitis and rhinitis indicating that this condition of chronic irritation is involved in the formation of a proportion of the nasal tumours in the Cpb:WU strain of rats. Maybe in rats nasal squamous cell carcinomas, in some instances, could be part of the malocclusion syndrome.

Discussion

Undoubtedly, chronic tissue damage, often accompanied by hyperproliferation, may play a role in the formation of cancer both in humans and experimental animals. Though many factors may be involved (deposition, clearance, metabolism, DNA-repair), lasting hyperproliferation as such seems to be a key factor. Therefore, in humans repeated and chronic tissue injury should be avoided where possible. In experimental carcinogenesis one should be aware of the impact tissue damage and hyperproliferation may have on the different steps of the process of carcinogenesis. Moreover, the occurrence of tissue damage and hyperproliferation should be taken into account in interpreting the results of carcinogenicity studies, particularly in predicting low-dose effects from high-dose findings, and in estimating human health risk.

As far as the nose is concerned, in man chronic inflammation is considered a predisposing factor for cancer of the maxillary sinus (Sato, 1987). Comparably, chronic necrotizing (peri)odontitis, rhinitis and sinusitis may be involved in the development of part of the nasal squamous cell carcinomas in untreated control rats of the Cpb:WU, Wistar random strain (Feron et al., 1989). A more detailed analysis of data on the possible association between malocclusion and squamous cell carcinoma seems to be indicated in this strain of rats and in other strains as well. Since rats suffering from the malocclusion syndrome may be more susceptible to nasal carcinogens than normal rats, data on degree and incidence of this syndrome in various strains of rats may be of relevance for the interpretation of data from rat carcinogenicity studies.

Chemical and mechanical irritation may contribute to the induction of nasal cancer in furniture industry workers (Mohtashamipur & Norpoth, 1983; Boysen, 1985). Studies in animals, aimed at elucidating the role of irritation in wood dust carcinogenesis, are necessary as scientific basis for setting occupational exposure limits for wood dust. A working hypothesis could be that wood dust levels not leading to (chemical or mechanical) irritation are low enough to avoid substantial risk of nasal cancer.

The crucial role of tissue damage followed by hyperplasia and metaplasia of the nasal respiratory epithelium in formaldehyde carcinogenesis has been demonstrated in a convincing way, has meanwhile been widely recognized, and should be included in human cancer risk assessment. Despite differences in anatomy and physiology of the nose between rats and humans, the respiratory tract defence systems are similar in both species. It is, therefore, reasonable to conclude that the response of the respiratory tract to formaldehyde will be qualitatively similar in rats and humans. If in humans exposure to formaldehyde were to be accompanied by recurrent tissue damage at the site of contact, formaldehyde may be assumed to have carcinogenic potential in man. It has been shown that in rats such recurrent tissue damage must be accompanied by exposure to high, cytotoxic concentrations of formaldehyde in order that nasal tumours be formed (Feron, 1988; Woutersen et al., 1989). Correspondingly, if the respiratory tract tissue is not recurrently injured, exposure of humans to relatively low, non-cytotoxic levels of formaldehyde can be assumed to represent a negligible cancer risk. Human exposure to formaldehyde should be minimized for its potency to damage tissue. At the workplace formaldehyde exposure should be controlled to levels below that likely to produce a significant irritant effect. Epidemiological cancer studies suggest that populations of workers showing an excess of nasal cancer have been exposed to formaldehyde levels higher than the threshold for tissue damage (Higginson et al., 1987). This threshold is about 1 ppm. Thus, an occupational exposure limit of 1 ppm would be likely to virtually eliminate any carcinogenic risk that might have existed at higher exposure concentrations in the past. For the general population an atmospheric exposure limit of e.g. 0.1 ppm would be a virtually safe level.

The role of tissue damage and hyperproliferation is clear in formaldehyde carcinogenesis; its role in acetaldehyde carcinogenesis seems similar but needs further study to justify inclusion in risk estimation. Compounds such as acrolein, furfural, dimethylamine and ethyl acrylate are very cytotoxic to the nasal mucosa and induced hyperproliferative changes but did not cause nasal tumours. These agents are not or only weakly genotoxic, which may explain their non-carcinogenicity for the nose. Apparently, the local tissue conditions created by these compounds did not meet the requirements for tumour initiation, promotion and progression. However, ethyl acrylate, being a nasal non-carcinogen, did cause carcinomas in the forestomach of rats and mice after administration by gavage (Maronpot, 1983). Such differences in response between organs may be due to differences in local tissue dose and/or to tissue-specific differences in sensitivity. Such differences can e.g. result in metabolic overload and the production of genotoxic metabolites in one tissue but not in another tissue (J.J. Clary, I.C. Munro and J. Orr, personal communication, 1988), or with one parent compound and not with another parent compound. Even if no genotoxic metabolites are involved, it is conceivable that hyperproliferation induced by one compound will lead to tumours on the basis of a certain epigenetic mechanism, whereas hyperproliferation induced by another substance will not lead to tumour

formation. For instance, differences in toxifying or detoxifying mechanisms, or in time available for DNA-repair ultimately determine whether or not "background" initiators and promoters will get hold of the tissue and eventually will result in neoplastic transformation. In brief, as far as neoplastic transformation is concerned, hyper- proliferative nasal tissue may easily behave differently from hyperproliferative forestomach tissue induced by the same agent; also hyperproliferative nasal epithelium induced e.g. by ethyl acrylate may behave very differently from hyperproliferative nasal epithelium induced by e.g. furfural (Feron & Kruysse, 1978), or electrocoagulation (Woutersen et al., 1989). Clearly, tissue- and compound-specific factors largely if not entirely determine the role of tissue injury in (nasal) carcinogenesis. Consequently, a status of hyperplasia may, but does not necessarily, lead to (nasal) neoplasia. On the other hand, for a compound causing (nasal) neoplasia only at concentrations also causing tissue injury followed by hyperplasia, induction of injury plus hyperproliferation most likely, but not necessarily, is a prerequisite for the manifestation of the compound's carcinogenic potential.

Conclusions

1. Chronic tissue injury may play a role in cancer development in man and experimental animals.
2. In humans chronic sinusitis is a predisposing factor for cancer of the paranasal sinuses.
3. The occurrence of tissue damage and hyperproliferation should be taken into account in interpreting results of carcinogenicity studies.
4. Nasal squamous cell carcinoma in rats may form part of the malocclusion syndrome.
5. It is hypothesized that wood dust levels not leading to chemical or mechanical irritation of the nasal mucosa are low enough to avoid substantial risk of nasal cancer in man.
6. Exposure of humans to non-cytotoxic levels of formaldehyde constitutes a negligible cancer risk.
7. Tissue- and compound-specific factors determine the role of tissue injury and hyperproliferation in (nasal) carcinogenesis.

References

Appelman, L.M., R.A. Woutersen, A. Zwart, H.E. Falke & V.J. Feron, 1988. One-year inhalation toxicity study of formaldehyde in male rats with a damaged or undamaged nasal mucosa. J. Appl. Toxicol. 8: 85-90.

Ashby, J., 1986. The prospects for a simplified and internationally harmonized approach to the detection of possible human carcinogens and mutagens. Mutagenesis 1: 3-16.

Bennett, D.E., W.F. Sasser & T.B. Ferguson, 1969. Adenocarcinoma of the lung in men. Cancer 23: 431-439.

Berenblum, I., 1944. Irritation and carcinogenesis. Arch. Pathol. 38: 233-244.

Bogdanffy, M.S., P.H. Morgan, T.B. Starr & K.T Morgan, 1987. Binding of formaldehyde to human and rat nasal mucus and bovine serum albumin. Toxicol. Letters 38: 145-154.

Bogdanffy, M.S., H.W. Randall & K.T. Morgan, 1987. Biochemical quantitation and histochemical localization of carboxylesterase in the nasal passages of the Fischer-344 rat and B6C3F1 mouse. Toxicol. Appl. Pharmacol. 88: 183-194.

Boysen, M., 1985. Histopathology of the nasal mucosa in furniture workers. Rhinol. 23: 109-113.

Boysen, M., R. Voss & L.A. Solberg, 1986. The nasal mucosa in softwood exposed furniture workers. Acta Otolaryngol. 101: 501-508.

Buckley, L.A., K.T. Morgan, J.A. Swenberg, R.A. James, T.E. Hamm & C.S. Barrow, 1985. The toxicity of dimethylamine in F-344 rats and B6C3F1 mice following a 1-year inhalation exposure. Fund. Appl. Toxicol. 5: 341-352.

Casanova, M. & H. d'A. Heck, 1987. Further studies of the metabolic incorporation and covalent binding of inhaled ^{3}H- and ^{14}C-formaldehyde in Fischer-344 rats: effects of glutathione depletion. Toxicol. Appl. Pharmacol. 89: 105-121.

DeBethizy, J.D., J.R. Udinsky, H.E. Scribner & C.B. Frederick, 1987. The disposition and metabolism of acrylic acid and ethyl acrylate in male Sprague-Dawley rats. Fund. Appl. Toxicol. 8: 549-561.

Feron, V.J., 1988. Recent inhalation and chronic oral studies with formaldehyde in rats. In: Proceedings of the Summer Toxicology Forum Meeting. Toxicology Forum Inc., Aspen. In press.

Feron, V.J., H.R. Immel, J.W.G.M. Wilmer, R.A. Woutersen & A. Zwart, 1987. Nasal Tumours in rats after severe injury to the nasal mucosa and exposure to formaldehyde vapour: preliminary results. In: E. Tyihak & G. Gullner (Eds.): The Role of Formaldehyde in Biological Systems. Sote Press, Budapest. p. 73-77.

Feron, V.J. & A. Kruysse, 1977. Effects of exposure to acrolein vapour in hamsters simultaneously treated with benzo(a)pyrene or diethyl-nitrosamine. J. Toxicol. Environ. Health 3: 379-394.

Feron, V.J. & A. Kruysse, 1978. Effects of exposure to furfural vapour in hamsters simultaneously treated with benzo(a)pyrene or diethyl-nitrosamine. Toxicology 11: 127-144.

Feron, V.J., R.A. Woutersen, A. van Garderen-Hoetmer & H.C. Dreef-van der Meulen, 1989. Upper respiratory tract tumors in Cpb;WU (Wistar random) rats. In: L. Uriah & K.T. Morgan (Eds.): Toxicologic pathology of the upper respiratory system. In press.

Ghanayem, B.I., R.R. Maronpot & H.B. Matthews, 1985. Ethyl acrylate-induced gastric toxicity. I. Effect of single and repetitive dosing. Toxicol. Appl. Pharmacol. 80: 323-335.

Grasso, P., 1987. Persistent organ damage and cancer production in rats and mice. Arch. Toxicol., Suppl. 11: 75-83.

Heck, H.d'A. & M. Casanova, 1987. Isotope effects and their implications for the covalent binding of inhaled ^{3}H- and ^{14}C- formaldehyde in the rat nasal mucosa. Toxicol. Appl. Pharmacol. 89: 122-134.

Heck, H.d'A. & M. Casanova-Schmitz, 1984. Biochemical toxicology of formaldehyde. Rev. Biochem. Toxicol. 6: 155-189.

Higginson, J., O.M. Jensen, L. Kinlen, W.H. Kirsten, B. MacMahon, G.M. Matanoski, T.J. Smith & D.C. Thomas, 1987. Epidemiology of chronic occupational exposure to formaldehyde. Report of the ad hoc panel on health effects of formaldehyde. Universities Associated for Research and Education in Pathology Inc., Bethesda.

Kerns, W.D., K.L. Pavkov, D.J. Donofrio, E.J. Gralla & J.A. Swenberg, 1983. Carcinogenicity of formaldehyde in rats and mice after long-term inhalation exposure. Cancer Res. 43: 4382-4392.

Laroye, G.J., 1974. How efficient is immunological surveillance against cancer and why does it fail? Lancer I: 1097-1100.

Ma, T.H. & M.H. Harris, 1988. Review of the genotoxicity of formaldehyde. Mutation Res. 196: 37-59.

Maronpot, R.R., 1983. NTP Technical report on the carcinogenesis bioassay of ethyl acrylate in rats and mice. National Toxicology Program. NTP TR No. 259.

Miller, R.R., J.A. Ayres, L.W. Rampy & M.J. McKenna, 1981. Metabolism of acrylate esters in rat tissue homogenates. Fund. Appl. Toxicol. 8: 549-561.

Miller, R.R., J.T. Young, R.J. Kociba, D.G. Keyes, K.M. Bodver, L.L. Calhoun & L.A. Ayres, 1985. Chronic toxicity and oncogenicity bioassay of inhaled ethyl acrylate in Fischer-344 rats and B6C3F1 mice. Drug Chem. Toxicol. 8: 1-42.

Mohtashamipur, E. & K. Norpoth, 1983. Zur Frage beruflich bedingter Tumoren in der holzverarbeitenden Industrie. Arbeitsmed. Sozialmed. Prä. 18: 49-52.

Morgan, K.T., E.A. Gross & D.L. Patterson, 1986. Distribution, progression, and recovery of acute formaldehyde-induced inhibition of nasal mucociliary function in F-344 rats. Toxicol. Appl. Pharmacol. 86: 448-456.

Morgan, K.T., X.Z. Jiang, T.B. Starr & W.D. Kerns, 1986. More precise localization of nasal tumors associated with chronic exposure of F-344 rats to formaldehyde gas. Toxicol. Appl. Pharmacol. 82: 264-271.

Sato, T., 1987. High-risk factors in the development of head and neck cancers. Gan To Kagaku Ryoko 14: 2626-2631.

Scheidt, R., H-P. Ehrhardt & R. Bartsch, 1987. Gehäuftes Auftreten von bösartigen Tumoren im Bereich der Nase und ihrer Nebenhöhlen bei Werktätigen der Holzindustrie und anderen Industriezweigen. Arch. Geschwulstforsch. 57: 393-399.

Swenberg, J.A., C.S. Barrow, C.J. Boreiko, H.d'A. Heck, R.J. Levine, K.T. Morgan & T.B. Starr, 1983. Non-linear biological responses to formaldehyde and their implications for carcinogenic risk assessment. *Carcinogenesis* 4: 945-952.

Wilhelmsson, B., H. Hellquist, J. Olofsson & C. Klintenberg, 1985. Nasal cuboidal metaplasia with dysplasia. Acta Otolaryngol 99: 641-648.

Woutersen, R.A., L.M. Appelman, A. van Garderen-Hoetmer & V.J. Feron, 1986. Inhalation toxicity of acetaldehyde in rats. III. Carcinogenicity study. Toxicology 41: 213-231.

Woutersen, R.A., L.M. Appelman, J.W.G.M. Wilmer, H.E. Falke & V.J. Feron, 1987. Subchronic (13-week) inhalation toxicity study of formaldehyde in rats. J. Appl. Toxicol. 7: 43-49.

Woutersen, R.A., A. van Garderen-Hoetmer, J.P. Bruyntjes, A. Zwart & V.J. Feron, 1989. Nasal tumours in rats after severe injury to the nasal mucosa and prolonged exposure to 10 ppm formaldehyde. J. Appl. Toxicol. In press.

EPIDEMIOLOGY OF NASAL CANCER IN NICKEL WORKERS

D.F. Easton

Institute of Cancer Research, Sutton, U.K.

Summary

The epidemiological evidence associating nickel refiners with an increased risk of nasal sinus cancer is described. The majority of nasal cancer cases can be related to dust exposure during the roasting and leaching of sulphide or oxide matte, suggesting a link with exposure to nickel sulphides or nickel oxides. There is also some indication of a risk in electrolysis areas and hence, perhaps, a risk due to soluble nickel exposure. To date there is no evidence of a nasal cancer hazard in other nickel related industries or nickel exposure in the general population.
Keywords: nickel, refineries, nasal sinus cancer

Introduction

The respiratory cancer risk to nickel refinery workers was first detected in 1928, when a general practitioner in Clydach, South Wales, noted two cases of nasal sinus cancer in workers. Since then marked excesses of nasal cancer (and lung cancer) have been observed in nickel refineries in many countries.

There is therefore a strong weight of evidence in favour of one or more of the compounds specifically involved in nickel refining being carcinogenic in man, but to date it is unclear which are the responsible agents. The principle exposures in the refineries are to nickel sulphides (particularly nickel subsulphide), nickel oxides, metallic nickel, and various soluble nickel salts. It should be noted that all of these nickel species have been found to be carcinogenic in laboratory animals, though primarily through causing sarcomas at the site of intramuscular or intrarenal injection (IARC, 1987). Only nickel subsulphide has proved to be consistently carcinogenic by inhalation.

We concentrate here therefore on reviewing the epidemiological evidence, particularly as it relates to the risks associated with different nickel species. In the tables that follow "Exp" refers to the number of deaths (or incident cancers as appropriate) expected on the basis of national rates, and "relative risk" to the ratio of observed to expected deaths.

Nickel refiners

Evidence of a nasal cancer excess in nickel refiners has now been observed in many countries, but the most important evidence comes from the cohorts studied at Clydach, South Wales (Peto et al,1984), Sudbury and Port Colbourne, Ontario, Canada (Roberts et al, 1984) and Kristiansaand, Norway

(Magnus et al,1982), summarised in table I. For the Welsh cohort I have included some updated data to appear shortly (Easton et al, in press). All these cohorts also show a substantial excess of lung cancer deaths, but no clear excess mortality from any other type of cancer or non-malignant cause.

Table 1. mortality results in the principle refinery cohorts (cancer incidence data only for Kristiansaand)
Colbourne and Sudbury, Ontario, and Kristiansaand, Norway.

Refinery	Nasal cancer Obs	Exp	Rel.Risk	Lung cancer Obs	Exp	Rel.Risk
Clydach+	73*	0.35	208.6	176	41.80	4.2
Port Colbourne	17*	0.33	51.5	62	33.09	1.9
Sudbury	5*	2.17	2.3	264	214.06	1.2
Kristiansaand	21	0.8	26.3	82	22.0	3.7

* Several additional nasal cancers are known to have occurred in these refineries, but were either not part of the cohorts or were coded to a different cause of death on the death certificate.
+ Workers first employed before 1930.

Table 2. Nasal cancer risk at Clydach, by duration of employment, time since first employment and age at first employment (Kaldor et al,1986). EMR = excess mortality ratio, allowing for the other factors and assumed multiplicative between factors.

Variable		EMR
Age at first employment	<20	1.0
	20-27.5	5.1
	27.5-35	10.9
	>35	36.6
Time since first employment	<20	1.0
	20-29	4.9
	30-39	6.6
	40-49	13.9
	>50	24.7
Duration of employment	<1	1.0
	1-4	4.5
	5-9	3.9
	10-14	13.3
	>15	21.2

The pattern of risk in relation to age at first exposure, time since first exposure and duration of exposure has been described in most detail for the Clydach cohort. No cases of nasal cancer have been observed within 15 years of first exposure, an observation replicated in all the cohorts. Thereafter the risk rises rapidly and is still increasing 40 years since first exposure. Kaldor et al (1986) also demonstrated an increasing risk with increasing age at first exposure, and strong relationship with duration of employment in high risk areas.

It should be noted that, in contrast to wood workers, where the risk appears to be entirely to adenocarcinoma (IARC, 1987), the majority (though not all) of the nasal cancer cases reported in nickel refiners are squamous cell carcinomas (L.G. Morgan, pers comm).

Type of exposure

All of the main cohorts have been able to provide information as to the type of exposure associated with the cancer risk. The situation is perhaps simplest for the Ontario refineries; here the excess of nasal cancer is largely or entirely confined to the areas involved in pretreating the sulphide ore (leaching, calcining and sintering; table 3). These areas would certainly have involved substantial exposures to nickel subsulphide, and this agent could plausibly be responsible for the entire excess (Roberts et al, 1984).

Table 3. Nasal cancer mortality at Sudbury and Port Colbourne, by occupation (Roberts et al, 1982).

Occupation	Obs	Exp	Rel.Risk
Sudbury sinter plant + Port Colbourne leaching, calcining and sintering	18	0.26	69.2
All other employees	4	2.24	1.8

At Kristiansaand there is also an excess in the roasting and smelting area, but in contrast to Ontario there is also a clear excess in the electolysis areas (table 4). The precise exposure in these areas is unclear, but is likely to be largely to soluble nickel rather than insoluble sulphides or oxides (Magnus et al,1982).

Table 2. Nasal cancer mortality at Kristiansaand by category of work (Magnus et al, 1982).

Category of work	Obs	Exp	Rel.Risk
Roasting and smelting	8	0.2	40.0
Electrolysis	8	0.3	26.7
Other processes	2	0.1	20.0
Administrative, service, unspec	3	0.2	15.0

The Clydach data is hardest to interpret because the job histories were less accurate and because men tended to work in many different areas. However a case control analysis by Kaldor et al (1986) clearly demonstrates a risk in the pre-refining stages of calcining and copper leaching, and in the furnaces. These areas would all have had substantial levels of nickel sulphide or oxide dust. There also however some suggestion of a risk in the "nickel sulphate" and "concentrates" plant, where exposure would be primarily to a soluble nickel aerosol.

It should also be noted that the Clydach cohort shows no association whatsoever with employment in the "nickel sheds", where the refining by the carbonyl process took place. This observation suggests that nickel carbonyl gas is unlikely to have been a major contributor to the cancer excess.

Other nickel exposure

Nickel refiners comprise a relatively small proportion of the total workforce with nickel exposure, most of whom are involved with use of the metal to produce alloys, or soluble salts. Several cohorts have have examined workers in such nickel user industries and none have found any evidence of a nasal cancer excess (Cragle et al, 1984; Cox et al, 1981; Enterline and Marsh, 1982; Redmond, 1984). It should be noted that the nickel exposures in such industries, of the order 0.1 mg/m^3 (Cox et al, 1981) are probably an order of magnitude lower than those in the refineries during the periods when the cancer risks occurred, so that the lack of an excess is not surprising, though certainly reassurring. Enterline and Marsh did find two probable cases of nasal cancer in their alloy plant but both may have had contaminating exposure from the calcining area of a neighbouring refinery.

No excess was observed in the one large study of nickel miners (Roberts et al, 1982).

Discussion

Despite the size of the nasal cancer excess, it is still very unclear which of the various nickel species are responsible. Indeed, a reasonable hypothesis is the ultimate carcinogen will prove to be the nickel ion, and hence that

all nickel compounds are carcinogenic at some level determined by the ability of the compound to make the nickel ion available to cells in the nasal (or bronchial) epithelium. There is at present a need for more long term inhalation experiments in laboratory animals to determine the relative carcinogenic potency of the different nickel compounds following a route of exposure relevant to the human problem.

In the meantime the epidemiological evidence relating to industrial nickel exposure , including substantial new data on many of the cohorts described above , is currently being assessed under an expert committee (Chairman: Sir Richard Doll) which is reporting shortly.

On the positive side it seems clear that, outside of the refineries, exposure to nickel compounds is such that no measurable risk (at least for nasal sinus cancer) is involved.

References

Cox, J.E., R. Doll, W.A. Scott & S. Smith, 1987. Mortality of nickel workers: experience of men working with metallic nickel. Br. J. Ind. Med. 38:235-239.

Cragle, D.L., D.R. Hollis, T.H. Newport, & C.M. Shy, 1984. A retrospective cohort mortality study among workers occupationally exposed to metallic nickel dust at the Oak Ridge gaseous diffusion plant. In: F.W. Sunderman Jr (Ed.): Cancer in the human environment. IARC sci. publ. 53. IARC, Lyon. p. 57-63.

Easton, D.F., J. Peto, L.G. Morgan, L. Metcalfe, V. Usher & R. Doll, 1989. Respiratory cancer mortality in Welsh nickel refiners. Advances in Environmental Science and Technology. In press.

Enterline, P.E. & G.M. Marsh, 1982. Mortality among workers in a nickel refinery and alloy manufacturing plant in West Virginia. J. Nat. Cancer Inst. 68:925-933.

IARC, 1987. Overall evaluations of carcinogenicity: an updating of IARC monographs volumes 1 to 42. IARC monographs on the evaluation of carcinogenic risk to humans, supplement 7. IARC, Lyon. p. 264-269, 380-383.

Kaldor, J., J. Peto, D. Easton, R. Doll, C. Hermon & L. Morgan, 1986. Models for respiratory cancer in nickel refinery workers. J. Nat. Cancer. Inst. 77:841-848.

Magnus, K., A. Andersen & A.C. Hogetvigeit, 1982. Cancer of respiratory organs among workers at a nickel refinery in Norway: a second report. Int. J. Cancer 30:681-685.

Peto, J., H. Cuckle, R. Doll, C. Hermon & L.G. Morgan, 1984. Respiratory cancer mortality of Welsh nickel refiners. In: F.W. Sunderman Jr (Ed.): Cancer in the human environment. IARC sci. publ. 53. IARC, Lyon. p. 37-46.

Redmond, C.K., 1984. Site-specific cancer mortality among workers involved in the production of high nickel alloys. In: F.W. Sunderman Jr (Ed.): Cancer in the human environment. IARC sci. publ. 53. IARC, Lyon. p. 73-86.

Roberts, R.S., J.A. Julian, D.C.S. Muir & H.S. Shannon, 1984. Cancer mortality associated with the high temperature oxidation of nickel subsulphide. In: F.W. Sunderman Jr (Ed.): Cancer in the human environment. IARC sci. publ. 53. IARC, Lyon. p.23-36.

Roberts, R.S., J.A. Julian, D.C. Muir, 1982. A study of cancer mortality in workers engaged in the mining, smelting and refining of nickel. Report to the Joint Occupational Committee of INCO and the United Steel Workers of America.

EPIDEMIOLOGY OF SINONASAL CANCER IN WOODWORKERS

B. Wilhelmsson

Department of Otorhinolaryngology, Huddinge Hospital, Karolinska Institute, Huddinge, Sweden

Summary

There is sufficient evidence from many countries that exposure to hard wood dust in the furniture-making industry has resulted in sinonasal cancer, predominently adenocarcinoma. However, some investigations during the last years have also indicated a notable incidence of nasopharyngeal squamous cell carcinoma in workers occupationally exposed to soft wood outside the furniture industry. Thus, it is not possible at this time to say that some particular wood is the one most likely to induce sinonasal cancer and that some other wood has no activity of this kind.
Key words: epidemiology, hardwood, sinonasal cancer, softwood, woodworker.

An association between adenocarcinoma of the nose and paranasal sinuses and occupational exposure to wood dust was first recognized by Hadfield, ENT surgeon of High Wycombe in Buckinghamshire, England. She noticed in her clinical work an unusually high incidence of adenocarcinoma of the nasal cavity and sinuses among workers in the furniture industry in that area. An ENT-colleague of hers, MacBeth (1965), reported this classical study, in which he described two groups of patients with sinonasal cancer, 40 diagnosed between 1951-1959 and 29 diagnosed from 1960-1964. Twenty of these nasal cancer patients came from High Wycombe, UK, where the principal industry for many years had been chair-making, and 15 of them (75%) were employed in the furniture industry. Subsequent epidemiological surveys in this area were then performed by them in fruitful collaboration with a clinical epidemiologist, E D Acheson, in Southampton, who directed the further studies.

After the first clinical report in the Lancet, 1967, Acheson et al. performed three thorough epidemiological studies in UK. In the first, 1968, they restricted the study to two groups of cases of carcinoma of the nasal cavity and accessory sinuses, found in the Oxford Hospital region, comprising Buckinghamshire, Oxfordshire and Berkshire. Group I consisted of 83 patients, 56 men and 27 women, identified during 1956 -1965. Group II consisted of 65 patients, 42 men and 23 women, identified either before January 1956 or after December 1965. Of the men in group I, 42 were employed at the time of the diagnosis, and 16 of these (38%) were woodworkers (14 cabinet- or chair-makers or wood machinists in the furniture industry, 1 crate maker and 1 cooper), compared with 3.3 % of the total population so employed. In group I, 23 male patients had adenocarcinomas; 14 were currently woodworkers in the furniture industry, 1 was a yard labourer in a furniture factory and 5 were ex-workers in the furniture industry (2 cabinet-makers, 2 wood-machinists and 1 French polisher). In group II, 10 of the 42 male patients had adenocarcinomas; 3 were woodworkers in the furniture industry, 1 was a French polisher, 1 was a yard labourer in a furniture

factory and 1 had been a clerk in the furniture industry. No cases were reported as carpenters or joiners, although these occupations comprised about half of the woodworkers in the area.

In a second epidemiological study, Acheson et al. (1972) identified all cases of adenocarcinoma, covering the years of 1961-1966, with all the cancer registries in England and Wales but excluding Oxford, covered in their first study in 1968. Controls were patients with other nasal cancers similarly ascertained, matched for age within five years, sex, year of death and area of registry. A total of 145 adenocarcinomas and 133 other nasal cancers, the latter used as controls, were ascertained. Slides of 107 adenocarcinomas and 98 control nasal tumours were analyzed and histological confirmation by a pathologist was carried out for 74 adenocarcinomas and 94 controls. Nineteen of 80 men with adenocarcinomas (24%) had been mainly employed as furniture workers, compaired with 5 of 85 (6%) men with other nasal cancers. About 0.2 cases of adenocarcinoma would have been expected in this group on the basis of incidence figures for England and Wales, giving an SMR of 9.500. Nine of the 80 men with adenocarcinomas (11%) were employed in other woodworking professions, compared with four of 85 controls with other nasal cancers (5%); 1.9 cases would have been expected, giving an SMR of 474. It is worth noting, however, that the risk of other nasal cancers in individuals in woodworking trades was also significantly higher than expected ($P < 0.001$) which suggests that wood dust exposure may induce other kinds of nasal cancers as well.

In a third epidemiological survey in 1976, Acheson reviewed the association between furniture occupations and nasal cancer. He estimated that the incidence of adenocarcinoma in High Wycombe cabinet- and chair-makers was 0.7 per 1000 per year during the decade 1956-1965, i.e. about 500 times the risk in the general male population in southern England. Twenty-eight of the 34 nasal tumours (82%) were adenocarcinomas, compared with 4 of 52 nasal tumours (8%) in men reporting other kinds of work; and 22 of the 28 adenocarcinomas occurred in wood-workers in the furniture industry and in other workers in that industry while almost half of all other nasal tumours occurred after the age of 65. The average age of adenocarcinoma patients was in general 10 years lower than that of patients with cancers of other cell types. The average period between commencement of employment and diagnosis was 43 years with a range of 27-69 years; however, adenocarcinomas developed after exposures as short as 4.5 and 7 years. It is worth mentioning that Esmé Hadfield (1970) reported a case whose period of exposure to wood dust within the furniture industry was only 18 months. There was no signifincant difference between the mean latent period for men who left the industry prior to diagnosis of their tumour and that for those who were still employed when diagnosed. After having analyzed all available information in case histories, Acheson (1976) also suggested that the factors inducing the nasal tumours where at least present in the furniture industry between 1920-1940 and that beech almost certainly and oak probably are associated with the disease. The risk of developing nasal cancer was highest for men in jobs with exposure to wood dust as turners, machinists and sanders rather than in jobs with exposure to polishes, varnishes etc. Acheson believed on the basis of occurrencies of nasal cancer in various types of woodworkers, that wood dust itself, rather than any of the other extraneous chemicals that might be met by a worker in the furniture industry, is the factor related to the workplace that contributes to the development of malignant neoplasms.

When making a summary of the three original epidemiological studies by Acheson, it is found that the first study limited to the Oxford Hospital region revealed an approximately twenty-fold increase in the incidence of nasal cancer, of which 75% of the cases being adenocarcinomas.

In the second study based on complete ascertainment of all cases of nasal cancer that occurred around Oxford during a decade, a ten-fold excess risk of nasal cancer was observed for furniture workers, with 90% of the cases being adenocarcinomas which should be compared to about 10% among other occupations. The third study comprised all cases of nasal cancer registered in the whole of England and Wales, excluding the Oxford region during a defined period, revealed a relative risk about 100 concerning nasal adenocarcinoma among furniture workers. A small but still significant increase in relative risk was also found for nasal cancers other than adenocarcinomas.

Studies in other countries also show excesses of nasal cancers in furniture workers. In the Netherlands, where Delemorre & Themans (1971) reported on 16 adenocarcinomas diagnosed during the years 1944-1967; 11 cases (69%) occurred in people working in the furniture industry. These cases were compared with 33 cases of other carcinomas of the nasal sinuses, among which 3 (9%) were found in workers in the furniture industry.

Hayes et al. (1986) presented a case-control study from the Netherlands, examining the relations between type of wood-working and extent of wood dust exposure to the risk for specific histologic types of sinonasal cancer; 116 male patients with primary malignant epithelial sinonasal tumours diagnosed between 1978-1981 were collected from the treatment centres of the country. Living controls were selected from the municipal registries, and diseased controls were selected from the national death registry. Interviews were performed for 91 (78%) cases and 195 (75%) controls. Work histories were coded concerning industry and occupation. Occupational exposure to wood dust was calculated and given an index. Adjustment for age and cigarette use was done. As a summary, it was found that risk for nasal adenocarcinoma was elevated by industry for wood and paper industry to an odds ratio of 11.9 and by occupation for employment in furniture-and cabinet-making to an odds ratio of 139.8; in factory joinary and carpentry work to an odds ratio of 16.3, and in association with high-level wood dust exposure to an odds ratio of 26.3. Further, it was found that other types of nasal cancer were not associated with wood-related industries or occupations. But, a moderate excess risk for squamous cell cancer with an odds ratio of 2.5, was associated with low-level wood dust exposure; however, no dose-response relation was evident. The association between wood dust exposure and adenocarcinoma was strongest for those employed in the wood dust related occupations between 1930-1941. The risk of adenocarcinoma in the sinonasal area did not appear to decrease for at least 15 years after termination of wood dust exposure. No cases of adenocarcinoma were observed in men whose first exposure to wood dust had occured after 1941.

In Sweden, Engzell et al. (1978) identified cases of carcinoma of the nose and paranasal sinuses from the Cancer Registry of the National Board of Health and Welfare of Sweden; adenocarcinomas registered between 1961 and 1971 and squamous cell and poorly differentiated carcinomas registered between 1965-1971. Occupation was then acertained

for each case by a questionnaire completed either by the patient or by a close relative. Nineteen of the 36 patients with adenocarcinomas (53%) were classified as joiners and/or cabinet-makers. Among 127 men with other nasal tumours, 5 (4%) were joiners in wood-related industries. Nineteen of the 32 woodworkers (59%) had adenocarcinomas compared with 17 of 131 patients (13%) who reported other occupations. The average age of men with adenocarcinomas was 62 years. The incidence of smoking and snuff-taking among patiens who completed the questionnaire was similar to that of the general population. When cases of adenocarcinoma were traced up to 1972, Engzell (1979) found that three out of 8 new cases were joiners. The latent period averaged 45 years with a range from 22 - 70 years for 21 joiners. Two workers employed in oak parquet flooring factories developed adenocarcinomas after only 8 and 9 years of employment.

There is one additional Swedish epidemiological study performed by Gerhardson et al (1985). They noticed that almost all published papers on sinonasal cancer and previous exposure to wood dust were based on case - control studies and case reports. Because of this finding they performed a cohort study of sinonasal adenocarcinoma among 8.141 furniture workers during a 19 years follow up period. They used the 1960 census in Sweden and the Swedish Cancer Registry in which all cases of cancer diagnosed in the population may be obtained for research. Each inhabitant living in Sweden has an individual identification number which is used in both registries. A cancer environment registry has been created by a record linkage between the 1960 census and the 1961 - 1979 Cancer Registry. Their study was based on 8.141 men aged 20-64 in 1960 and classified as furniture workers in the 1960 census. The reference group of 1.4 million consists of all employed men, except those classified as furniture workers, aged 20-64 in 1960. Only blue collar workers were included in the study. Expected numbers were calculated based on the number of furniture workers and on the cumulative incidences in the reference group. Stratification was made by year of birth (5 year group) and county. To quantify the increase in risk, if any, the standardized morbidity ratio (SMR) was calculated as the observed number of cases divided by the expected number. Among the furniture workers sinonasal carcinoma was 7 and sinonasal-adenocarcinoma 44 times more common than expected. There were 14 cases of adenocarcinoma in furniture workers, but of the 17 cases of adenocarcinoma of the sinonasal cavities in the reference population, 5 were woodworkers but not workers in the furniture industry.

Estimates of the relative risk varies widely between different case-control studies, from 2.5 to more than 100 (Acheson et al, 1972). In the only previous reported cohort study, performed by Rang and Acheson (1981), the relative risk was estimated to 133, based on 8 cases of sinonasal adenocarcinoma in furniture workers in England.

Cases of sinonasal cancer and adenocarcinoma among woodworkers have also been reported from the U.S.A. by Brinton et al. (1977), from Belgium by Debois (1969), from France by Gignoux & Bernard (1969), and by Haguenauer et al. (1977), from the GDR by Löbe & Ehrhardt (1978) and from Denmark by Andersen (1975), Andersen et al (1976, 1977) and by Olsen & Sabroe (1979). Also from the other hemisphere, in Australia, it was reported by Ironside & Mattheus (1975), cases of adenocarcinoma of the nose and paranasal sinuses in woodworkers in the state of Wictoria. They also found a significantly higher proportion of wood workers among

their cases than in the general population.

In 1981 the International Agency for Research on Cancer (IARC) concluded that there is sufficient evidence that nasal adenocarcinomas have been caused by employment in the furniture-making industry. The excess risk occurs mainly among those exposed to wood dust. Although adenocarcinomas predominate, an increased risk of other nasal cancers among furniture workers was also suggested. Further, it was concluded that at that time, epidemiological data were not sufficient to make a definite assessment of the carcinogenic risks of employment in the lumber and sawmill industries. There were some studies suggesting that the incidences of nasal cancers and Hodgkin´s disease may be increased. There were neither sufficient epidemiological data to make a definite assessment of the carcinogenic risks of employment as acarpenter or joiner. A number of studies, however, raised the possibility of an increased risk of Hodgkin´s disease. In 1981 there was conflicting evidence about an association between nasal adenocarcinoma and work as a carpenter.

After these conclusions by the IARC (1981), the interest in performing further epidemiological studies on this subject naturally decreased. But from 1981 and further on, there are some reports indicating that softwood dust exposure appears to be associated with a squamous cell and anaplastic carcinomas. Apart from a case-referent study from British Columbia by Elwood (1981), Hernberg et al. (1983), showed in a joint Danish-Finish-Swedish case-referent investigation on nasal and sinonasal cancer, apart from indicating that hard wood dust exposure was associated with adenocarcinoma, that softwood dust exposure alone was associated with epidermoid and anaplastic carcinomas. In this study only primar malignant tumours in the nasal cavity and paranasal sinuses were included, according to the international certification of diseases (ICD-8) 160.00-160.99. This study showed sinonasal cancers in 13 workers exposed only to softwood dust, giving an odds ratio of 3.3. There were only two cases of sinonasal tumours in people exposed to hardwood dust only, giving an odds ratio of 2.0. Further there were 12 persons with sinonasal cancers exposed to both hardwood and softwood dusts, giving an odds ratio of 12.0. The most interesting finding in this study was the association between softwood dust and sinonasal cancer. In this study exposure to hardwood dust often occurred together with exposure to softwood dust. Most of these workers had been employed in the furniture industry and their main type of tumours were adenocarcinomas as expected from other studies. Those with exposure to softwood only had a completely different occupational distribution. Most of them were construction or sawmill workers. Such categories of workers do not usually handle hardwood in the Nordic countries. However, it should be noted that birch dust was classified as a soft type of wood. In the Nordic countries exposure to spruce and pine dust is almost always combined with exposure to birch dust, but pine and spruce are much more prevalent and therefore dust from these woods constitutes the dominating component of this type of mixed exposure.

There is another paper on sinonasal cancer and exposure to softwood published by Voss et al. (1985), from Norway. They reviewed the occupational histories and histological sections of 70 patients treated at the ENT department of the National Hospital in Oslo. Data information concerning previous occupations was obtained by telephone interviews according to a standardized questionnaire. The census of 1946 was used as a control material. The study revealed a strong association between

wood dust exposure and sinonasal cancer (SMR 300) and, which was most interesting, of 12 wood dust exposed men, 11 had been exposed exclusively to softwood. Squamous cell carcinoma was the predominant type of cancer in joiners, carpenters and loggers, while non-Hodgkin lymphomas appeared to be associated with employment in saw- and planningmill firms. It should be emphasized that in this study also nasopharyngeal carcinomas were included in sinonasal cancers.

Thus, there are now a few studies suggesting that the risk of sinonasal cancer, when including nasopharyngeal carcinoma according to the WHO classification from 1978, is increased also after exposure to softwood dust.

References

Acheson, E.D., E.H. Hadfield & R.G. Macbeth, 1967, Carcinoma of the nasal cavity and accessory sinuses in woodworkers. Lancet, i, 311-312.

Acheson, E.D., E.H. Hadfield & R.G. Macbeth, 1968. Nasal cancer in woodworkers in the furniture industry. Br.Med J. i, 587-596.

Acheson, E.D., R.H. Cowdell & E.Rang, 1972. Adenocarcinoma of the nasal cavity and sinuses in England and Wales. Br.J.Ind.Med. 29: 21-30.

Acheson, E.D., 1976. Nasal cancer in the furniture and boot and shoe manufacturing industries. Prev.Med. 5:295-315.

Andersen, H.C., 1975. Exogenous causes of cancer of the nasal cavities (Dan.). Ugeskr. Laeg. 137:2567-2571.

Andersen, H.C., J Solgaard & I.Andersen, 1976. Nasal cancer and nasal mucus-transport rates in woodworkers. Acta Otolaryngol. (Stockh) 82:263-265.

Andersen, H.C., I Andersen & J.Solgaard, 1977. Nasal cancers, symptoms and upper airway function in woodworkers. Br.J.Ind. Med. 34:201-207.

Brinton, L.A., W.J. Blot, B.J. Stone & J.F. Fraumeni Jr., 1977. A death certificate analysis of nasal cancer among furniture workers in North Carolina. Cancer Res. 37:3473-3474.

Debois, J.M., 1969. Tumours of the nasal cavities among woodworkers (Flem,). Tijdschr.v.Geneeskd. 2:92-93.

Delemarre, J.F.M. & H.H. Themans, 1971. Adenocarcinoma of the nasal cavities (Dutch). Ned.T.Geneeskd., 115:688-690.

Elwood, J.M., 1981. Wood exposure and smoking: association with cancer of the nasal cavity and paranasal sinuses in British Columbia. Can.Med.Assoc.J. 124:1573-77.

Engzell, U., A. Englund & P. Westerholm, 1978. Nasal cancer associated with occupational exposure to organic dust. Acta Otolaryngol. (Stockh) 86:437-442.

Engzell, U., 1979. Occupational etiology and nasal cancer. Acta Otolaryngol. (Stockh), Suppl. 360:126-128.

Gignoux, M. & P. Bernard, 1969. Malignant tumours of the ethmoid in woodworkers (Fr). J.Méd.Lyon. 50:731-736.

Gerhardsson, M.R., S.E. Norell, H.J. Kiviranta & A. Ahlbom, 1985. Respiratory cancers in furniture workers. Brit.J.Ind.Med. 42:403-405.

Hadfield, E.H., 1970. A study of adenocarcinoma of the paranasal sinuses in woodworkers in the furniture industry. Ann.R.Coll.Surg. (UK). 46:301-319.

Haguenauer, J.P., P. Romare, J.C. Duclos & R.Guinchard, 1977. Occupational cancers of the ethmoid. (Fr.). Arch.Mal.Prof. 38:819-823.

Hayes, R.B., M.Gerin, J.W.Raatgever & A. de Bruyn, 1986. Wood-related occupations, wood dust exposure, and sinonasal cancer. Am.J.Epidemiol. 124:569-77.

Hernberg, S., P. Westerholm, K. Schultz-Larsen, R. Degerth, E. Kuosma, A. Englund, U. Engzell, H. Sand Hansen, P. Mutanen, 1983. Nasal and sinonasal cancer: Connection with occupational exposures in Denmark, Finland and Sweden. Scand J Work Environ Health 9. p. 315-326.

International Agency for Research on Cancer, 1981. IARC monograph on the evaluation of the carcinogenic risk of chemicals to humans. Wood, leather and some associated industries. Vol. 25, Lyon: IARC.

Ironside, P. & J. Matthews, 1975. Adenocarcinoma of the nose and paranasal sinuses in woodworkers in the state of Victoria, Australia. Cancer 36:1115-21.

Löbe, L.-P. & H.-P. Erhardt, 1978. Adenocarcinoma of the nose and paranasal sinuses - an occupational disease in workers in the wood industry (Ger.). Dtsch. Gesundheitswes. 33:1037-1040.

Macbeth, R., 1965. Malignant disease of the paranasal sinuses. J. Laryngol. 79:592-612.

Olsen, J. & S. Sabroe, 1979. A follow-up study of non-retired and retired members of the Danish Carpenter/Cabinet Makers´ Trade Union. Int. J. Epidemiol. 8:375-382.

Rang, E.H. & E.D. Acheson, 1981. Cancer in the furniture workers. Int. J. Epidemiol. 10:253-61.

Voss, R., T. Stenersen, B. Roald Oppedal & M. Boysen, 1985. Sinonasal cancer and exposure to softwood. Acta Otolaryngol (Stockh). 99:172-178.

HUMAN STUDIES OF FORMALDEHYDE EXPOSURE AND CANCERS OF THE RESPIRATORY TRACT

Leslie Thomas Stayner

National Institute for Occupational Safety and Health, Cincinnati, Ohio

Summary

Over the past decade, a considerable debate has developed over the possible relationship between formaldehyde exposure and cancers of the respiratory tract in humans. This controversy is largely due to reports of an excess of nasal squamous cell carcinomas in rats exposed to formaldehyde. This report presents a summary and synthesis of the findings from over 20 epidemiologic investigations which have examined the possible relationship between formaldehyde exposure and cancers of the respiratory tract.

The findings from the studies which have been performed to date appear to be inconsistent with a large increase in the relative risk of lung cancer due to formaldehyde exposure. Lung cancer mortality was less than expected in most of the studies, and only moderately increased in a few of the cohort studies. It is worth noting, however, that due to the high background rate of lung cancer and the large number of individuals who are exposed to formaldehdye, even a small increase in relative risk would be important from a public health viewpoint.

The epidemiologic evidence is suggestive for a relationship between formaldehyde exposure and cancers of the upper respiratory tract. Elevated odds ratios were reported in 3 of the 5 nasal cancer case-control studies. In two of these studies the odds ratio was statistically significant and greater than 2. Nasopharyngeal cancer was found to be significantly associated with formaldehyde exposure in one (OR=2.3), and nonsignificantly associated with formaldehyde exposure in another (OR=2.1) of the 3 case-control studies. A statistically significant excess in buccal or buccal-pharyngeal cancer mortality was reported in 2 of the 6 cohort studies of industrial workers exposed to formaldehyde, and a significant excess of nasopharyngeal cancer was reported in 1.

Inconsistencies among these studies should be expected given the large variations in the dose, form and pattern of the formaldehyde exposures in the populations studied. Future research efforts should be directed towards studies which better define formaldehyde dose, and which examine the possible interactions between formaldehyde, particulates and other exposures found in the workplace.
Keywords: formaldehyde, respiratory tract cancer, nasal cancer, human studies

Introduction

Over the last decade, a considerable controversy has developed over the possible relationship between formaldehyde exposure and cancer of the respiratory tract in humans. The intensity of this debate is largely a reflection of the commercial importance of this chemical, and the large number of individuals who are potentially exposed to formaldehyde. In the United States alone, approximately 9 billion pounds of formaldehyde are produced annually (SRI, 1985), and 1.6 million workers are potentially exposed to formaldehyde (NIOSH, 1981).

Concern over the possible carcinogenicity of formaldehyde has largely developed as a result of reports from 2 independent research laboratories of an excess of nasal squamous cell carcinomas in rodents exposed to formaldehyde. In early work by investigators at New York University (NYU), approximately 25 % of the rats exposed to a combination of 10.7 ppm hydrochloric acid (HCL) and 14.6 ppm formaldehyde developed nasal squamous cell carcinomas (Albert 1982). Although this experiment was initially designed to test whether these two chemicals would combine to form bis-chloromethyl ether (BCME), the tumors observed were histologically distinct from what had been reported in experimental studies involving BCME exposures. Subsequent experiments by investigators at New York University (NYU), and at the Chemical Industry Institute of Toxicology (CIIT) have demonstrated similar findings of nasal squamous cell carcinomas among rats exposed to formaldehyde alone (Swenberg, et al 1980 and Albert, et al 1982).

Table 1. Results from Studies of Inhalation Exposures in Rodents.

Ref	Species	Exposure	Findings
Albert (1982)	Rats	14.6 ppm CH2O + 10.7 ppm HCL	*25 % of the Rats Developed Nasal Squamous Cell Carcinomas
Swenberg (1980)	Rats & Mice	0, 2, 6 OR 15 ppm CH2O	*At 15 ppm 39 % of the rats and 2 % of the mice developed nasal squamous cell carcinomas *At 6 ppm 1% of the rats developed Nasal Squamous Cell Carcinomas
Albert (1982)	Rats	1) 14.6 ppm CH2O 2) 14.6 ppm CH2O + 10.6 ppm HCL 3) 10.6 ppm HCL	*10 % of group 1, and 9 % of group 2 developed Nasal Squamous Cell Carcinomas *No nasal tumors were observed in Group 3 or in any of the controls

In response to the findings from the experimental studies described above, over 20 epidemiologic investigations designed to examine the carcinogenicity of formaldehyde have been completed. This presentation will attempt to summarize and synthesize the findings from these studies concerning respiratory tract cancer. Although the animal studies described above only reported an excess of nasal cancer, it is reasonable to expect, that due to differences in the anatomy and physiology between rodents and humans other respiratory

sites in humans might be affected. In addition, it is important to recognize that nonrespiratory neoplasms have been reported to be in excess among human populations exposed to formaldehyde. Particularly noteworthy are excesses of brain cancer and leukemia which have been observed to be in excess in studies of anatomists (Stroup, 1984), pathologists (Harrington, 1975) and embalmers (Walrath and Fraumeni, 1983 and 1984, Levine, 1984), but generally not in studies of industrial workers (Marsh, 1982, Liebling et al, 1983, Acheson et al, 1984, Blair et al, 1986, Stayner et al, 1988).

Methods

Three basic types of epidemiologic studies have been used to examine the potential relationship between formaldehyde and respiratory tract cancer in humans. In cohort mortality studies, populations of workers exposed to formaldehyde have been identified retrospectively and followed to identify deaths due to a particular disease. A modified life-table analysis approach has been generally used to compute expected numbers of death by applying the age, race, sex and calendar time specific person-years of observation from the study group to the corresponding death rates from a comparison population. The standardized mortality ratio (SMR) is then calculated, which is simply the ratio of observed to expected number of deaths.

In proportionate mortality studies, sources of death information (e.g. a union death benefit insurance fund) have been used to identify deaths among a population of workers exposed to formaldehyde. This is similar to a cohort study, except that information is unavailable on individuals who did not die and thus person-years at risk for the entire study population can not be estimated. Expected numbers of death from a particular cause may be estimated by multiplying the age, race, sex, and calendar time specific total number of deaths in the study population by the corresponding proportion of deaths from a particular cause in a comparison population. Similar to the SMR, the proportionate mortality ratio (PMR) is calculated by dividing the number observed by the number expected. The PMR has a potential bias which does not apply to the SMR, due to the fact that an excess or deficit of one cause of death may result in an spuriously deflated or inflated PMR for another cause of death.

Finally, case-control studies have been conducted in which cases of respiratory tract cancer and controls, who are hopefully representative of the population from which the cases arose, are selected. These studies have generally identified incident cases from sources such as a tumor registry. The odds of exposure among the cases is compared with odds of exposure in the controls in the form of an odds ratio (OR). Control of confounding by age or other covariates is generally achieved by either matching cases to controls on these factors, or by using a stratified analysis and producing summary estimates.

The SMR, PMR and OR may all be thought of as estimates of relative risk (RR), which is the ratio of the risk (cumulative incidence) among the exposed population, to the risk among the nonexposed

population. These measures may also be referred to as measures of the magnitude of an association between the disease and exposure (formaldehyde), which is not to suggest that such associations are necessarily causal. For all 3 of the relative risk estimators (SMR, PMR, or OR) a ratio of 1 suggests an absence of effect, a ratio of more than 1 suggests an increased risk of disease, and a ratio of less than 1 suggests a decreased risk of disease in the exposed population relative to the referent population.

The statistical significance of the relative risk estimators will be discussed; however, statistically nonsignificant findings will also be considered in this discussion in order to best summarize the weight of the epidemiologic evidence. As has been pointed out by Rothman (1986), statistical significance is only one element which should be used in evaluating the results of an epidemiologic investigation, or in summarizing the evidence from a number of studies. Consistency in the magnitude and direction of the relative risk estimates is also an important consideration.

Following is a review and synthesis of the findings from the epidemiologic studies which have been performed to date. Highlights of the strengths and limitations of these studies are presented. It should be recognized, that many of the results presented by the authors did not take into account a latency period (time since first exposure). It is frequently expected that occupational cancers may only become manifest after an adequate latency period (e.g. 10-20 years). Results with a latency assumption will be presented in this paper where available. It is also worth noting, that few of the studies had quantitative estimates of formaldehyde exposure available for analysis.

Study Findings

In this paper the results of cohort studies of industrial workers are presented separately from the results of studies of professional groups (i.e. anatomists, pathologists and embalmers). This was done because industrial and professional workers differ greatly in regard to their extent and pattern of formaldehyde exposure. They also differ with respect to their socioeconomic status, which may be related to other risk factors for respiratory tract cancer (e.g. smoking).

Table 2 summarizes the findings of cohort and PMR studies which have evaluated the relationship between lung cancer and formaldehyde exposure among anatomists, pathologists and embalmers. All 3 of these groups are exposed to solutions of formaldehyde and other chemicals which are used for fixing human tissues. Although these groups only have intermittent exposures to formaldehyde, their peak exposures tend to be high. A deficit in mortality from lung cancer was evident in all but one of these studies (Walrath and Fraumeni, 1983) for which the relative risk estimates (OR=1.08) was close to one. The relative risk estimate for lung cancer decreased in this study when a 35 year latency period was assumed.

Table 2. Relative Risk (RR) Estimates and 95% Confidence Intervals (95%CI) for Lung Cancer Mortality from Cohort and PMR Studies of Embalmers, Pathologists and Anatomists (Professionals) With and Without Latency[a]

REFERENCE	GROUP STUDIED	STUDY DESIGN	STUDY SIZE	OVERALL OBS (95%CI)	OVERALL RR (95%CI)	LATENCY OBS (95%CI)	LATENCY RR (95%CI)
Walrath (1983)	New York Embalmers	PMR	1106	72 (0.84-1.36)	1.08	35 (0.70-1.40)	1.01
Walrath (1984)	California Embalmers	PMR	1007	41 (0.69-1.30)	0.96	17 (0.62-1.70)	1.06
Levine (1983)	Ontario Embalmers	SMR	1477	19 (0.57-1.47)	0.89	-	-
Harrington (1975)	British Pathologists	SMR	2079	11 (0.31-1.00)	0.39	-	-
Harrington (1984)	British Pathologists	SMR	2307	9 (0.19-0.78)	0.40	-	-
Logue (1986)[b]	American Pathologists	SMR	5585	-	0.24	-	-
Stroup (1984)	American Anatomists	SMR	2239	12 (0.14-0.49)	0.28	-	-

a. Relative risk estimates using a latency period assumption were not always reported by the authors, and the latency period assumed varied between 20-35 years.
b. The observed and expected numbers of death, and confidence intervals were not reported for this study.

Table 3 presents a summary of the findings for lung cancer among industrial groups exposed to formaldehyde. The first 2 studies by Marsh (1982) and Liebling et al (1982) were of workers at the same Massachusetts formaldehyde plant, but covered different time periods at this facility. All of the studies were of workers exposed to formaldehyde in the chemical industry, except for one study which concerned workers exposed to formaldehyde in a permanent press garment facility (Stayner et al, 1988).

The largest excess of lung cancer was reported among Italian chemical workers exposed to formaldehyde (SMR=1.36); however, an even greater risk of lung cancer was reported among workers exposed to other chemicals (SMR=1.48) in this study (Bertazzi et al, 1986). A significant excess in lung cancer was reported in 1 of the 6 facilities included in Acheson's (1984) study, and in 1 of the 3 facilities included in the study by Stayner et al (1988). In the Blair study (1986) a statistically significant excess of lung cancer (SMR=1.32) was reported among wage workers with more than 20 years since their first exposure to formaldehyde (latency). Lung cancer mortality did not appear to increase with latency in any of the other studies of industrial workers. Lung cancer mortality also did not appear to increase with duration or dose of formaldehyde exposure in any of these studies.

Table 3. Relative Risk (RR) Estimates and 95% Confidence Intervals (95%CI) for Lung Cancer Mortality from Cohort and PMR Studies of Industrial Workers With and Without Latency[a]

REFERENCE	GROUP STUDIED	STUDY DESIGN	STUDY SIZE	OVERALL OBS (95%CI)	OVERALL RR	LATENCY OBS (95%CI)	LATENCY RR
Marsh (1982)	American Chemical	PMR	136	6 (0.29-1.73)	0.80	3 (0.17-2.41)	0.83
Liebling (1982)	American Chemical	PMR	24	3 (0.27-3.81)	1.29	-	-
Wong (1983)	American Chemical	SMR	2026	11 (0.47-1.69)	0.94	6 (0.32-1.90)	0.87
Acheson E (1984)	British Chemical	SMR	17836	205 (0.83-1.09)	0.95	-	-
Blair A (1986)	American Chemical	SMR	26561	220 (0.95-1.24)	1.08	151 (1.12-1.55)	1.32*
Bertazzi P (1986)	Italian Chemical	SMR	1332	5 (0.44-3.19)	1.36	2 (0.14-4.66)	1.29
Stayner L (1988)	American Garment	SMR	11030	39 (0.81-1.56)	1.14	8 (0.38-1.71)	0.87

a. Relative risk estimates with a latency assumption were not always reported by the authors, and the latency period assumed varied between 15-20 years.

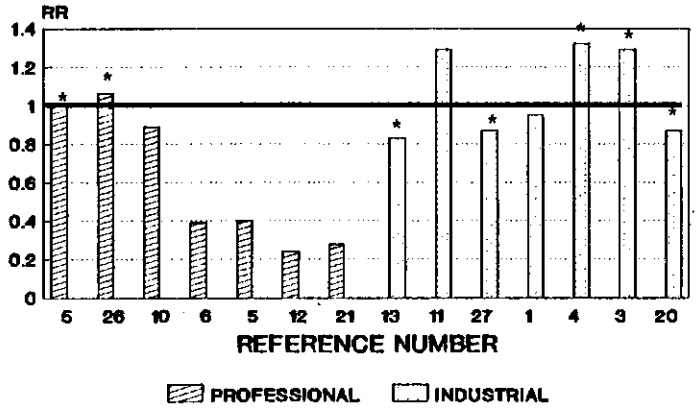
*p < .05 (one tail)

Figure 1 graphically summarizes the results for lung cancer for all of the cohort and PMR studies. Overall, in the majority (10 of 14) of the studies conducted the relative risk estimate for lung cancer was less than or equal to 1. The relative risk estimate was greater than 1 in 3 of 7 of the studies of industrial groups, in contrast with the studies of professional groups in which the relative risk was greater than 1 in only 1 of 7 studies. In the studies demonstrating an excess, however, the relative risk estimates were only moderately increased. It is important to recognize that cigarette smoking information was not controlled for in any of these investigations. Thus, differences in smoking prevalence may in part explain the discrepancies in the findings between studies of professional and industrial workers.

Although an excess of nasal cancer was not observed in any of the cohort or PMR studies, these studies were all lacking in statistical power for this relatively rare tumor. Statistically significant excesses of other upper respiratory tract cancer sites have been reported in 3 of the 6 studies of industrial workers exposed to formaldehyde, but not in any of the studies of professional workers. Liebling et al (1982) reported a statistically significant excess of buccal-pharyngeal cancer among chemical workers exposed to formaldehyde, which was based on only 2 cases. Blair et al (1986) reported a statistically significant 3-fold excess of nasopharyngeal cancers in his study of chemical workers exposed to formaldehyde. This excess was reported to increase with cumulative formaldehyde

exposure among workers exposed to particulates, but not among workers exposed to formaldehyde alone (Blair et al, 1987). Finally, Stayner et al (1988) in a study of garment workers exposed to formaldehyde reported a significant 3-fold excess of buccal cavity cancer.

FIGURE 1
LUNG CANCER COHORT STUDIES



* Latency adjusted RRs presented when available

This table presents a summary of the results from the case-control studies which have been conducted to evaluate the possible association between formaldehyde exposure and respiratory tract cancer. The potential confounding influence of cigarette smoking was controlled for in only four of these investigations (Vaughan et al, 1986, Hernberg et al, 1983, Partanen et al, 1986, and Hayes et al, 1986). The first 2 studies concerned the association between lung cancer and formaldehyde exposure. Jensen and Andersen (1982) detected no association (OR=1.0) between lung cancer and formaldehyde exposure among Finnish woodworkers. The other lung cancer case-control study by Partanen (1985) detected only a slight statistically nonsignificant association (OR=1.4) between lung cancer and employment as a pathologist among Danish physicians.

The earliest nasal cancer case-control study (Hernberg, 1983) reported no association between formaldehyde exposure and nasal cancer; however, according to the authors this study was not specifically designed to address this question. Olsen et al (1984) and Hayes et al (1986) both reported a significant association between formaldehyde exposure and nasal cancer (OR=2.8 and OR=2.5 respectively). In both of these investigations, the association with formaldehyde exposure appeared to be independent of wood dust exposure. Vaughan et al (1986) in a case-control study in Washington State, examined the association between formaldehyde exposure and

nasal, nasopharyngeal, and oro-hypopharyngeal cancer. They reported a statistically nonsignificant association between occupational exposure to formaldehyde and nasopharyngeal (OR=2.1), and oro-hypopharyngeal cancer (OR=1.5). Vaughan et al (1986) also reported a statistically significant association (OR=2.3) between nasopharyngeal cancer and possible residential formaldehyde exposure (mobile home residence), which is not shown in Table 5. Finally, Rousch et al (1987) reported a statistically significant association between formaldehyde exposure and nasopharyngeal cancer (OR=2.3), and a statistically nonsignificant association (OR=1.5) with nasal cancer among individuals after a 20 year latency period.

Table 4. Odds Ratio (OR) Estimates and Confidence Intervals (CI) from Respiratory Cancer Case-Control Studies With and Without a Latency[a]

REFERENCE	CANCER SITE	NUMBER OF CASES	NUMBER OF CONTROLS	OVERALL ODDS RATIO (95%CI)	LATENCY ODDS RATIO (95%CI)
Jensen (1982)	Lung	84	252	1.0	-
				(0.4-2.4)	-
Partanen (1985)	Lung	57	171	1.4	1.3
				(0.7-3.0)[b]	(0.5-3.0)[b]
Hernberg (1983)[c]	Nasal	167	167	1.0	-
				-	-
Olsen (1985)	Nasal	525	2465	2.8*	3.1*
				(1.8-4.3)	(1.8-5.3)
	Nasopharynx	314	2465	0.7	-
				(0.5-14.3)	-
Hayes (1986)[d]	Nasal	116	259	2.5*	-
				(1.5-4.3)[b]	-
Vaughan (1986)	Nasal	53	552	0.3	0.0
				(0.0-2.3)	-
	Nasopharynx	27	552	2.1	2.1
				(0.6-7.8)	(0.4-10.0)
	Oro-Hypopharynx	205	552	1.5	1.3
				(0.7-3.0)	(0.6-3.1)
Rousch (1987)	Nasal	198	605	1.0	1.5
				(0.5-2.2)	(0.6-3.9)
	Nasopharynx	173	605	1.4	2.3*
				(0.6-3.1)	(0.9-6.0)

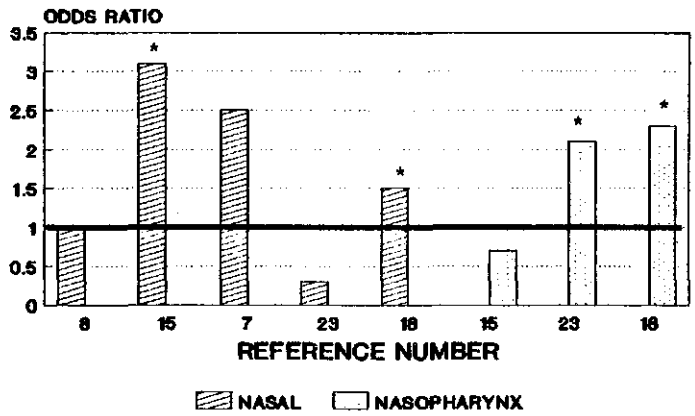
a. Odds ratios with a latency assumption were not always reported, and the latency period assumed varied between 10-20 years.
b. 90 % confidence interval were reported and presented here.
c. An odds ratio for formaldehyde exposure was not reported, however, the authors indicated that the frequency of possible formaldehyde exposure was evenly distributed between the cases and controls.
d. A second analysis was performed using another exposure classification which resulted in an odds ratio estimate of 1.9 (90%CI=1.2-3.0).

*p < .05 (one tail)

The pattern of the findings from the upper respiratory tract cancer case-control studies is graphically represented in Figure 2. In the majority (5 of 8) of these studies the relative risk (odds ratio)

estimate was greater than one. In two of the studies of nasopharyngeal cancer the odds of formaldehyde exposure was approximately twice as high among the cases than among the controls. However, in the 3rd nasopharyngeal cancer case-control study the odds ratio was less than 1. The results were also mixed for nasal cancer for which 3 of the 5 studies reported odds ratios which were greater than 1.

FIGURE 2
CASE-CONTROL STUDIES



*Latency adjusted ORs presented when available

Conclusions

Despite the completion of over 20 epidemiologic investigations, a considerable controversy still exists regarding the possible relationship between formaldehyde exposure and cancers of the respiratory tract in man. The findings from the studies which have been performed to date appear to be inconsistent with a large increase in the relative risk of lung cancer due to formaldehyde exposure. It is worth noting, however, that due to the high background rate of lung cancer and the large number of individuals who are exposed to formaldehdye, even a small increase in relative risk would be important from a public health viewpoint. Lung cancer mortality was less than expected in most of the studies, and only moderately increased in a few of the cohort studies. In addition, the odds ratio estimate was close to 1.0 in the two lung cancer case-control studies which have been conducted. Although failure to control for cigarette smoking in these studies clouds the interpretation, it seems unlikely that smoking could fully explain these findings.

The evidence is suggestive for a relationship between exposure to formaldehyde and upper respiratory tract cancer in humans. An

elevated odds ratio for formaldehyde exposure was reported in 3 of the 5 nasal cancer case-control studies. In two of these studies the odds ratio was statistically significant and greater than 2. A statistically significant association with formaldehyde exposure was reported in 1 of the nasopharyngeal case-control studies, and a positive but statistically nonsignificant association was reported in another. A statistically significant excess in buccal or buccal-pharyngeal cancer mortality was reported in 2 of the 6 cohort studies of industrial workers exposed to formaldehyde, and a significant excess of nasopharyngeal cancer was reported in 1.

It should be recognized that inconsistencies between these studies might be expected, given the large variations in the dose, form and pattern of the formaldehyde exposures in the study populations. The findings from the NCI study which suggest that formaldehyde may interact with particulate exposures in the causation of upper respiratory tract cancers in man are particularly intriguing, and may explain why several of the other studies failed to detect an excess risk. Future research efforts should be directed towards studies which better define formaldehyde dose, and which examine potential interactions between formaldehyde, particulates and other exposures in the workplace in the causation of cancers of the upper respiratory tract.

References

1) Acheson, E.D., Barnes, H.R., Gardner, M.J., Pannett, B., Barnes, H.R., Osmond, C., and Taylor, C.P., 1984. Formaldehyde in the British chemical industry: An occupational cohort study. Lancet i 611-616.
2) Albert, R.E., Sellakumar, A.R., Laskin, S., Kuschner, M., Nelson, A., and Snyder, C.A., 1982. Nasal cancer in the rat induced by gaseous formaldehyde and hydrogen chloride. J. Natl. Cancer Inst. 68:597-603.
3) Bertazzi, P., Pesatori, A., Radice, L., Zocchetti, C., and Vai, T., 1986. Exposure to formaldehyde and cancer mortality in a cohort of workers producing resins. Scand. J. Work Env. Health, 12: 461-468.
4) Blair, A., Stewart, P., O'Berg, M., Gaffey, W., Walrath, J., Ward, J., Bales, R., Kaplan, S., and Cubit, D., 1986. Mortality among industrial workers exposed to formaldehyde. J. Natl. Cancer Inst 37(4):487-492.
5) Harrington, J.M. & Oakes, D., 1984. Mortality among British pathologists. Br. J. Ind. Med. 41:188-191.
6) Harrington, J.M. & Shannon, H.S., 1975. Mortality study of pathologists and medical laboratory technicians. Br. Med. J. 4:329-332.
7) Hayes, R.B., Raatgever, J.W., de Bruyn, A., 1986. Tumors of the nose and nasal sinuses: A case-control study. Int. J. Cancer, 37:487-492
8) Hernberg, S., Westerholm, P., Schultz-Larsen, K., Degerth, R., Kuosma, E., Englund, A., Engzell, U., Hansen, H.S., and Mutanen, P., 1983. Nasal and sinonasal cancer. Connection with occupational exposures in Denmark, Finland and Sweden. Scand. J. Work Env. Hlth., 9:315-326.
9) Jensen, O., Andersen, ?., 1982. Lung cancer risk from formaldehyde. Lancet i:913.

10) Levine, R.J., Andjelkovitch, D.A., Shaw, L.K., & Dal Corso, R.D., 1984. Mortality of Ontario undertakers and a review of formaldehdye-related mortality studies. J. Occ. Med., 26:740-746.
11) Liebling, T., Rosenman, K.D., Pastides, H., Griffith, R.G., & Lemeshow, S., 1983. Mortality in relation to occupation: a study of workers at the Monsanto-Springfield chemical plant. Am. J. Ind. Med., 5:423-428.
12) Logue, J, Barrick, M, Jessup, G., 1986. Mortality of radiologists and pathologists in the radiation registry of physicians. J. Occ. Med., 28(2): 91-99.
13) Marsh, G.M., 1982. Proportional mortality patterns among chemical plant workers exposed to formaldehyde. Br. J. Ind. Med., 39:313-322.
14) National Institute for Occupational Safety and Health, 1981. Current Intelligence Bulletin 34. Formaldehyde: Evidence of Carcinogenecity. U.S. Department of Health and Human Services, Public Health Service, Centers for Disease Control, National Institute for Occupational Safety and Health, Cincinnati, OH 45226
15) Olsen, J.H., Jensen, S.P., Mogens, H., Faurbo, K., Breum, N.O., & Jensen, M., 1984. Occupational formaldehyde exposure and increased nasal cancer risk in man. Int. J. Cancer 34:639-644.
16) Partanen, T., Kauppinen, T., Nurminen, M., Nickels, J., Hernberg, S., Hakulinen, T., Pukkala, E., Savonen, E., 1985. Formaldehyde exposure and respiratory and related cancers. Scand. J. Environ. Health 11:409-415.
17) Rothman, K., 1986. Modern Epidemiology. Little Brown and Company, pp. 115-119.
18) Rousch, G., Walrath, J., Stayner, L., Kaplan, S., Flannery, J., Blair, A., 1987. Nasopharyngeal cancer, sinonasal cancer, and occupations related to formaldehyde: A case-control study. JNCI, 79(6):1221-1224.
19) SRI International, 1985. Directory of Chemical Producers. SRI International, Menlo Park, CA, p. 617.
20) Stayner, L., Elliot, L., Blade, L., Keenlyside, R., Halperin, W., 1988. A retrospective cohort mortality of workers exposed to formaldehyde in the garment industry. Am. J. Ind. Med., 13:667-681.
21) Stroup, N.E., 1984 A retrospective follow-up study of mortality in anatomists. Ph.D. Dissertation. Yale University, New Haven, Connecticut.
22) Swenberg, J.A., Kerns, W.D., Mitchell, R.E., Gralla, E.J., & Pavkov, K.L., 1980. Induction of squamous cell carcinomas of the rat nasal cavity by inhalation exposure to formaldehyde vapor. Cancer Res. 40:3398-3402.
23) Vaughan, T.L., Strader, C., Davis, S. & Daling, J.R., 1986. Formaldehyde and cancers of the pharynx, sinus and nasal cavity: I. Occupational exposures. Int. J. Cancer 38:677-683.
24) Vaughan, T.L., Strader, C., Davis, S. & Daling, J.R., 1986. Formaldehyde and cancers of the pharynx, sinus and nasal cavity: II. Residential exposures. Int. J. Cancer 38:685-688.
25) Walrath, J. & Fraumeni, J.F., 1983. Mortality patterns among embalmers. Int. J. Cancer 31 407-411.
26) Walrath, J. & Fraumeni, J.F., 1984. Cancer and other causes of death among embalmers. Cancer Res. 44 4638-4641.

27) Wong, O. An epidemiologic mortality study of a cohort of chemical workers potentially exposed to formaldehyde. In: Formaldehyde Toxicitiy (J.E. Gibson, Ed.), Hemisphere, New York, pp. 256-272.

Part 3

Mechanisms of nasal carcinogenesis

DEPOSITION AND ABSORPTION OF INHALED VAPORS IN THE NASAL CAVITY

J.B. Morris

Toxicology Program, University of Connecticut, Storrs, CT, U.S.A.

Summary

The upper respiratory tract (URT) deposition of selected non-reactive vapors has been investigated in the guinea pig, hamster, mouse (B6C3F1), and two strains of rats (F344 and Sprague-Dawley). In all animals deposition efficiency was highly dependent on the inspiratory flow rate. Deposition efficiencies as low as 5-32% were observed for acetone and ethanol vapors despite the fact that these solvents are miscible with water, indicating high water solubility alone is not sufficient for efficient URT deposition. Metabolism within the nasal mucosa can enhance URT deposition efficiency, however, this effect 1) was not observed for all vapors which were metabolized by the nasal mucosa in vitro, and 2) when observed, was dependent on the flow rate at which the vapor was inspired. Large species differences were observed in URT acetone deposition, with deposition being most efficient in the Sprague-Dawley rat, least efficient in the hamster and guinea pig and of intermediate efficiency in the F344 rat and mouse. Species differences did not correlate with nasal cavity surface areas but may be due to differences in nasal perfusion rates.
Keywords: vapor deposition, species differences, nasal metabolism

Introduction

The most fundamental principle of the science of toxicology is dose-response. Intrinsic in this principle is the tenet that the response is proportional to the concentration of toxicant received at the target site. That regional deposition is important in determining the concentration of inhaled toxicant received in potential respiratory tract target tissues and, therefore, in determining the local response has long been realized. Deposition of inhaled materials in the upper respiratory tract (URT) serves to protect the lower respiratory tract from their potential toxic effects. This phenomenon, however, places the nasal tissues at high risk for injury. The physical and physiologic factors which influence inhaled aerosol deposition are well understood and several excellent reviews of this field have been published (e.g., Schreider, 1983). Our understanding of the factors which influence regional gas or vapor deposition is minimal.

Theoretical considerations

While URT vapor deposition is poorly understood, the phenomena involved in gas-liquid interactions and in the alveolar uptake of vapors are well described (Henderson and Haggard, 1943). With appropriate qualifications the principles of these fields can be applied to the URT. Briefly, airborne vapor molecules may diffuse to the fluid interface and may, if sufficiently soluble dissolve in this fluid. In the ideal case vapor solutes reach equilibrium between gaseous and liquid solvents in

accordance with Henry's Law. The critical factor in this regard is that the liquid (tissue) phase becomes saturated with vapor molecules. The concentration in the tissue phase at saturation is determined by the airborne concentration and the tissue-air partition coefficient. Once tissues become equilibrated (saturated), continued uptake of vapor can only occur if vapor molecules are removed from the interface. Vapor molecules may be removed by: 1) reaction with water or tissue components (either enzymatically or non-enzymatically) or, 2) diffusion to deeper tissues or to the capillaries and removal via the bloodstream. In URT tissues removal via mucociliary transport may occur, but the volumetric flow rate of URT mucus is so low that this can not offer a significant removal pathway (Morris et al., 1987;Morgan et al., 1984).

When measured under constant velocity inspiratory flow conditions, URT deposition efficiency rapidly reaches a plateau level which is maintained for as long as 2-3 hours (Aharonson et al. 1974; Stott & McKenna, 1984). Because of its small volume, URT tissues must equilibrate with inspired vapor quickly. Continued deposition can not be explained by diffusion throughout URT tissues but must be due to continued removal via chemical reaction or via the bloodstream. At steady state, the net amount of vapor entering URT tissues (e.g. depositing) must exactly balance the amount of vapor leaving the tissues. This can be expressed mathematically as follows:

Deposition rate = amount removed via bloodstream +
amount metabolized + amount directly reacted (1).

This relationship is of fundamental importance. There are no assumptions involved in its derivation, it is merely an expression of conservation of mass at steady state. Our research has focussed on this equation and has been limited to vapors which do not directly react with water or tissue components thus removing the "amount directly reacted" term. For information on deposition mechanisms of directly reactive gases the reader is referred to the work of Overton and Miller (1988).

The maximal capacity for removal of vapors from the URT via the bloodstream or via metabolism is fixed. Metabolic clearance rates can not exceed the maximal velocity (Vmax) of the enzymes present in the nose. The capacity for circulatory removal is determined by the local volumetric blood flow rate and by the solubility of vapor in the blood, which is dependent on the blood-air partition coefficient. While beyond the scope of this text, the partitioning of inspired vapors between the airstream and the bloodstream would be predicted to be dependent on the inspiratory flow rate due to the greater volume of air drawn through the URT (per unit time) at high flow rates compared to low flow rates (Morris et al., 1986; Morris and Cavanagh, 1986, 1987).

The experimental approach utilized in our studies was straightforward. Vapor laden, heated-humidified air was drawn through the surgically isolated URT of anesthetized animals under constant velocity flow conditions at selected flow rates ranging from 50% to 275% of the minute ventilation (Vm) as predicted by the equation of Guyton (1947). Analysis of vapor concentration in air entering and leaving the URT allowed for calculation of deposition efficiency. Multiple repetitive air samples were drawn through the URT of each animal to confirm the attainment of steady state deposition conditions.

Non-Metabolized vapors

Our initial studies focussed on non-reactive, non-metabolized vapors, utilizing acetone and ethanol for model vapors. The selection of these vapors was based in part upon the studies of Landahl and Herrmann (1950) who examined the deposition of these vapors in the human URT. To date, we have examined deposition of one or both of these vapors in the Hartley guinea pig, Syrian golden hamster, Sprague-Dawley (S-D) rat, Fischer 344 (F344) rat and B6C3F1 mouse. Deposition of these vapors rapidly attained an apparent steady state in all of these animals. Shown in Table 1 are the average URT steady state deposition efficiencies observed at selected flow rates in the F344 rat.

Table 1. Steady state URT deposition efficiencies in the F344 rat

	Inspiratory flow rate (% of minute ventilation-Vm)		
	50% Vm	100% Vm	275% Vm
Acetone	45%	34%	12%
Ethanol	84%	59%	32%

These data indicate that deposition efficiency is highly dependent on the inspiratory flow rate at which it was measured. This phenomenon has been observed in every species examined to date in our laboratory and also in the dog (Aharonson et al., 1974) and the human (Landahl and Hermann, 1950). This is a critical concept. A complete knowledge of vapor deposition in any species requires investigations utilizing multiple flow rates. Furthermore, it is necessary to perform some flow rate normalization to compare URT deposition efficiencies among species with differing ventilation rates. It is not known whether any of these flow rates is reflective of the average flow under normal cyclic respiratory conditions. The duration of the inspiratory phase is roughly one-half of the total respiratory cycle suggesting the best estimate would be a flow rate equal to or greater than twice the predicted Vm. It is apparent that further work is necessary to clarify this point, however, this line of reasoning suggests deposition efficiencies of 12 and 32% would be expected to be most reflective of that occurring normally. The literature is replete with references that URT deposition efficiency correlates with aqueous solubility and that highly soluble vapors would be expected to deposit efficiently in that site. Such statements are overly simplistic. Both acetone and ethanol are miscible with water, they are infinitely soluble. Despite this, deposition efficiencies of only 12 and 32 % are observed. Deposition efficiencies as low as 5-7% were observed in the hamster and guinea pig (see below). High water solubility alone is not sufficient to cause extremely efficient deposition. URT Deposition efficiencies of 90% or greater are typically observed only for those vapors which are directly reactive with water or tissue components. In these circumstances it appears that deposition is controlled by the rate of reactivity rather than by solubility (Overton and Miller, 1988). Deposition depends on the ability of nasal circulation to remove vapors as determined by the

local perfusion rate and the solubility of vapor in blood (as reflected by the blood-air partition coefficient). The enhanced deposition of ethanol compared to acetone is due to its greater blood-air partition coefficient. The deposition efficiencies of both vapors are low because nasal perfusion is not fast enough to support high deposition rates (Morris et al., 1986; Morris & Cavanagh, 1986, 1987).

Metabolized vapors

Stott and McKenna (1984) have shown that URT deposition efficiencies for ethyl acrylate, a substrate for carboxylesterases, can be reduced by approximately 50% by administration of a carboxylesterase inhibitor. Our studies provide a framework with which to understand and quantitate the effect of metabolism on URT vapor deposition. Local metabolism in URT tissues provides an alternate source for removal of vapors and, therefore, would be expected to enhance deposition efficiencies. At tissue concentrations above enzyme saturation, metabolism is a zero-order process, however, transfer of vapor molecules to the bloodstream is a first-order process. Consequently, examination of the effects of inspired vapor concentration on deposition efficiency provides a way to quantitate apparent in vivo URT metabolism rates. (For a complete discussion of the theory see Morris & Cavanagh, 1987).

In vitro studies indicated the presence of significant levels of alcohol dehydrogenase in the nasal mucosa of the hamster. (Significant levels of this enzyme were not observed in other species.) The nasal mucosa of the hamster metabolized propanol with a Vmax of 2.2 ug/min. A strong concentration dependence on URT deposition efficiency of propanol vapor was observed at an inspiratory flow rate equivalent to 275% Vm in the hamster. From the deposition-inspired concentration relationship an apparent in vivo metabolism rate of 3.8 $\pm$ 1.3 ug/min (Mean $\pm$ SE) was calculated. This value did not differ significantly from the metabolism rate observed in vitro. Surprisingly, no concentration dependence on deposition was observed at low inspiratory flow rates (100% or 50% Vm) suggesting that when inspired at these flows propanol vapor was not metabolized in nasal tissues. Thus, the extent of local metabolism is apparently dependent on the inspiratory flow rate. It is not known if this effect is specific to propanol or applies to other vapors as well. This effect may be due to nasal ventilation patterns. At low inspiratory flow rates the airstream tends to follow the ventral meatus; at high flows it follows the dorsal meatus and may actually penetrate into the olfactory epithelial-lined ethmoturbinates (Dawes, 1952). Perhaps propanol metabolizing enzymes are concentrated in the dorsal meatus and/or ethmoturbinates. This result was not predictable from in vitro studies and indicates that in vitro information alone is not sufficient to predict effects occurring in vivo. This concept has been confirmed in our recent studies on acetone deposition in the B6C3F1 mouse. Nasal tissues from this mouse metabolize acetone vapor via a NADPH dependent pathway at a high rate in vitro. (Metabolism of acetone was not apparent in nasal mucosa of any other species.) In vivo deposition studies, however, failed to detect a concentration dependence on acetone deposition in this species suggesting that metabolism of this vapor did not occur in the in vivo setting. Perhaps acetone metabolizing enzymes are located in cells which are not in direct communication with the airstream. These data provide further indication of the dangers involved in extrapolating data obtained in vitro to the

in vivo condition and indicate we have much to learn about the nasal ventilation and deposition process.

Species Differences

A principal aim of our studies has been to provide detailed information on species-specific variability in nasal vapor deposition. As outlined above such comparisons can only be made if a complete understanding of the effect of inspiratory flow rate is known and if some normalization with respect to flow rate is performed. For the sake of comparison, inspiratory flow rate was normalized to predicted Vm (Guyton, 1947). The results are shown in Table 2 along with the nasal cavity surface area of each animal model (if known) and the blood-air partition coefficient for each species. Nasal cavity surface area was also normalized to predicted Vm to allow comparison between these species with markedly different ventilation rates.

Table 2. URT acetone deposition in several species

	Species				
	S-D rat	F344 rat	B6C3F1 mouse	Hartley guinea pig	Syrian hamster
100% Vm	37%	34%	25%	13%	19%
275% Vm	21%	12%	14%	7%	5%
Surface area mm2/ml/min	8	9	10	10	--
Blood-air Partition Coeff.	275	260	282	270	275

Data from species which are contained within the same box are not statistically different, those in different boxes are different at the $p<0.05$ level (Two-factor ANOVA, Newman-Keuls test).

Significant species differences were observed. URT acetone deposition efficiency was 2-3 fold lower in the hamster and guinea pig than the S-D rat. Efficiencies were intermediate in the F344 rat and B6C3F1 mouse. The mouse data were included in this comparison despite the existence of in vitro metabolism because no evidence of in vivo metabolism was observed. Species differences were not due to differing blood-air partition coefficients as this parameter was similar in all species. Species differences did not correlate with the structural complexity of the nasal cavity as quantitated by surface area, viz., deposition was most efficient in the S-D rat yet this strain of rat had the lowest surface area. Landahl and Herrmann (1950) measured acetone deposition efficiency in the human nose and reported values of roughly 20% (at 250-300% Vm). While comparison to the data of Landahl and Herrman (1950) must be made with caution due to differing experimental methodologies, these data suggest the human nose has a relatively high

capacity for scrubbing inspired non-reactive vapors despite its low surface area (2 mm2/ml/min). At least superficially it would appear that the S-D rat is a good model for the human. The physiologic basis for these species differences is not known, however, based on our theoretical understanding of the URT deposition process is seems likely that these differences are attributable to species-specific variation in nasal perfusion rates. Whatever the physiologic basis, it is apparent that potential species differences in URT deposition efficiency will need to be taken into consideration when comparing toxicity data between species or when extrapolating such data to the human.

Acknowledgments

This work was supported by NIH grants 1R01 ES03676 and 2R01 ES03676.

References

Aharonson, E.F., H. Menkes, G. Gurtner, D.L. Swift & D.F. Proctor, 1974. Effect of respiratory airflow rate on removal of soluble vapors by the nose. J. Appl. Physiol. 37:654-657.

Dawes, J.D.K., 1952. The course of nasal airstreams. J. Laryngol. Otol. 66:583-593.

Gross, E.A., J. Swenberg, S. Fields & J.A. Popp, 1982. Comparative morphometry of the nasal cavity in rats and mice. J. Anat. 135:83-88.

Guyton, A.C., 1947. Measurement of the respiratory volumes of laboratory animals. Amer. J. Physiol. 150:70-77.

Henderson, Y. & H.W. Haggard, 1943. Noxious gases and the principles of respiration influencing their action. Reinhold, New York.

Landahl, H.D. & R.G. Herrmann, 1950. Retention of vapors and gases in the human nose and lung. A.M.A. Arch. Ind. Health 1:36-45.

Morgan, K.T., X.-Z. Jiang, D.L. Patterson & E.A. Gross, 1984. The nasal mucociliary apparatus. Correlation of structure and function in the rat. Amer. Rev. Resp. Dis. 130:275-281.

Morris, J.B. & D.G. Cavanagh, 1986. Deposition of ethanol and acetone vapors in the upper respiratory tract of the rat. Fundam. Appl. Toxicol. 6:78-88.

Morris, J.B., R.J. Clay and D.G. Cavanagh, 1986. Species differences in upper respiratory tract deposition of acetone and ethanol vapors. Fundam. Appl. Toxicol. 7:671-680.

Morris, J.B. & D.G. Cavanagh, 1987. Metabolism and deposition of propanol and acetone vapors in the upper respiratory tract of the hamster. Fundam. Appl. Toxicol. 9:34-40.

Overton, J.H. & F.J. Miller, 1988. Absorption of inhaled reactive gases. In: D.E. Gardner, J.D. Crapo & E.J. Massaro (Ed.): Toxicology of the lung. Raven Press, New York. p. 477-507.

Schreider, J.P., 1983. Nasal airway anatomy and inhalation deposition in experimental animals and people. In: G. Reznik and S.F. Stinson (Ed.): Nasal tumors in animals and man. Vol. III. CRC Press, Boca Raton. p. 1-26.

Stott, W.T. and M.J. McKenna, 1984, The comparative absorption and excretion of chemical vapors by the upper respiratory tract of rats. Fundam. Appl. Toxicol. 4:594-602.

UPTAKE AND IRREVERSIBLE BINDING OF XENOBIOTICS IN THE NASAL MUCOSA

E.B. Brittebo

Dept. of Pharmacology and Toxicology, Faculty of Veterinary Medicine, the Swedish University of Agricultural Sciences, Uppsala Biomedical Centre, Box 573, S-751 23 Uppsala, Sweden

Summary

* Various rodent epithelial linings, including those of the nasal cavity, have been shown to have a marked ability to metabolize and irreversibly bind xenobiotics such as short-chained and cyclic halogenated hydrocarbons, N-nitrosamines and certain drugs. The metabolic activities of the epithelia in many cases exceed those of the liver.

* The nasal carcinogen phenacetin has been used to determine the cellular sites of activation in glutathione-depleted nasal septa incubated in vitro. The Bowman's glands, the supporting cells in the olfactory mucosa and the nonciliated cells and goblet-cells in the respiratory epithelium were found to irreversibly bind this drug.

* The olfactory mucosa of late gestational rat fetuses had a phenacetin O-dealkylating ability that increased with fetal age and almost reached the adult level on the first day of life.

Keywords: halogenated hydrocarbons, N-nitrosamines, phenacetin, Bowman's glands, supporting cells, nonciliated cells, fetal and neonatal olfactory mucosa.

Introduction

Many xenobiotics induce a variety of toxic effects including necrosis and carcinogenesis following transformation to chemically reactive metabolites that become irreversibly bound to the tissue (Miller and Miller, 1978). Such metabolites may be formed in different tissues by a number of enzyme systems - the cytochrome P-450 dependent monooxygenase system being the dominating one. In many cases, organ-specific toxicity is dependent upon metabolic events that occur at the target site. This presentation will focus on the metabolic activation of some compounds in the olfactory mucosa of fetal and adult mice and rats.

Sites of irreversible binding in the body

By the use of various autoradiographic techniques the distribution of irreversibly bound metabolites in the tissues of animals dosed with radiolabelled xenobiotics has been studied. As can be seen in Table 1, a variety of xenobiotics are metabolized and irreversibly bound in the nasal olfactory mucosa in vivo and in vitro. The autoradiographic studies have also demonstrated a pronounced irreversible binding of metabolites in several other surface epithelia of the body, predominantly in the tracheo-bronchial epithelium, the epithelium of the upper alimentary tract and in the cervico-vaginal epithelium. This pattern of irreversible metabolite-binding is a frequent finding after administration of

short-chained and cyclic halogenated hydrocarbons and N-nitrosamines. A typical example is shown in Fig. 1.

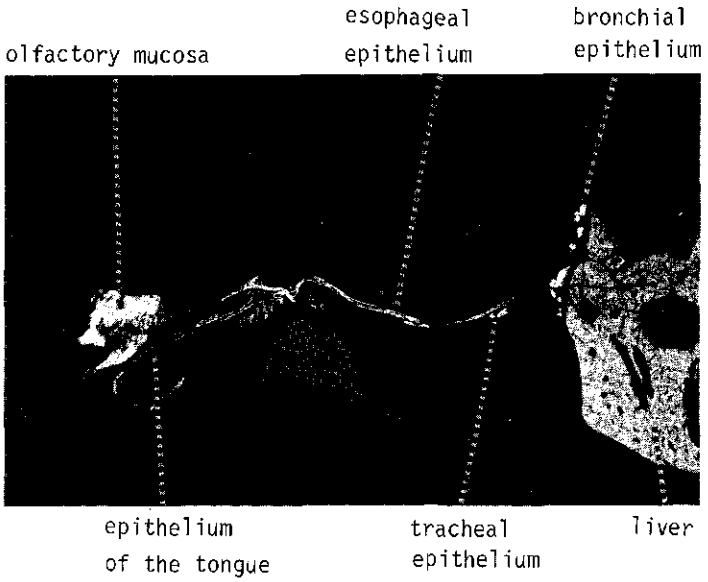


Fig. 1. Autoradiogram showing the distribution of metabolites 30 min. after an i.v. injection of ^{14}C-1,2-dibromoethane in a C57Bl mouse. There is an intense labelling of the olfactory and tracheo-bronchial mucosa, the mucosa of the tongue, esophagus and of the liver.

It thus appears that the mucosal linings of the portals of entry into the body have a high ability to activate certain xenobiotics into reactive metabolites. The concentration of bound metabolites in the epithelia in many cases markedly exceeds that of the liver. With the exception of the nasal mucosa and bronchial epithelium, the drug metabolizing activity in surface epithelia and certain subepithelial glands is not generally acknowledged, however. In homogenates of a whole organ, e.g. of the esophagus, the enzyme system of the metabolically active epithelial cells is diluted with inactive tissue in excess. Consequently, the metabolic activities in the studied organ will be underestimated and even considered insignificant. However, in homogenates prepared from the nasal mucosa, the dilution of the metabolically active cells with inactive tissue (such as nerve bundles and connective tissue) is less pronounced and the high drug-metabolizing activity is more easily observed. A high rate of metabolic activation of the nasal carcinogens N-nitrosonornicotine, 1,2-dibromoethane and phenacetin in nasal homogenates has been demonstrated (Brittebo and Tjälve, 1981; Kowalski et al., 1985; Brittebo, 1987).

In some cases, the irreversible *in vivo* binding of metabolites in the nasal mucosa is highly selective and not only exceeds that of the liver but also all other tissues and epithelial linings of the body. This was observed after administration of the halogenated herbicides 2,6-dichloro-benzamide, -tiobenzamide and -benzonitrile. The reason for the pronounced and selective binding of these herbicides in the nasal mucosa has not been determined (Bakke et al., in press).

Table 1. Xenobiotics which are irreversibly bound in the olfactory mucosa of adult mice and/or rats.

Compounds	In vivo	In vitro	Reference
Halogenated hydrocarbons			
1,2-dichloroethane	+	+	Brittebo et al., unpubl.
1,2-dibromoethane	+	+	Kowalski et al., 1985
1,2-dichloroethylene	+	NE	Brittebo et al., unpubl.
1,1,2,2-tetrachlorethane	+	+	- " -
Carbon tetrachloride	+	+	Bergman, 1979 Tjälve and Löfberg, 1983
Chloroform	+	+	Bergman, 1979 Löfberg and Tjälve, 1986
Halothane	+	+	Ghantous et al., 1988
Chlorobenzene	+	+	Brittebo and Brandt, 1984
Bromobenzene	+	+	Brittebo et al., unpubl.
2,6-dichlorobenzamide	+	NE	Bakke et el., in press
2,6-dichlorothiobenzamide	+	NE	- " -
2,6-dichlorobenzonitrile	+	NE	- " -
γ-hexachlorocyclohexane	+	NE	Brittebo et al., 1987
N-nitrosamines			
N-nitrosodiethylamine	+	+	Brittebo et al., 1981a
N-nitrosopyrrolidine	+	+	Brittebo et al., 1981b
N-nitrosodibutylamine	+	+	Brittebo and Tjälve, 1982
N-nitrosonornicotine	+	+	Brittebo and Tjälve, 1981 Brittebo et al., 1983
Miscellaneous			
Phenacetin	+	+	Brittebo, 1987
Cocaine	+	+	Brittebo, in press

NE = Not examined.

Sites of irreversible binding in the nasal mucosa

The different cell types in the nasal mucosa as well as in other heterogeneous tissues may activate xenobiotics at quite different rates and may differ both qualitatively and quantitatively in this respect. Therefore, the cellular metabolic ability cannot be determined unless each cell type in the nasal mucosa is separated and independently measured. An easier, although less exact way of estimation is autoradiography of the nasal septum or turbinates, which have been incubated in vitro with a radiolabelled compound. Since the morphological and cellular integrity of the tissue is maintained during the incubation, the obtained result represents the composite metabolic activity of the several cell types which make up the nasal mucosa. When using this approach it is essential that the diffusion of the parent compound into the nasal mucosa

is free and not rate limiting. It is also important that the formed metabolites are reactive, so that they do not migrate but become bound to macromolecules in the vicinity of their site of formation.

Autoradiographic studies of a number of compounds have shown that the concentration of irreversibly bound metabolites may vary in the different cell types of the nasal mucosa. As seen in Fig. 2, irreversibly bound metabolites were present in the Bowman's glands but also in the supporting cells of the olfactory epithelium and in the nonciliated cells and goblet-cells of the respiratory epithelium after incubation of glutathione-depleted nasal septa with ^{3}H-phenacetin (Brittebo, 1987). Thus, these cell types can metabolically activate phenacetin. After i.v. injection of ^{3}H-phenacetin into rats, bound metabolites were detected only in the Bowman's glands, however. This discrepancy is probably due to the different ways of exposure of ^{3}H-phenacetin in the nasal mucosa in vitro and in vivo. In vitro the surface epithelial cells are directly exposed to high levels of ^{3}H-phenacetin present in the incubation medium, whereas after parenteral administration the diffusion of ^{3}H-phenacetin to the surface epithelial cells probably is poor as compared to the better perfused subepithelial glands of Bowman.

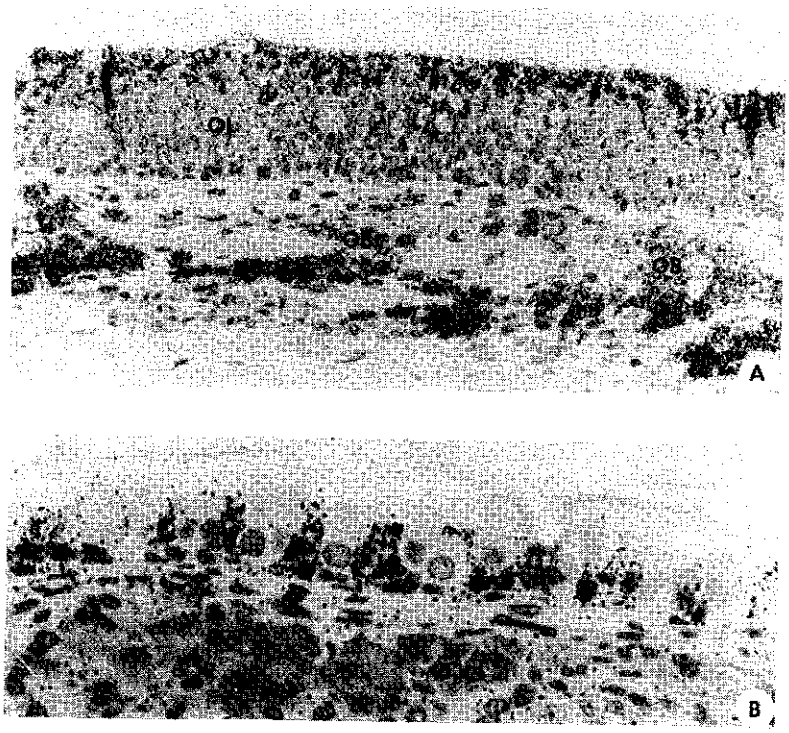


Fig. 2. Microautoradiograms of a glutathione-depleted rat nasal septum which was incubated with ^{3}H-phenacetin for 60 min. A. Olfactory mucosa. B. Respiratory mucosa. In A, tissue-bound metabolites are present in Bowman's glands (GB) and in cells in the outer surface of the olfactory epithelium (OL) - most likely the supporting cells. In B, tissue-bound metabolites are present in goblet-cells and in nonciliated cells, whereas the ciliated cells are not labelled. (Cancer Res. 1987, 47, 1449.)

The underlying basis for the cell specific metabolic activation of phenacetin in the nasal mucosa remains to be elucidated. The presence of certain isozymes of cytochrome P-450 in the respiratory surface epithelium, in the supporting cells of the olfactory surface epithelium and in the subepithelial glands of Bowman is probably of importance (Voigt et al., 1985; Foster et al., 1986).

Usually the irreversible binding of reactive metabolites to tissue macromolecules does not occur until the most abundant cellular nucleophile, glutathione (GSH), has been depleted. This was true also for the nasal binding of phenacetin. After a high dose of phenacetin (100 mg/kg) there was, however, only a limited decrease of the GSH-level in the olfactory mucosa (≈ 70 % of control value). As pointed out previously, the olfactory mucosa is a heterogeneous tissue. A phenacetin-induced depletion of GSH in a restricted cell population may therefore be disguised and underestimated by unchanged GSH-levels in other cell types. It should be noted that cell specific changes may be masked in all types of biochemical determinations that are done on a heterogeneous tissue, such as the nasal mucosa.

Drug metabolism in the fetal and neonatal nasal mucosa

Generally, the capacity of the fetus to metabolize xenobiotics is low or absent as compared to the adult tissues. In certain cases, however, the late gestational fetus is capable of activating the xenobiotic in a tissue-specific manner similar to that of the adult. When phenacetin was injected into rat fetuses *in utero*, a selective labelling of the fetal olfactory mucosa was observed (Brittebo and Åhlman, 1984). *In vitro* experiments confirmed that the fetal olfactory mucosa had an ability to O-dealkylate phenacetin; the activity exceeded considerably that of the fetal liver and increased with fetal age. An irreversible binding of certain N-nitrosamines and short-chained halogenated hydrocarbons in the fetal nasal mucosa of mice has also been observed (Brittebo et al., 1981; Kowalski et al., 1986; Danielsson et al., 1986). Thus, these studies demonstrate the presence of a drug-metabolizing activity in the nasal mucosa before birth.

It is known that olfaction plays a critical role for the behaviour of the neonatal rodent and that the process of olfaction is relatively mature at birth. Already on day 1 of life, the O-dealkylation of phenacetin almost reached the adult level. Thus, the high neonatal drug-metabolizing activity in the olfactory mucosa seems to be linked with the well developed process of olfaction of airborne odorants by the neonatal rodent.

Acknowledgements

Financial support was given by the Swedish Work Environment Fund.

References

Bergman, K., 1979. Whole-body autoradiography and allied tracer techniques in distribution and elimination studies of some organic solvents. Scand. J. Work, Environ. and Hlth 5(Suppl.1):1-263.

Brittebo, E.B., 1987. Metabolic activation of phenacetin in rat nasal mucosa: Dose-dependent binding to the glands of Bowman. Cancer Res. 47:1449-1456.

Brittebo, E.B. and M. Åhlman, 1984. Nasal mucosa from rat fetuses and neonates metabolizes the nasal carcinogen phenacetin. Toxicol. Lett. 23:279-285.
Brittebo, E.B. and I. Brandt, 1984. Metabolism of chlorobenzene in the mucosa of the murine respiratory tract. Lung 162:79-88.
Brittebo, E.B., A. Castonguay, K. Furuya and S.S. Hecht, 1983. Metabolism of tobacco-specific nitrosamines by cultured rat nasal mucosa. Cancer Res. 43:4343-4348.
Brittebo, E.B., P.G. Högman and I. Brandt, 1987. Epithelial binding of hexachlorocyclohexanes in the respiratory and upper alimentary tracts: A comparison between the α-, β- and γ-isomers in mice. Fd Chem. Toxic. 25:773-780.
Brittebo, E.B., A. Lindgren and H. Tjälve, 1981. Foetal distribution and metabolism of N-nitrosodiethylamine in mice. Acta Pharm. Tox. 48: 355-363.
Brittebo, E.B., B. Löfberg and H. Tjälve, 1981a. Sites of metabolism of N-nitrosodiethylamine in mice. Chem.-Biol. Interactions 34:209-221.
Brittebo, E.,B. Löfberg and H. Tjälve, 1981b. Extrahepatic sites of metabolism of N-nitrosopyrrolidine in mice and rats. Xenobiotica 11: 619-625.
Brittebo, E.B. and H. Tjälve, 1981. Formation of tissue-bound N'-nitrosonornicotine metabolites by the target tissues of Sprague-Dawley and Fisher rats. Carcinogenesis 11:959-963.
Brittebo, E.B and H. Tjälve, 1982. Tissue-specificity of N-nitrosodibutylamine metabolism in Sprague-Dawley rats. Chem.-Biol. Interactions 38:231-242.
Danielsson, B.R.G., H. Ghantous and L. Dencker, 1986. Distribution of chloroform, methyl chloroform and their metabolites in pregnant mice. Biol. Res. Pregn. 7:77-83.
Foster, J.R., C.L. Elcombe, A.R. Boobis, D.S. Davies, D. Sesardic, J. McQuade, R.T. Robson, C. Hayward and E. Lock, 1986. Immunocytochemical localization of cytochrome P-450 in hepatic and extra-hepatic tissues of the rat with a monoclonal antibody against cytochrome P-450 c. Biochem. Pharmacol. 35:4543-4554.
Ghantous, H., B. Löfberg, H. Tjälve, B.R.G. Danielsson and L. Dencker, 1988. Extrahepatic sites of metabolism of halothane in the rat. Pharmacol. Toxicol. 62:135-141.
Kowalski, B., E.B. Brittebo and I. Brandt, 1985. Epithelial binding of 1,2-dibromoethane in the respiratory and upper alimentary tracts of mice and rats. Cancer Res. 45:2616-2625.
Kowalski, B., E.B. Brittebo, R. D'Argy, G.O. Sperber and I. Brandt, 1986. Fetal epithelial binding of 1,2-dibromoethane in mice. Carcinogenesis 7:1709-1714.
Löfberg, B. and H. Tjälve, 1986. Tracing tissues with chloroform-metabolizing capacity in rats. Toxicology 39:13-35.
Miller, J.A. and E.C. Miller, 1978. Metabolic activation of chemicals to reactive electrophiles: an overview. In: Y. Cohen (Ed.): Toxicology, Advances in Pharmacology and Therapeutics. Pergamon Press, Oxford, 3-12.
Tjälve, H. and B. Löfberg, 1983. Extrahepatic sites of metabolism of carbon tetrachloride in rats. Chem.-Biol. Interactions 46:299-316.
Voigt, J.M., F.P. Guengerich and J. Baron, 1985. Localization of a cytochrome P-450 isozyme (cytochrome P-450 PB-B) and NADPH-cytochrome P-450 reductase in rat nasal mucosa. Cancer Lett. 27:241-247.

NASAL TISSUE ACTIVATION AND DETOXICATION OF INHALANTS

Alan R. Dahl, James A. Bond, Margaret L. Crews, William M. Hadley, Patrick J. Sabourin

Inhalation Toxicology Research Institute, Lovelace Biomedical and Environmental Research Institute, Albuquerque, NM USA

The presence of cytochrome P450-dependent monooxygenases in the rat nasal cavity was suspected when, after inhalation of tritium and iron-59 dual-labeled ferrocene vapor, the tritiated organic component cleared from the nasal cavity and from the body in a few hours while the iron component remained in the nasal cavity for several months (Dahl and Briner, 1980). Obviously, the ferrocene molecule had decomposed, but, since ferrocene is a very stable molecule, decomposition could only have occurred after metabolism to an unstable compound. Because it had been reported that hepatic P-450 could metabolize ferrocene to an unstable hydroxylated species, the observed fate of ferrocene in the nasal cavity suggested that such a system existed there also. Subsequent research showed this to be true (Hadley and Dahl, 1982; Dahl et al., 1982). Since nasal P-450 was reported, other nasal xenobiotic metabolizing enzymes have been reported, including: aldehyde dehydrogenases, flavin containing monoxygenase, rhodanese, glutathione S-transferases, UDP-glucuronyl transferase, carboxylesterases, and epoxide hydrolase. Recent reviews describing the tissue distribution and activities of nasal xenobiotic metabolizing enzymes and their contribution to the toxicity of inhaled material are available (Dahl et al., 1988; Dahl, 1988a, b, c).

Since the first recognition of the involvement of nasal P-450 in determining the fate of inhaled materials, the list of reports of compounds that produce toxic effects after nasal metabolism has grown steadily. Efforts to localize and identify the enzymes responsible have increased to where it is now possible to describe more specifically: the types of enzymes present in the nasal cavity; how these vary in their specific catalytic capacity; how they vary among animal species; and, how knowledge of these enzymes can be used to predict the activation or detoxication of inhaled compounds.

The Nature of Reactive Metabolites and Their Target Cells

The cell in which a molecule is activated to a toxic metabolite need not be the exclusive target cell. Toxic metabolites have half-lives ranging from fractions of a second to hours (Table 1). The longer the half-life, the more likely that cells and tissues remote from the site of activation will be affected. Thus, dimethylnitrosamine (DMN) and benzo(a)pyrene (BaP) are both nasal tumorigens that form reactive metabolites capable of escaping the cell of origin (Umbenhauer and Pegg, 1981; Dahl et al., 1985). It is probable that upon inhalation both are metabolized largely in olfactory Bowman's gland cells or other olfactory tissue cells (compare Tables 2 and 3). Inhaled DMN causes aesthesioneuroepitheliomas, thus affecting the olfactory sensory cells (Druckrey et al., 1967), where little metabolic activity is expected (Hadley and Dahl, 1982). Inhaled BaP causes tumors in the Syrian hamster, both in the nasal cavity and in the alimentary tract (Thyssen et al., 1981),

Table 1. Some types of reactive metabolites of importance in nasal toxicology.

Parent compound	Reactive metabolite	Half-life of metabolite	Prominent site of toxicity
Dihydrosafrole[a]	Carbene	< 1 second to seconds	Activating enzyme
Dimethylnitrosamine[b]	Hydroxy Azo compound	Seconds to minutes	Metabolizing tissue
Benzo(a)pyrene[c]	Diol epoxide	Minutes to hours	Vicinity of metabolizing tissue
Butadiene[d]	Epoxide	Hours to days	Escapes body

[a]Dahl and Brezinski (1985).
[b]Dahl et al. (unpublished).
[c]Dahl et al. (1985).
[d]Filser and Bolt (1984).

Table 2. Distribution of P-450 isozymes in the nasal mucosa.[a]

Tissue	P-450 form	Inducer[b]	Species[c]
Respiratory epithelium	IIB1	PB	Rat ++, Rabbit +
	IIIA	PCN	Rat +
	IV	Clofibrate	Rabbit ++
Olfactory epithelium	IA2	3MC	Rabbit +
	IIB1	PB	Rabbit +
	IIE	Ethanol	Rabbit +++
	IIIA	PCN	Rat ++
	IV	Clofibrate	Rabbit +
Bowman's gland	IA1	3MC	Rat ++

[a]Rat data adapted from Baron et al. (1988). Rabbit data, except for form IIE, from Sabourin et al. (1988). Rabbit IIE data from Ding et. al. (1986). Nomenclature adopted from Nebert et al. (1987).
[b]A recognized inducer of the liver form of the isozyme is given to assist in identification (Nebert et al., 1987). PB = phenobarbital; PCN = pregnenolone 16α-carbonitrile; 3MC = 3-methylcholanthrene.
[c]Number of plus signs are intended to indicate concentrations relative to other forms in the nasal cavity.

although activating enzymes are present only at low levels in the alimentary tract tissues. The induction of toxic metabolites in the nasal cavity has been implicated in the production of alimentary tract tumors (Dahl et al., 1985).

Table 3. Activities of P-450 isozymes reported in the nasal cavity.[a]

Activity[b]	Isozymes					
	IA1	IA2	IIB1	IIE	IIIA	IV
p-NA O-DM	++	+	++			
d-BP N-DM	++	++	+++		+	
AP N-DM	++	++	+++		+	
7-EC O-DE	++	+	+	+	±	
EM N-DM	+	+	++		+	
Acetan 4-H	++	+	+		+	
BaP H	+	+	+		+	
An H	+	+	+	++	±	
DMN N-DM	+	+	+	++	+	
7-ER O-DE	+	+				
Acetam O	+	+		+		
1-BuOH O				++		
Benz O				+		
2-AF N-O						+++

[a]Catalytic properties reported are for rat tissue isozymes from Guengerich et al. (1982), except for rabbit IIE which are from Coon and Koop (1987) and references therein (except for benzene), and rabbit IV from Vanderslice et al., 1987. Benzene metabolism by IIE is from Koop et al., 1988.

[b]Activities are, in order: p-nitroanisole o-demethylase; d-benzphetamine N-demethylase; aminopyrine-N-demethylase; 7-ethoxycoumarin O-deethylase; ethylmorphine N-demethylase; acetanilide 4-hydroxylase; benzo(a)pyrene hydroxylase; aniline hydroxylase; dimethylnitrosamine N-demethylase; 7-ethoxyresorufin O-deethylase; acetaminophen oxidase; 1-butanol oxidase; benzene oxidase; 2-aminofluorene N-oxidase.

Not only is the presence of an activating enzyme system not a prerequisite for the sensitivity of a cell to the effects of an activatable toxicant, but, cells other than the activating cells may even be the more sensitive. Thus, both olfactory and respiratory tissues of the nasal cavity contain the repair enzyme O^6-methylguanine DNA methyltransferase (J. A. Bond, personal communication). If the olfactory tissue DNA repair activity is located mainly in the sustentacular cells or Bowman's gland cells, as is likely the case for the activating system, these cells may nevertheless suffer less permanent DNA damage from activated DMN than will the sensory cells where little activation is expected (Hadley and Dahl, 1982).

Cells and tissues differ not only in their ability to repair damage, but also in their ability to detoxicate potentially harmful compounds. Thus, epoxide hydrolase (EH) occurs at higher concentrations in the olfactory epithelium than in the respiratory epithelium of rats (Table 4).

Table 4. Distribution of glutathione S-transferases (GSH-T) and epoxide hydrolase (EH) in the nasal mucosa.[a]

Enzyme/Tissue[b]	Typical Substrates[c]	RE[d]	OE[d]
GSH-T 1,1	Δ^5-Androstene-3,17-dione	+++	++
GSH-T 3,3	1,2-Dichloro-4-nitrobenzene	±	+
	Bromosulfophthalin		
GSH-T 5,5	1,2 Epoxy-3-(p-nitrophenoxy)propane	+	+
	Iodomethane		
EH Form A	Styrene oxide ++	±	++
	BaP-4,5-oxide ++		
	BaP-7,8-oxide +		
	Benzanthracene-5,6-oxide +		
	3MC-11,12-oxide ±		
	2-Chlorethylene oxide +		

[a]Adapted from Baron et al. (1988). Nomenclature used here is that of Mannervik (1985) for GSH-T and of Guengerich et al. (1979b) for EH. Plus signs indicate relative concentrations.
[b]Enzyme nomenclature is that of Mannervik (1985) for GSH-T and Guengerich et al. (1979b) for EH.
[c]From Mannervik (1985) for GSH-T and Guengerich et al. (1979a) for EH. Plus signs indicate relative V_{max} Values.
[d]RE = respiratory epithelium; OE = olfactory epithelium. Plus signs indicate relative concentrations.

Therefore, although the olfactory epithelium has the greater capacity to metabolize BaP to reactive products (Dahl et al., 1982, 1985; Table 5), the increased activation may be counteracted by the increased deactivation brought about by the higher levels of EH. Differences in levels of detoxicating activities for aldehydes also occur at the cell and tissue level (Table 5). Carboxylesterase, a potentially detoxicating enzyme for substrates such as vinyl acetate and a potentially activating enzyme for substrates such as acrylate esters, also shows cell and tissue specific localization in the nasal cavity (Table 5). Thus, in evaluating a cell type as a potential target for an activatable inhalant, knowing the location of detoxicating enzymes is important.

The results of in vitro nasal enzymology can be used to explain effects observed in vivo and to some degree can be used to predict toxic effects. This is illustrated by three examples.

Table 5. Cellular and tissue localization of some xenobiotic metabolizing activities in the nasal cavity.

Tissue/Cell	Activity: α-Naphthyl-butyrate carboxyl-esterase[a]	Acet-aldehyde dehydro-genase[b]	Benzo(a)-pyrene hydroxylase[c]
Squamous epithelial cells		+	
Respiratory mucosa			+
ciliated epithelial cells	++	+++	
nonciliated epithelial cells	+	++	
goblet cells	±	+	
cuboidal cells	++	±	
basal cells	+		
seromucous glands	±		+
Olfactory mucosa			++
Bowman's glands	+++	±	++
sustentacular cells	++	−	
basal cells	±	+	
sensory cells	−	−	

[a]From Bogdanffy et al. (1987).
[b]From Bogdanffy et al. (1986).
[c]From Baron et al. (1988).

Dimethylnitrosamine (DMN)

Whole-body autoradiographs of mice administered radiolabeled DMN intravenously showed relatively high concentrations of bound radiolabel in the olfactory region (Johansson et al., 1978). As mentioned previously, tumors arose from the olfactory sensory cells in rats exposed to DMN intermittently for 19 weeks at 0.08 parts per million (Druckrey et al., 1967). We are investigating the relationship of the concentration of inhaled ^{14}C-labeled DMN and the amount of covalent binding to tissues in the respiratory and olfactory mucosa of rats and hamsters. Some results are shown in Figure 1.

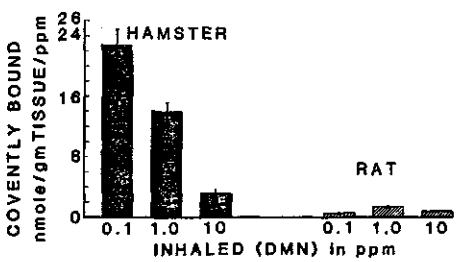


Fig. 1. Efficiency of production of covalently-bound ^{14}C in the ethmoturbinates of hamsters and rats exposed for two hours by the nose-only mode to stepped concentrations of dimethylnitrosamine (DMN). Turbinates were removed, homogenized and exhaustively dialyzed. The nondialyzable (bound) activity was determined by liquid scintillation spectroscopy after dissolving the tissue in tetraethylammonium hydroxide.

The efficiency of conversion of inhaled DMN to tissue-bound metabolites in the ethmoturbinates is much greater in the hamster than in the rat at inhaled concentrations of 0.1, 1 and 10 ppm. Such a result is predicted from the much greater aniline hydroxylase activity in the hamster nasal cavity relative to that in the rat nasal cavity (Hadley and Dahl, 1983) because aniline hydroxylase and DMN N-demethylase are activities of the same P-450 isozymes (Table 3). The decreased efficiency of production of tissue-bound metabolites with increased concentrations of inhaled DMN is pronounced in the hamster and indicates that the metabolic pathway is saturable.

Benzo(a)pyrene (BaP)

BaP inhaled at 10 mg/m^3 produces nasal and upper alimentary tract tumors in Syrian hamsters (Thyssen et al., 1981). To determine if BaP deposited in the nasal cavity is metabolized to toxicants that might be swallowed, thus exposing the alimentary tract, ^{14}C-labeled BaP was placed on the nasal mucosa of Syrian hamsters. The mucus was collected by means of an esophageal catheter (Dahl et al., 1985). BaP-7,8-diol (BaPD), the penultimate carcinogen, was found in the mucus in relatively high concentrations. Evidence for the ultimate carcinogen, BaP-7,8-diol-9,10-epoxide (BaPDE), was also found. BaP is an example of a compound that is activated in the nasal cavity and which subsequently affects tissues in the same proximity. The carcinogenic metabolite, BaPDE, is formed stepwise through the progression of BaP-7,8-epoxide, BaPD and then BaPDE. Thus, formation of BaPD in the nasal cavity means that the weakly activating esophagus tissue needs to cycle the molecule through its P-450 system only once to produce the ultimate carcinogen, BaPDE.

Ferrocene

Ferrocene belongs to the organometallic class of compounds. Ferrocene and similar compounds are used increasingly in industry for production of heat-stable polymers and as fuel additives. Currently, the potential inhalation toxicity of ferrocene is being investigated in the U. S. National Toxicology Bioassay Program. In preliminary inhalation toxicity studies, a single 6-hr exposure of rats to 40 mg/m^3 of ferrocene vapor produced moderate to severe olfactory lesions (N. Gillett, personal communication). Prompted by this finding, we investigated the rate of metabolism of ferrocene in the nasal tissues as well as in the liver (Table 6).

Increased rates of metabolism in the olfactory and the respiratory regions relative to those in liver indicate that iron would be released in the nasal tissues at a faster rate compared to liver. The released iron may catalyze peroxidation of nasal mucosa leading to the observed lesions (Dahl and Briner, 1980).

Table 6. Apparent kinetic values for the metabolism of ferrocene by nasal and liver microsomes.[a]

Tissue	V_{max} (nmoles/min/gm tissue)	Km (μM)	V_{max} Km
Maxilloturbinates	3.9 ± 0.5	6 ± 2	0.7
Ethmoturbinates	8.8 ± 0.8	8 ± 2	1.1
Liver	1.2 ± 0.2	15 ± 2	0.08

[a]Values for V_{max} and Km and estimated 95% confidence intervals are from a plot of rate and concentrations of ferrocene using ten points with a range of 2 to 300 μM ferrocene. Protein concentration was 0.5-1 mg microsomal protein which was then normalized to a per gm tissue basis. The assay consisted of 20 min incubations with iron-59 labeled ferrocene, followed by raising the pH to 11 and exhaustively extracting unmetabolized ferrocene from the water soluble metabolites.

Conclusion

Research on specific xenobiotic metabolism activities in the nasal cavity is bringing this important area of inhalation toxicology into ever sharper focus. Past observations of toxic effects from inhalants are explained; toxic effects from current studies are predicted, sometimes accurately. By examination of potential activation pathways and consideration of the stabilities of reactive metabolites, assessments of cells and tissues at risk can be made. Research into nasal metabolism is still in the early stages, however. Relationships between activation, detoxication and repair enzyme activities need to be examined. A major gap is still the lack of a data base regarding human nasal xenobiotic metabolism. Finally, the contribution of nasal metabolism to the fate of inhalants should be quantitated for a range of compounds to provide the basis for estimations of the relative degree of metabolism among structurally related compounds.

Acknowledgments

This research was supported by National Institute of Environmental Health Sciences Grant No. ES04422 and by the Office of Health and Environmental Research of the Department of Energy in facilities provided by the Office of Health and Environmental Research of the U.S. Department of Energy under Contract Number DE-AC04-76EV01013.

References

Baron, J., J.P. Burke, F.P. Guengerich, W.B. Jakoby & J.M. Voigt, 1988. Sites for xenobiotic activation and detoxication within the respiratory tract: Implications for chemically induced toxicity. Toxicol. Appl. Pharmacol. 93:493-505.

Bogdanffy, M.S., H.W. Randall & K.T. Morgan, 1987. Biochemical quantitation and histochemical localization of carboxylesterase in the nasal passages of the Fischer-344 rat and B6C3F$_1$ mouse. Toxicol. Appl. Pharmacol. 88:183-194.

Bogdanffy, M.S., H.W. Randall & K.T. Morgan, 1986. Histochemical localization of aldehyde dehydrogenase in the respiratory tract of the Fischer-344 rat. Toxicol. Appl. Pharmacol. 82:560-567.
Coon, M.J. & D.R. Koop, 1987. Alcohol-inducible cytochrome P-450 ($P\text{-}450_{ALC}$). Arch. Toxicol. 60:16-21.
Dahl, A.R., 1988a. The effect of cytochrome P-450-dependent metabolism and other enzyme activities on olfaction. Molecular neurobiology of the olfactory system (FL Margolis and TV Getchell, eds). Plenum, NY (1988) pp 51-70.
Dahl, A.R., 1988b. Comparative metabolic basis for the disposition and toxic effects of inhaled materials. In: Inhalation toxicology. Springer, Verlag, Heidelberg.
Dahl, A.R., 1988c. Metabolic characteristics of the respiratory tract. In: R.O. McClellan & R.F. Henderson (Eds.): Concepts in inhalation toxicology. Hemisphere, Washington, DC.
Dahl, A.R. & D.A. Brezinski, 1985. Inhibition of rabbit nasal and hepatic cytochrome P-450-dependent hexamethylphosphoramide (HMPA) N-demethylase by methylenedioxyphenyl compounds. Biochem. Pharmacol. 34:631-636.
Dahl, A.R. & T.J. Briner, 1980. Biological fate of a representative lipophilic metal compound (ferrocene) deposited by inhalation in the respiratory tract of rats. Toxicol. Appl. Pharmacol. 56:232-239.
Dahl, A.R., W.M. Hadley, F.F. Hahn, J.M. Benson & R.O. McClellan, 1982. Cytochrome P-450-dependent monoxygenases in olfactory epithelium of dogs: Possible role in tumorigenicity. Science 216:57-59.
Dahl, A.R., D.S. Coslett, J.A. Bond & G.R. Hesseltine, 1985. Metabolism of benzo(a)pyrene on the nasal mucosa of Syrian hamsters: Comparison to metabolism by other extrahepatic tissues and possible role of nasally produced metabolites in carcinogenesis. JNCI 75:135-139.
Dahl, A.R., J.A. Bond, J. Petridou-Fischer, P.J. Sabourin & S.L. Whaley, 1988. Effects of respiratory tract on inhaled materials. Toxicol. Appl. Pharmacol. 93:484-492.
Ding, X., D.R. Koop, B.L. Crump & M.J. Coon, 1986. Immunochemical identification of cytochrome P-450 isozyme 3a ($P\text{-}450_{ALC}$) in rabbit nasal and kidney microsomes and evidence for differential induction by alcohol. Mol. Pharmacol. 30:370-378.
Druckrey, H.R., R. Preussman, S. Ivankovic, D. Schmähl, J. Afkham, G. Blum, H.D. Mennel, M. Müller, P. Petropoulos & H. Schneider, 1967. Organotrope carcinogene wirkungen bei 65 verschiedenen N-nitrosoverbindungen an BD-ratten. Zeitschrift für Krebsforschung 69:103-201.
Filser, J.G. & H.M. Bolt, 1984. Inhalation pharmacokinetics based on gas uptake studies. VI. Comparative evaluation of ethylene oxide and butadiene monoxide as exhaled reactive metabolites of ethylene and 1,3-butadiene in rats. Arch. Toxicol. 55:219-223.
Guengerich, F.P., P. Wang, M.B. Mitchell & P.S. Mason, 1979a. Rat and human liver microsomal epoxide hydratase: Purification and evidence for the existence of multiple forms. J. Biol. Chem. 254:12248-12254.
Guengerich, F.P., P. Wang, P.S. Mason & M.B. Mitchell, 1979b. Rat and human microsomal epoxide hydratase: Immunological characterization of various forms of the enzyme. J. Biol. Chem. 254:12255-12259.
Guengerich, F.P., G.A. Dannan, S.T. Wright, M.V. Martin & L.S. Kaminsky, 1982. Purification and characterization of liver microsomal cytochromes P-450: Electrophoretic, spectral, catalytic, and immunochemical properties and inducibility of eight isozymes isloated from rats treated with phenobarbital or β-naphthoflavone. Biochem. 21:6019-6030.

Hadley, W.M. & A.R. Dahl, 1982. Cytochrome P-450 dependent monoxygenase activity in rat nasal epithelial membranes. Toxicol. Letts. 10:417-422.

Hadley, W.M. & A.R. Dahl, 1983. Cytochrome P-450-dependent monoxygenase activity in nasal membranes of six species. Drug. Metab. Dispos. 11:275-276.

Jakoby, W.B., B. Ketterer & B. Mannervik, 1984. Glutathione transferases: Nomenclature. Biochem. Pharmacol. 33:2539-2540.

Johansson, E.B. & H. Tjälve, 1978. The distribution of [^{14}C]dimethylnitrosamine in mice. Autoradiographic studies in mice with inhibited and noninhibited dimethylnitrosamine metabolism and a comparison with the distribution of [^{14}C]formaldehyde. Toxicol. Appl. Pharmacol. 45:565-575.

Koop, D.R., C.L. Orlandi & G.G. Schier, 1988. Identification and induction of P-450 isozymes 3a and 6 in rabbit bone marrow microsomes. Abstract from NIEHS International Symposium on Benzene Metabolism, Toxicity and Carcinogenesis, March 14-16, 1988.

Mannervik, B., 1985. The isoenzymes of glutathione transferase. In: A. Meister (Ed.): Advances in enzymology and related areas of molecular biology. John Wiley & Sons, New York, Vol. 57, pp. 357-406.

Nebert, D.W., M. Adesnik, M.J. Coon, R.W. Estabrook, F.J. Gonzalez, F.P. Guengerich, I.C. Gunsalus, E.F. Johnson, B. Kemper, W. Levin, I.R. Phillips, R. Sato & M.R. Waterman, 1987. The P450 gene superfamily: Recommended nomenclature. DNA 6:1-11.

Sabourin, P.J., R.E. Tynes, R.M. Philpot, S. Winquist & A.R. Dahl, 1987. Distribution of microsomal monooxygenases in the rabbit respiratory tract. Drug Metab. Dispos. 16:557-562.

Thyssen, J., L. Althoff, G. Kimmerle & U. Mohr, 1981. Inhalation studies with benzo(a)pyrene in Syrian golden hamsters. JNCI 66:575-577.

Umbenhauer, D.R. & A.E. Pegg, 1981. Alkylation of intracellular and extracellular DNA by dimethylnitrosamine following activation by isolated rat hepatocytes. Cancer Res. 41:3471-3474.

Vanderslice, R.R., B.A. Domin, G.T. Carver & R.M. Philpot, 1987. Species-dependent expression and induction of homologues of rabbit cytochrome P-450 isozyme 5 in liver and lung. Mol. Pharmacol. 31:320-325.

BIOCHEMICAL RESPONSES OF THE RAT NASAL EPITHELIA TO INHALED AND INTRAPERITONEALLY ADMINISTERED ACROLEIN

R.-A. Walk and H.-J. Haussmann

INBIFO Institut für biologische Forschung, Köln, FRG

Summary

The acute inhalation of sublethal concentrations of acrolein (0.7 to 4 ppm) caused a decrease of the total glutathione (GSH plus GSSG) pool in both the rat nasal respiratory and the olfactory epithelium as well as in the trachea and the lungs. This glutathione content decrease was associated with complex changes of the enzymic activities of GSSG reductase, GSH peroxidase, and GSH-S-transferase. A single intraperitoneal (i.p.) administration of acrolein at doses corresponding to the systemic dose calculated for the inhalation did not decrease the total glutathione content but did affect the 3 enzymic activities. The relevance of the results observed with acrolein regarding the glutathione-dependent detoxification system to the toxicity/carcinogenicity of xenobiotics in the nasal epithelia and other respiratory tract tissues in general is still under evaluation.

Keywords: nasal epithelia, glutathione, acrolein, inhalation, intraperitoneal.

Introduction and Concept

The purpose of the ongoing investigations is to contribute to a better understanding of the molecular mechanisms of toxicity/carcinogenicity in the nasal epithelia, the trachea, and the lungs. The simultaneous, comparative investigation of several tissues of the rat respiratory tract is thought to be especially useful, because an understanding of the target site specificity may be possible.

The concept behind our investigations is to evaluate a matrix of biochemical and morphological responses thought to be of importance for chemical toxicity/carcinogenicity in the respiratory tract following acute, subchronic, and chronic exposure of rats to gases and aerosols of several model xenobiotics. In order to study the influence that the test substance administration route has on biochemical responses, we compared the responses observed following inhalation to those observed following i.p. administration.

The following parameters are being investigated in the present series of studies:

model compounds:
- acrolein
- formaldehyde
- acetaldehyde
- mixtures of aldehydes
- ammonia
- benzo(a)pyrene
- paraquat

respiratory tract tissues and organs:
- nasal cavity, respiratory epithelium (NRE)
- nasal cavity, olfactory epithelium (NOE)
- trachea, total and epithelium
- lungs, total and bronchial epithelial cells

biochemical assay parameters:

protective systems:
- glutathione-dependent detoxification
- phagocytosis

xenobiotic metabolism:
- aryl hydrocarbon monooxygenase activity
- NADPH-cytochrome P-450-reductase activity

modification of DNA:
- alkaline elution
- DNA aneuploidy
- SCE
- chromosome aberrations

proliferation:
- ornithine decarboxylase

The topic of this presentation will be restricted to the effects of a selected model compound, i.e., acrolein, on the glutathione-dependent protective system in the nasal epithelia, trachea, and lungs of SPF-bred and barrier housed Sprague Dawley rats following acute treatment with acrolein either by inhalation or by a single i.p. administration. Other aspects of our investigations such as chromosome aberrations and SCE in primary rat nasal and tracheal epithelial cells as well as the histopathological effects observed in rat nasal epithelia after the inhalation of aldehydes and ammonia have been shown in posters during this symposium by my colleagues Doris Bachmayer and Ashok Teredesai. Additional details to this presentation as well as the results of the effect of formaldehyde administration on the rat respiratory tract glutathione metabolism are presented in a poster by H.-J. Haussmann. A new, highly sensitive technique to investigate single strand breaks in the nasal epithelium of a single rat and also a new fluorescence detection technique for HPLC-separated metabolites of polycyclic aromatic hydrocarbons have been developed recently by Thomas Miller and by H.-J. Haussmann in our laboratory and are currently being applied to aldehyde-exposed rats.

In rodents, acute or subchronic exposure to acrolein is cytotoxic to the respiratory tract epithelial cells. In the nose, acrolein causes hyperplastic changes, keratinizing squamous metaplasia, and atrophy in the respiratory or olfactory epithelium at concentrations below 1 ppm (Beauchamp et al., 1985). The LC50 value for mice and rats was found to be approx. 10 ppm based upon a 4- or 6-hour exposure (Beauchamp et al., 1985). The LD50 following i.p. administration to rats was 4 mg/kg (Murphy et al., 1983).

Although acrolein is genotoxic in vitro (e.g., Lam et al., 1985), it was not found to be carcinogenic in hamsters (inhalation 4 ppm, Feron & Kruysse, 1977). This could be related to the action of efficient protective systems operating in vivo. In vitro, acrolein reacts rapidly and irreversibly with sulfhydryl groups including those of glutathione (Esterbauer et al., 1975). Depletion of non-protein sulfhydryl groups was observed in the NRE following acrolein inhalation (Lam et al., 1985). Therefore, it is of major importance to investigate whether the modification of the glutathione content is a primary event in the mechanism of toxicity of acrolein in the nasal tissues. The modification of the glutathione content may be of major importance when acrolein is administered together with carcinogens which are also detoxified by the glutathione-dependent protection system (Lam et al., 1985).

Acrolein exposure:

(1) Inhalation: 4 h, head-only exposure
0.7, 1.4, 2.3, 4, 9, and 17 ppm
estimated doses: 0.2 to 5 mg/kg body weight (BW)
dissection immediately after the end of the exposure
7 to 8 rats/concentration

(2) I.p. administration:
single injection: 0.2 and 1.0 mg/kg BW
dissection at 3, 9, and 24 h after administration
4 to 9 rats/time point

From the 4 tissues, homogenates and postmitochondrial supernatants were used for the biochemical assays:

total glutathione (GSH + GSSG) content (cycling assay)
GSSG reductase activity
GSH peroxidase activity (substrate: hydrogen peroxide)
GSH-S-transferase activity (substrate: CDNB)

Results and Discussion

Both nasal epithelia and the lungs showed a similar glutathione content of approx. 13 umol/g protein. The content in the trachea was approx. half of that in the other tissues. Following the 4-hour inhalation of 1 to 17 ppm acrolein, a concentration-dependent decrease was observed in all 4 tissues and organs (Fig. 1). At 4 ppm (equivalent to 1 mg/kg BW), the glutathione content was reduced to 40, 60, 80, and 90 % as compared to sham-exposed controls in NRE, trachea, NOE, and lungs, respectively. At 3, 9, and 24 h after a single i.p. administration of sublethal acrolein doses, an effect on the glutathione concentration in these tissues and organs was not observed (Fig. 2).

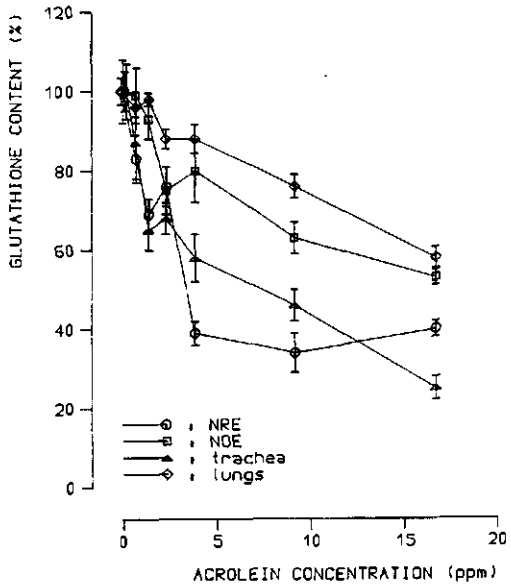


Fig. 1. Effect of acrolein inhalation on the total glutathione content in the tissues/organs of the rat respiratory tract.

The means of 7 to 8 rats/group with the standard error of the mean (SE).

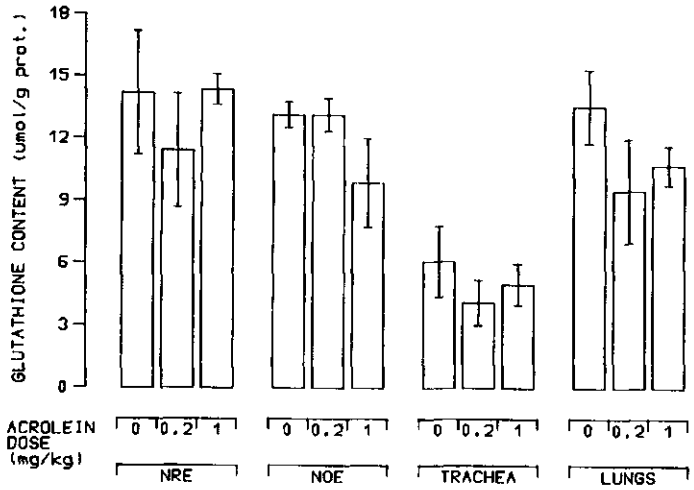


Fig. 2. Effect of a single i.p. administration of acrolein on the total glutathione content in the tissues/organs of the rat respiratory tract.

The means of 4 rats with SE.

Although the total glutathione content in the 2 nasal epithelia was very similar, the activities of the 3 glutathione-dependent enzymes were markedly different. The GSSG reductase activity in the 4 tissues was: NOE > NRE = trachea > lungs. It was different by a factor of approx. 3 (25 to 70 U/g protein). The GSH peroxidase activity in the NOE and the lungs was found to be lower than that in NRE and the trachea (30 vs. 60 U/g protein). The GSH-S-transferase activity in NRE (500 U/g protein) and NOE (900 U/g protein) was 5- to 10-fold higher than that in the trachea and the lungs.

Although a uniform decrease of the total glutathione content was observed following the inhalation of sublethal doses of acrolein (0.7 to 4 ppm) in both nasal tissues, this decrease was associated with a differential pattern of responses of the activities of the enzymes in NRE and NOE: In NRE it was associated with a decrease of the reductase and peroxidase activities (approx. 50 % of the sham-exposed control), in NOE with an increase in the reductase, peroxidase, and transferase activities (maximally to 150 % of the sham-exposed control) (Table 1).

Table 1. Comparison of effects of acrolein inhalation and i.p. administration at doses up to 1 mg/kg BW on glutathione and glutathione-dependent enzymes.

Tissue/ organ	Assay parameter	Effect following acrolein inhalation	i.p. injection
nasal respiratory epithelium (NRE)	glutathione,	D	0
	GSSG reductase,	D	0
	GSH peroxidase,	D(?)	I
	GSH-S-transferase	0	0
nasal olfactory epithelium (NOE)	glutathione,	D	0(D?)
	GSSG reductase,	I	D
	GSH peroxidase,	I	I
	GSH-S-transferase	I	0
trachea	glutathione,	D	0
	GSSG reductase,	D	0
	GSH peroxidase,	I(?)	I
	GSH-S-transferase	I	0(I?)
lungs	glutathione,	D	0
	GSSG reductase,	I	I
	GSH peroxidase,	I	I
	GSH-S-transferase	I	I

I = increase; D = decrease; 0 = no effect; (?) = questionable

The response of the enzymes in the lungs and trachea to inhaled acrolein was similar to that in NOE although in the trachea the reductase activity was found to be decreased.

Following i.p. administration of doses of acrolein corresponding to the systemic dose calculated for the inhalation, the total glutathione content was found to be unaffected, although the peroxidase activity was increased in all 4 tissues and the reductase activity was also affected in the NOE (decrease to approx. 50 %) and in the lungs (increase to approx. 140 %). The changes of the activities of the enzymes observed in the present investigation following i.p. administration indicate that acrolein or metabolites derived from it could either reach and affect all 4 tissues even when administered at a distant site or unspecific hormone-mediated modulations of enzyme activities are operating (Murphy, 1965). Acrolein inhalation was shown to induce liver enzymes via glucocorticoids. The changes of the enzyme activities in the absence of a change of the glutathione content also indicate that their determination may be a more sensitive indicator of the in vivo modification of the glutathione-dependent protection against xenobiotics.

In order to evaluate whether the changes of the glutathione content and the activities of enzymes dependent on it in the nasal and other respiratory tract organs are of toxicological relevance, we are currently investigating the level to which the glutathione content in these tissues must be reduced so that the protective role against genotoxic materials such as formaldehyde is lost.

It appears likely that the differential responsiveness of the glutathione-dependent enzymes may offer explanations for the mechanism of toxicity/carcinogenicity in the respiratory tract tissues. We are currently investigating whether changes of the glutathione content as well as of the activities of glutathione-dependent enzymes are restricted to target organs/tissues/cells.

References

Beauchamp, R.O., D.A. Andjelkovich, A.D. Klugerman, K.T., Morgan & H. d'A. Heck, 1985. A critical review of the literature on acrolein toxicity. CRC Crit. Rev. Toxicol. 14:309-380.

Esterbauer, H., H. Zollner & N. Scholz, 1975. Reaction of glutathione with conjugated carbonyls. Z. Naturforsch. c 304:466-473.

Feron, V. & A. Kruysse, 1977. Effects of exposure to acrolein vapor in hamsters simultaneously treated with benzo(a)pyrene or diethylnitrosamine. J. Toxicol. Environm. Health 3:379-394.

Lam, C.-W., M. Casanova & H. d'A. Heck, 1985. Depletion of nasal mucosal glutathione by acrolein and enhancement of formaldehyde induced DNA-protein crosslinking by simultaneous exposure to acrolein. Arch. Toxicol. 58:67-71.

Murphy, S.D., 1965. Mechanism of the effect of acrolein on rat liver enzymes. Toxicol. Appl. Pharmacol. 7:833-843

Murphy, M.J., D.A. Dunbar & L.S. Kaminsky, 1983. Acute toxicity of fluorinated ether anesthetics: role of 2,2,2-trifluoroethanol and other metabolites. Toxicol. Appl. Pharmacol. 71:84-92.

DNA ADDUCT CHEMISTRY AND *IN VIVO* BINDING TO RAT NASAL MUCOSA OF SEVERAL DIRECT-ACTING ALKYLATING AND ACYLATING AGENTS[1]

Jerome J. Solomon, Alvin Segal, Carroll A. Snyder and Frank Mukai

New York University Medical Center, Institute of Environmental Medicine, 550 First Avenue, New York, New York, 10016

Summary

Several years ago we observed that the potency of bis(chloromethyl)ether (BCME), epichlorohydrin (ECH), and dimethylcarbamyl chloride (DMCC) for the induction of nasal tumors in rats after inhalation exposure for 30 days appeared to be related to their chemical reactivity as measured by their rates of hydrolysis. Recent studies extended this observation by finding that concentrations of methyl methanesulfonate (MMS) and β-propiolactone (BPL) which produced similar nasal cancer yields were inversely proportional to their hydrolysis rates, i.e. potency increased with reactivity. Since these agents include both alkylating and acylating carcinogens which can react by different mechanisms with DNA, the types, persistence, and repair of specific adducts in the nasal mucosa were expected to be variable. This paper reports on our initial study of the DNA binding and the persistence of specific adducts in rat nasal mucosa for several rat nasal carcinogens. Total binding was found to be linear with inhaled dose for DMCC, MMS, and isopropyl methanesulfonate (IPMS). Isopropyl (IP) adducts (O^6-IP-dGuo and 7-IP-Gua) were much more stable in rat nasal DNA than methyl adducts (3-Me-Ade and 7-Me-Gua). The binding of the SN1 agents IPMS and DMCC to DNA was much lower than for the SN2 agents MMS and BPL. The SN2 agents MMS and BPL bound to DNA roughly in proportion to their hydrolysis rates.

Key words: alkylating agents, acylating agents, inhalation carcinogenesis, rat nasal tumors, *in vivo* DNA binding.

Introduction

In a series of earlier studies at our Institute (Kuschner et al., 1975; Laskin et al., 1980; Sellakumar et al., 1983), we have shown that inhalation exposure to electrophilic compounds resulted in nasal tumors in male Sprague-Dawley rats after only 30 days (6 hr/d x 5 d/wk) exposure. Inhalation exposures using this brief protocol have been conducted for the following direct-acting alkylating and acylating agents: BCME, DMCC, BPL, MMS, ethylchloroformate (ECF), dichloroacetyl chloride (DCAC), ECH, and propylene oxide (PO). Exposure concentrations were chosen to be inversely proportional to the compounds' published hydrolysis rates. All compounds except PO, the slowest reacting substance ($t_{1/2}$=5000 min), produced nasal cancer in rats. The hydrolysis rates of these compounds span six orders of magnitude. When initially comparing the tumor response for BCME, ECH, and DMCC, it was found that similar tumor responses were

1. This research was supported by Public Health Service grants ES00260 and ES03563 from the National Institute of Environmental Health Sciences, and by grant CA13353 from the National Cancer Institute.

observed when comparing exposure levels that were inversely proportional to the hydrolysis rates of the three compounds. This observation was supported by recent experiments (Sellakumar et al., 1987) with BPL and MMS, where, again, similar nasal cancer yields were produced when exposure concentrations were chosen to reflect the differences in reactivity as measured by hydrolysis rates. This relationship did not hold for all agents studied (Sellakumar et al., 1987).

The agents studied have other significant chemical differences in addition to hydrolysis rate. They include both alkylating and acylating agents and agents that react by a unimolecular SN1 mechanism as well as agents which react by a bimolecular SN2 mechanism. For an initial attempt to characterize how these agents bind to DNA of the rat nasal mucosa, we began a study to determine the in vivo DNA adduct pattern and persistence of four compounds, two SN2 alkylating agents, BPL and MMS, and two SN1 agents, DMCC (an acylating agent) and IPMS (an alkylating agent).

Results and Discussion

Before in vivo DNA adduct formation can be studied, it is necessary to characterize the adducts formed with 2'-deoxyribonucleosides and in vitro with DNA. We have previously determined the adducts formed by in vitro reaction of BPL with calf thymus DNA (Segal et al., 1981). These carboxyethyl (CE, $-CH_2CH_2COOH$) adducts are shown in Figure 1. The relative amounts of these adducts after a 6 h reaction at pH 7 and 37°C is given under each structure. Alkylation occurred at endocyclic nitrogens, as expected for an SN2 alkylating agent (Singer & Grunberger, 1983). In studies with other rodent carcinogens, the CE adducts 1-CE-Ade and 3-CE-Cyt were detected after in vitro reaction of DNA with acrylonitrile (Solomon & Segal, 1985) and acrylamide (Solomon et al., 1985). The potential importance of the adenine adduct has previously been discussed (Hochwalt et al., 1988) with relation to an A ---> T H-ras mutation observed in a BPL-induced mouse skin squamous cell carcinoma.

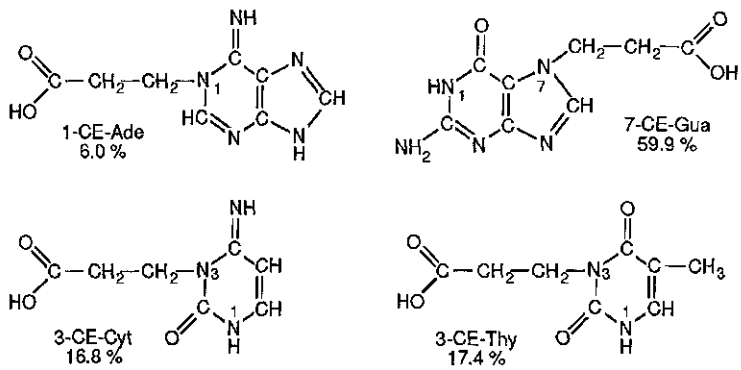


FIGURE 1. Carboxyethyl (CE) DNA adducts of β-propiolactone with calf thymus DNA (pH 7, 37°C, 6 h).

DMCC, an SN1 acylating agent, would be expected to have a completely different adduct profile from MMS or BPL. We showed (Segal et al., 1982) that DMCC exclusively acylates exocyclic oxygens at O^6 of guanine and O^4 of thymine. These adducts can undergo further reaction and Figure 2 gives the adducts detected in vitro at pH 7 and 37°C after a 4 h

reaction. The dimethylamino (DMA) adducts 6-DMA-dGuo and 4-DMA-dThd probably result from a nucleophilic aromatic substitution reaction with dimethylamine which forms in the reaction mixture from the hydrolysis of DMCC. The *in vitro* DNA adducts of MMS (Singer & Grunberger, 1983) and IPMS (Lawley et al., 1975) have been reported by others.

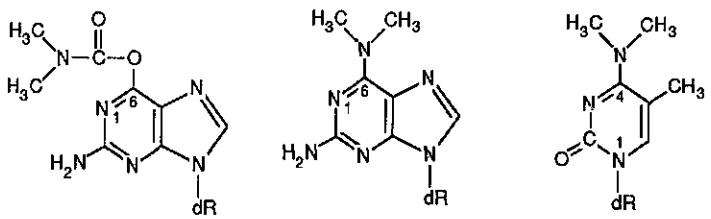


FIGURE 2. *In vitro* products of dimethylcarbamyl chloride with calf thymus DNA (pH 7, 37°C, 4 h), dR=2'-deoxyribose.

An inhalation exposure system (Snyder & Bowers, 1987) was constructed to determine the inhaled dose to rats of radiolabeled carcinogen. Dose response experiments were performed with DMCC (0.13-2.7 ppm), MMS (2-20 ppm), and IPMS (0.2-6.6 ppm)$_3$. Groups of three rats were exposed for 2 h to a given concentration of [^{3}H]-carcinogen and the nasal mucosal DNA was subsequently isolated, purified, and counted. Total DNA binding (DPM/µg DNA) was found to be linear with respect to mCi of inhaled carcinogen. This is shown in Figure 3 for MMS and IPMS. The binding of DMCC to nasal mucosal DNA vs mCi inhaled by individual rats has been reported (Snyder & Bowers, 1987) and was 3.19 DPM/µg per mCi inhaled, which is essentially equal to the DNA binding of the other SN1 agent IPMS (3.1 DPM /µg/mCi).

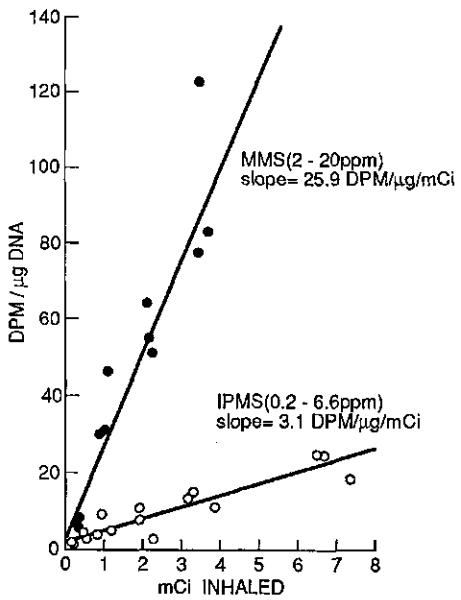


FIGURE 3. Dose/response curves for total DNA binding of [^{3}H]-MMS or [^{3}H]-IPMS in rat nasal mucosa.

Both SN1 agents bind to DNA to a lesser extent (1/8) than MMS (25.9 DPM/μg per mCi inhaled). Binding dose response studies on BPL have not been completed at this time. Rats previously exposed to DMCC or IPMS for 29 d followed by a single 2 h exposure to [^{3}H]-carcinogen exhibited similar dose response curves. This indicates that adduct formation in DNA is a linear function of inhaled dose even after repeated exposures.

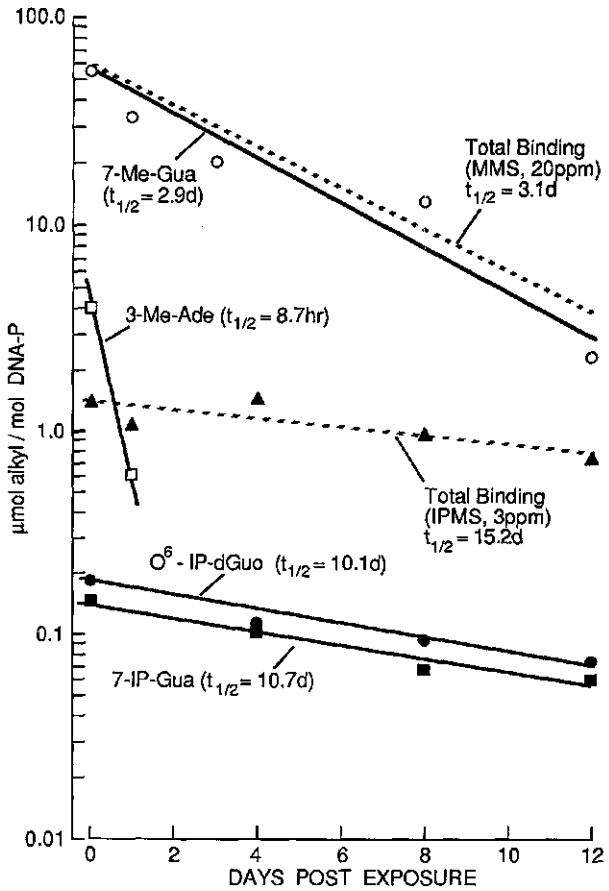


FIGURE 4. Persistence of total DNA binding and specific adducts in rat nasal mucosa after 2 h inhalation exposure to MMS (20 ppm) or IPMS (3 ppm)

Experiments were conducted to determine the rate of disappearance of the total binding to nasal DNA after initial 2 h exposure and to measure the stability of specific adducts. Figure 4 shows the decay of total rat nasal DNA binding following 2 h exposure to 20 ppm [^{3}H]-MMS (208 μCi/μmol). The major adduct 7-Me-guanine (93% of recovered activity), decayed *in vivo* with a half-life of 2.9 d and accounted for the observed decay of total binding ($t_{1/2}$=3.1 d). 3-Me-adenine (7%) decayed so rapidly ($t_{1/2}$=8.7 h) that it could not be detected after 24 h. The half lives we determined in rat nasal mucosa are comparable to the half-lives of 7-Me-Gua (2.4 d) and 3-Me-Ade (6.5 h) measured *in vivo* in rat liver

in rat liver DNA exposed to dimethylnitrosamine (Den Engelse et al., 1986). Figure 4 also shows the decay of total binding of ^{3}H-IPMS (3 ppm, 1.3 mCi/μmole) as well as the DNA adducts O^{6}-IP-dGuo and 7-IP-Gua. The total binding decays 5 times slower than for MMS with a half-life of 15.2 days. The specific IP adducts at 7-Gua and O^{6}-dGuo have half-lives of 10.1 d and 10.7 d, respectively. When comparing the persistence of methyl vs ethyl adducts in rat liver, the bulkier ethyl adducts had longer half-lives (Den Engelse et al., 1986). In our study, the repair of the even bulkier IP adducts appear to follow that pattern. O^{6}-IP-dGuo and 7-IP-Gua account for only 10% and 14%, respectively, of the total DNA binding. Isopropylphospotriesters probably account for much of the remaining binding since ethylnitrosourea, an SN1 alkylating agent with a similar reactivity to IPMS, results in 60% of the total in vivo DNA binding to rat liver (Den Engelse et al., 1986) as ethylphosphotriesters which are repaired very slowly ($t_{1/2}$=32 d). Studies are currently underway to determine the carcinogenic activity of IPMS in the rat nose. DMCC which, like IPMS, binds to DNA about 1-2 orders of magnitude less than the SN2 agents BPL and MMS, nevertheless is a much more potent rat nasal carcinogen than either BPL or MMS. When comparing BPL and MMS, the hydrolysis rates are in the ratio of about 10:1 and 5 ppm BPL produced a tumor response (Sellakumar et al., 1987) roughly equal to that for 50 ppm MMS. In addition, preliminary results indicate that BPL binds to rat nasal DNA seven times more than MMS per mCi inhaled (188 DPM/μg/mCi inhaled). This is consistent with a prediction of DNA binding using the Swain-Scott relationship (Lawley, 1984) which considers only the hydrolysis rate and the Swain-Scott substrate constant (s) for the electrophile. Thus, for certain SN2 compounds with similar s values, hydrolysis rates may predict the level of DNA binding which is in turn related to carcinogenic potency.

References

Den Engelse, L., Menkveld, G.J., de Brij, R-J., and Tates, A.D. 1986. Formation and stability of alkylated pyrimidines and purines (including imidazole ring-opened 7-alkylguanine) and alkylphosphotriesters in liver DNA of adult rats treated with ethylnitrosourea or dimethylnitrosamine. Carcinogenesis 7:393-403.

Hochwalt, A.E., Solomon, J.J., and Garte, S.J. 1988. Mechanism of H-ras oncogene activation in mouse squamous carcinoma induced by an alkylating agent. Cancer Res. 48:556-558.

Kuschner, M., Laskin, S., Drew, R., Capiello, V., and Nelson, N. 1975. Inhalation carcinogenicity of alpha-halo ethers. III. Lifetime and limited period inhalation studies with bis(chloromethyl)ether at 0.1 ppm. Arch. Environ. Health 30:73-77.

Laskin, S., Sellakumar, A.R., Kuschner, M., Nelson, N., La Mendola, S., Rusch, G.M., Katz, G., Dulak, N.C. and Albert, R.E. 1980. Inhalation carcinogenicity of epichlorohydrin in non inbred Sprague-Dawley rats. JNCI 65(4):751-757.

Lawley, P.D. 1984. Carcinogenesis by alkylating agents. In: C.E. Searle (ed.): Chemical Carcinogens, second edition, Vol. 1, ACS Monograph 182, Washington, DC, p. 325-484.

Lawley, P.D., Orr, D.J., and Jarman, M. 1975. Isolation and identification of products from alkylation of nucleic acids: ethyl- and isopropyl purines. Biochem. Journal 145:73-84.

Segal, A., Solomon, J.J., Mate, U., VanDuuren, B.L. 1982. Formation of 6-dimethylcarbamyloxy-dGuo, 6-dimethylamino-dGuo, and 4-dimethylamino-d-Thd following in vitro reaction of dimethylcarbamyl chloride with

calf thymus DNA, and 6-diethylcarbamyloxy-dGuo following in vitro reaction of diethylcarbamyl chloride with calf thymus DNA. Chem. Biol. Interact. 40:209-231.

Segal, A., Solomon, J.J., Mignano, J., and Dino, J. 1981. The isolation and characterization of 3-(2-carboxyethyl)cytosine following in vitro reaction of β-propiolactone with calf thymus DNA. Chem.-Biol. Interactions 35:349-361.

Sellakumar, A., Albert, R., and Kuschner, M. 1983. The pathogenesis of nasal neoplasia induced by alkylating and aldehyde compounds. In: G. Reznik (ed.): Nasal Tumors in Man and Animals,, Vol. III, CRC Press, Boca Raton, FL, p. 27-45.

Sellakumar, A., Snyder, C.A., and Albert, R.E. 1987. Inhalation carcinogenesis of various alkylating agents. J. Natl. Cancer Inst. 79:285-289.

Singer, B. and Grunberger, D. 1983. Molecular Biology of Mutagens and Carcinogens, Plenum Press, New York.

Snyder, C.A. and Bowers, A.B. 1987. A new inhalation exposure system for the determination of inhaled doses in laboratory rats. Arch. Toxicol. 61:3-6.

Solomon, J.J., Fedyk, J., Mukai, F., and Segal, A. 1985. Direct alkylation of 2'-deoxynucleosides and DNA following in vitro reactions with acrylamide. Cancer Research 45:3465-3470.

Solomon, J.J. and Segal, A. 1985. Direct alkylation of calf thymus DNA by acrylonitrile. Environ. Health Perspect. 67:227-230.

ROLE OF DNA ADDUCT FORMATION AND CELL PROLIFERATION IN THE INDUCTION OF NASAL TUMORS IN THE RAT BY THE TOBACCO SPECIFIC CARCINOGNEN 4-(N-METHYL-N-NITROSAMINO)-1-(3-PYRIDYL)-1-BUTANONE

S.A. Belinsky[1], V.E. Walker[2], L.C. Uraih[1], R.R. Maronpot[1], J.A. Swenberg[2] and M.W. Anderson[1]

[1]National Institute of Environmental Health Sciences and [2]Chemical Industry Institute of Toxicology, Research Triangle Park, North Carolina, USA

Summary

The dose response for O^6-methylguanine (O^6MG) formation and cytotoxicity was determined in the respiratory and olfactory regions from Fischer 344 rats during multiple dose administration of the tobacco-specific nitrosamine 4-(N-methyl-N-nitrosamino)-1-(3-pyridyl)-1-butanone (NNK). The dose response for O^6MG formation differed considerably between the respiratory and olfactory mucosa from the nasal passages of the rat. The dose response was nonlinear in respiratory mucosa but linear in olfactory mucosa. The alkylation efficiency increased dramatically only in the respiratory mucosa as the dose of NNK was decreased. These studies suggest that a low K_m pathway for NNK activation is present in the nose and that this pathway is localized predominantly in the respiratory region. In contrast to alkylation data, greater sensitivity to toxicity by NNK was observed in the olfactory region. Only mild metaplasia and a 5% incidence of malignant tumors were observed in the respiratory region. However, treatment with NNK resulted in marked necrosis of the Bowman's glands and olfactory epithelium. Subsequently, basal cell hyperplasia and squamous cell metaplasia was observed in the olfactory mucosa resulting ultimately in a 45% incidence of tumors in this area of the nose. These data suggest that both the formation of promutagenic adducts and cell proliferation secondary to toxicity are required for the induction of neoplasia by NNK within the nose.

Keywords: 4-(N-methyl-N-nitrosamino)-1-(3-pyridyl)-1-butanone, O^6-methylguanine, respiratory epithelium, olfactory epithelium.

Introduction

One of the major carcinogenic constituents of tobacco products is NNK (Hoffmann et al., 1979). Carcinogenicity studies with Fischer 344 rats have demonstrated that exposure to high doses of NNK is associated with a high incidence of lung, liver and nasal cavity tumors, while at low doses of carcinogen, the majority of tumors were of lung origin (Hoffmann et al., 1984). The potent carcinogenicity of NNK may result from metabolic activation via cytochrome P-450 to a methylating agent (Hecht et al., 1980). Treatment of rats with multiple administrations of a high dose of NNK (100 mg/kg/day) results in the alkylation of DNA in target tissues (Belinsky et al., 1986). After 1 day of treatment, the concentration of the promutagenic adduct O^6MG was greatest in

nasal mucosa followed by liver and lung. However, during the next 5 days of treatment, alkylation of DNA in nasal epithelium declined to one half the initial amount and remained constant for the remaining 6 days of carcinogen administration. The decrease in O^6MG concentration appeared to result from cytotoxicity induced by exposure to NNK. Following only 4 days of treatment, severe necrosis of the olfactory mucosa and early metaplasia of the respiratory mucosa was evident. These data suggest that both the high concentration of DNA methylation and cytotoxicity may contribute to the induction of nasal tumors by NNK. The purpose of the studies presented below was to determine the dose response for DNA methylation and cytotoxicity in the respiratory and olfactory regions of the nose and their relationship to the induction of neoplasia.

Results and Discussion

The dose response for O^6MG in respiratory and olfactory mucosa was determined 4 h after treatment of rats with doses of NNK ranging from 0.3 to 100 mg/kg. The molecular dosimetry of O^6MG formation differed considerably for the two regions of the nose (Belinsky et al.,1987a). The relationship between dose and O^6MG formation was nonlinear in respiratory mucosa. The dose-response curve was very steep from 0.3 to 3 mg/kg but declined markedly in the dose range of 10 to 100 mg/kg (Figure 1A). In contrast, the dose response for the olfactory mucosa did not demonstrate such a large change in slope over the same dose range (Figure 1B). Differences in the shape of the dose response curves were accompanied by two to four times greater concentration of O^6MG in respiratory than olfactory region after treatment with doses of NNK ranging from 0.3 to 3.0 mg/kg, while the concentration of this adduct was similar in both regions of the nose after treatment with higher doses of carcinogen (30 or 100 mg/kg). The difference in the shape of the dose-response curves for O^6MG in respiratory and olfactory regions may stem from the localization of metabolic pathways for biotransformation of NNK. The amount of O^6MG formed per unit dose of NNK, an index of alkylation efficiency, increased 8-fold in respiratory mucosa as the dose of carcinogen was decreased from 100 to 0.3 mg/kg (Figure 1C). Alkylation efficiency in the olfactory mucosa was not affected as the dose of NNK was altered (Figure 1D). An alkylation efficiency curve similar to that for the respiratory mucosa has been observed in lung under similar conditions (Belinsky et al., 1987b). Based on the two component nature of this curve, we hypothesized previously (Belinsky et al., 1987b) that low and high K_m pathways exist for activation of NNK to a methylating agent in the lung. The fact that the concentration of O^6MG was greater in respiratory than olfactory mucosa and that the efficiency for alkylation increased in respiratory mucosa but remained constant in the olfactory mucosa after low dose exposure suggests that a low K_m pathway for NNK activation is also present in the nose and that this pathway is localized predominantly in the respiratory region. This conclusion is supported by autoradiographic studies which demonstrated that silver grains were more heavily concentrated over respiratory than olfactory epithelium 4 h after treatment of rats with 1 mg/kg NNK (Belinsky et al., 1987a). Furthermore, recent studies (Belinsky et al., 1987b; Devereux et al., 1988.) have demonstrated not only that a high affinity pathway exists in the Clara cell of the lung for activation of NNK but that specific P-450 isozymes are involved in the metabolism of this carcinogen. The isozyme which was found to metabolize 80% of NNK was cytochrome $P\text{-}450_b$, an isozyme which is also more concentrated in the respiratory than olfactory region of the nose (Baron et al.,

1988). Thus, the localization of this isozyme is associated with high concentrations of O^6MG in both the Clara cell and the respiratory region following metabolic activation of NNK.

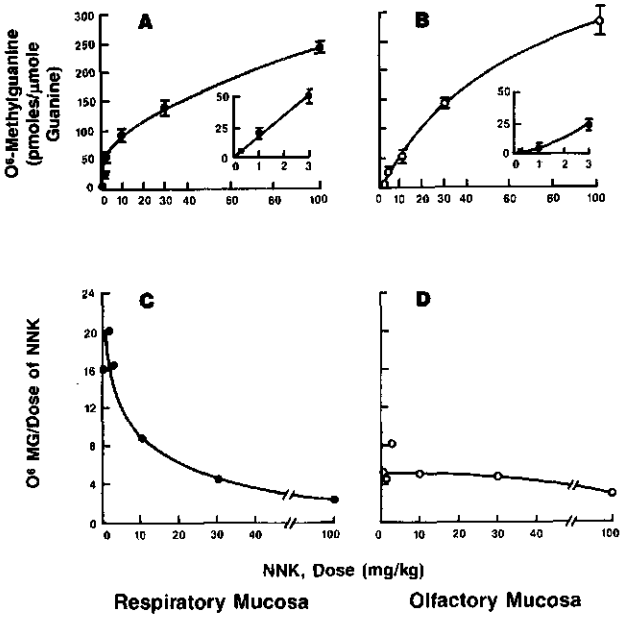


Figure 1. Dose response (A,B) and efficiency of O^6-methylguanine formation (C,D) in nasal mucosa. O^6MG was determined in respiratory (A) and olfactory (B) mucosa from rats 4 hours after treatment with 0.3, 1, 3, 10, 30 or 100 mg/kg NNK. The concentration of O^6MG (pmoles/µmole guanine) in respiratory (C) and olfactory (D) 4 hr after treatment with NNK was divided by the dose of carcinogen (mg/kg) and plotted against dose.

Since DNA isolated from respiratory mucosa is selectively alkylated after low doses of NNK, one would hypothesize that this region would also exhibit greater sensitivity for cytotoxicity and neoplasia than the olfactory mucosa following carcinogen exposure. However, no acute toxicity was observed in either portion of the nasal passages (Table 1) using the dosing regimen which gave the largest differences in alkylation between the two regions of the nose (1 mg/kg). The earliest morphological changes were obseved after treatment for 2 days with 10 mg/kg NNK and involved the selective necrosis of the secretory cells of the lateral nasal glands (Steno's gland, Table 1). Necrosis of Bowman's glands underlying the olfactory mucosa was evident after 2 days of treatment with 30 mg/kg NNK (Table 1). Damage to both Steno's and Bowman's glands progressed in a dose and time dependent manner (Belinsky et al., 1987a).

Serous glands underlying the respiratory mucosa were histologically normal during treatment with NNK. Degeneration of the olfactory epithelium was observed after treatment for 4 days with 30 mg/kg NNK. Higher cumulative doses of carcinogen resulted in complete replacement of the olfactory epithelium by plump metaplastic cells. The respiratory mucosa was relatively resistant to toxicity by NNK (Table 1). These data indicate that the olfactory region is significantly more sensitive to toxicity induced by NNK than the respiratory region. Based on autoradiography and alkylation studies, it is apparent that the differential toxicity to the nasal passages induced by NNK cannot be attributed to either the local concentration of DNA adducts or to the NNK itself.

Table 1. Dose response for toxicity in the nasal passages of the Fischer 344 rat during treatment with NNK.

The dose response for toxicity in the nasal passages was determined in rats treated with 1, 10, 30 or 100 mg/kg NNK for 2,4, or 12 days. Three to four rats were used for each dose and time point.

Location of lesion[a]	Dose (mg/kg)	Time		
		2 Days	4 Days	12 Days
Steno's gland	1	-	-	-
	10	+	++	+++
	30	++	+++	++++
	100	+++	++++	++++
Bowman's glands	1	-	-	-
	10	-	+	++
	30	+	++	++++
	100	+++	++++	++++
Olfactory epithelium	1	-	-	-
	10	-	-	++
	30	-	+	++++
	100	+	++++	++++
Respiratory epithelium	1	-	-	-
	10	-	-	-
	30	-	-	+
	100	-	+	++

[a]Degree of necrosis or metaplasia graded from - to ++++.

A carcinogenicity study was initiated to determine whether chronic treatment with NNK produces morphological changes and neoplasia which parallel the localization of acute cytotoxicity. Since significant toxicity to the olfactory region was observed after treatment with either 30 or 100 mg/kg, an intermediate dose of 50 mg/kg was chosen for the carcinogenicity study. Based on the dose response for O^6MG formation (Figure 1), this concentration of NNK should result in similar alkylation in both regions of the nose. Male Fischer 344 rats were treated 3 times a week for 20 weeks (5) with either NNK (50 mg/kg, s.c.) or vehicle (trioctanoin). Nasal tumors were not present in any of the

vehicle-treated animals. The majority (28/31) of malignant neoplasms originated in the olfactory region (Table 2). Gland like structures were present in some of these carcinomas, but most were comprised of sheets of anaplastic epithelial cells (Belinsky et al., 1987a). Many of these malignant neoplasms extended anteriorly into the respiratory region of the nose and/or infiltrated through the cribriform plate into the brain. In contrast, the majority of the benign neoplasms (18/21) were localized in the respiratory region of the nose and were polypoid adenomas.

Table 2. Tumor incidence and distribution in the nasal passages of the Fischer 344 rat following chronic treatment with NNK.

Sixty-two rats received NNK (50 mg/kg, s.c.) dissolved in trioctanoin three times a week for 20 weeks while 17 rats were treated with vehicle (trioctanoin) following the same dosing regimen. Animals in the treatment group were killed after a 10-15% loss of body weight over the course of 35 weeks following the cessation of treatment.

	Number of tumors per group			
	NNK		Vehicle	
Location of lesion	Benign	Malignant	Benign	Malignant
Respiratory region				
Steno's gland	0	1	0	0
Serous gland	4	1	0	0
Respiratory epithelium	14	1	0	0
Olfactory region				
Bowman's gland	3	15	0	0
Olfactory epithelium	0	13	0	0

Recent studies (L. Uraih et al., unpublished) have characterized the time course and identified the morphological changes induced during chronic exposure to NNK. During the first 6 weeks of exposure, the majority of histological changes were similar to those observed following acute exposure to NNK (Table 1). After 10 weeks of treatment, the olfactory region exhibited mild squamous metaplasia and basal cell hyperplasia. These changes increased in severity over the next 10 weeks and after 20 weeks of exposure, dysplasia and marked squamous metaplasia of the olfactory mucosa was evident. In the animals which were sacrificed over a 35 week period following the cessation of treatment, the majority of lesions were also present in the olfactory region (Belinsky et al., 1987a). These included basal cell hyperplasia and possibly hyperplasia of Bowman's glands as well as squamous metaplasia and dysplasia of the olfactory mucosa. Based on these data, it is difficult to determine the cell of origin for the carcinomas which were localized in the olfactory mucosa.

These studies indicate that at least two factors are important in the initiation of neoplasia in the nose, DNA alkylation and cytotoxicity. If one attempted to predict the tumor response in the nose based solely on the localization of DNA adducts and alkylation efficiency, then the respiratory region would be considered the major target site for neoplasia. However, the majority

of tumors induced by NNK appear to originate from the olfactory region and occur only after chronic treatment with high doses of NNK (Hoffmann et al., 1984). The steep dose response curve for induction of tumors (Hoffmann et al., 1984) as well as the localization of lesions in the nasal passages can be explained by a difference in sensitivity to the cytotoxicity of NNK. Although the concentration of O^6MG was greater in respiratory regions after low dose exposure to NNK, no cytotoxicity was observed in either region of the nose. In contrast, after exposure to high doses of the carcinogen, adduct concentrations were similar in both regions of the nose, however marked cytotoxicity was localized to the olfactory region. Following cytotoxicity, cell proliferation was induced in the olfactory region as demonstrated by severe basal cell hyperplasia. Taken together, these data indicate that both the formation of promutagenic adducts and cell proliferation secondary to toxicity are required for the induction of neoplasia by NNK within the nose.

References

Baron, J., J.P. Burke, F.P. Guengerich, W.B. Jakoby & J.M. Voigt, 1988. Sites for xenobiotic activation and detoxication within the respiratory tract: Implications for chemically induced toxicity. Toxc. Applied Pharm. 93: 493-505.

Belinsky, S.A., V.E. Walker, R.R. Maronpot, J.A. Swenberg & M.W. Anderson, 1987a. Molecular dosimetry of DNA adduct formation and cell toxicity in rat nasal mucosa following exposure to the tobacco specific nitrosamine 4-(N-methyl-N-nitrosamino)-1-(3-pyridyl)-1-butanone and their relationship to induction of neoplasia. Cancer Res. 47: 6058-6065.

Belinsky, S.A., C.M. White, J.A. Boucheron, F.C. Richardson, J.A. Swenberg & M.W. Anderson, 1986. Accumulation and persistance of DNA adducts in respiratory tissue of rats following multiple administrations of the tobacco specific carcinogen 4-(N-methyl-N-nitrosamino)-1-(3-pyridyl)-1-butanone. Cancer Res. 46: 1280-1284.

Belinsky, S.A., C.M. White, T.R. Devereux, J.A.Swenberg & M.W. Anderson, 1987b. Cell selective alkylation of DNA in rat lung following low dose exposure to the tobacco specific carcinogen 4-(N-methyl-N-nitrosamino)-1-(3-pyridyl)-1- butanone. Cancer Res. 47: 1143-1148.

Devereux, T.R., M.W. Anderson & S.A. Belinsky, 1988. Factors regulating activation and DNA alkylation by 4-(N-methyl-N-nitrosamino)-1-(3-pyridyl)-1-butanone and nitrosodimethylamine in rat lung and isolated lung cells, and the relationship to carcinogenicity. Cancer Res. 48: 4215-4221.

Hecht, S.S., R. Young & C.B. Chen, 1980. Metabolism in the F344 rat of 4-(N-methyl-N-nitrosamino)-1-(3-pyridyl)-1-butanone. Cancer Res. 40: 298-302.

Hoffmann, D., J.D. Adams, K.D. Brunnemann & S.S. Hecht, 1979. Assessment of tobacco specific nitrosamines in tobacco products. Cancer Res. 39: 2505-2509.

Hoffmann, D., A. Rivenson, S. Amin & S.S. Hecht, 1984. Dose-response study of the carcinogenicity of tobacco-specific N-nitrosamines in F344 rats. J. Cancer Res. Clin. Oncol. 108: 81-86.

IMMUNOCYTOCHEMICAL STUDY OF FORMATION AND REPAIR OF NNK-INDUCED DNA ADDUCTS IN NASAL EPITHELIUM

J. van Benthem[1], J.W.G.M. Wilmer[2], H.H.K. Winterwerp[1], W.R. Leeman[2], E. Scherer[1] and L. den Engelse[1]

1. The Netherlands Cancer Institute, Division of Chemical Carcinogenesis, Plesmanlaan 121, 1066 CX Amsterdam, The Netherlands
2. TNO-CIVO Toxicology and Nutrition Institute, Department of Biological Toxicology, P.O. Box 360, 3700 AJ Zeist, The Netherlands

Summary

The cellular localization of DNA methylation (O^6- and 7-methylguanine) by the nicotine-derived carcinogen 4-(N-methyl-N-nitrosamino)-1-(3-pyridyl)-1-butanone (NNK) has been established immunocytochemically in male Sprague-Dawley rats treated i.p. with 30 mg NNK/kg. The strongest nuclear staining was observed in the Bowman glands of the olfactory submucosa. In the olfactory epithelium staining was weak and restricted to the sustentacular and basal cells. DNA adducts could also be visualized in the cells of the respiratory epithelium and to some extent in the secretory ducts of the serous glands of the respiratory submucosa. Accumulation and persistence were studied in rats and golden hamsters treated twice a week for 8 weeks with 3.5 mg NNK/kg i.p. or 10 mg NNK/kg s.c., respectively. In the rat DNA adducts (O^6- and 7-meGua) accumulated in the Bowman gland cells and in the respiratory epithelium. After the cessation of NNK treatment, the DNA adduct level decreased within 4 weeks to the detection limit. In the hamster, accumulation occurred in the same cell types and in addition in the sensory cells of the olfactory epithelium.

Introduction

The capacity of N-nitrosamines to induce various types of cancer has experimentally been well established (Druckrey et al., 1967; Lijinsky, 1987). There is abundant evidence that humans are exposed either occupationally or environmentally to a variety of N-nitrosamines (Bartsch and Montesano, 1984). The highest estimated exposure is caused by tobacco use (Bartsch and Montesano, 1984). Tobacco and tobacco smoke contain considerable levels of N-nitroso compounds formed by nitrosation of nicotine and other alkaloids during fermentation (Hecht and Hoffmann, 1983). Among the tobacco-specific N-nitroso compounds 4-(N-methyl-N-nitrosamino)-1-(3-pyridyl)-1-butanone (NNK) is the most potent carcinogen in experimental animals (Hecht et al., 1988). It causes lung, trachea, nasal cavity and liver tumors in the rat as well as lung, trachea and nasal cavity tumors in the hamster (Hecht et al., 1980a; Hoffmann et al., 1981). Induction of nasal tumors is largely independent of the route of administration. NNK needs bioactivation for carcinogenicity and the formation of electrophilic metabolites which can react with DNA (Hecht et al., 1980b). DNA methylation (O^6-meGua, 7-meGua and O^4-meThy) has been reported in

Abbreviations used: 4-(N-methyl-N-nitrosamino)-1-(3-pyridyl)-1-butanone, NNK; O^6-methylguanine, O^6-meGua; 7-methylguanine, 7-meGua).

the target organs of NNK in rats and in hamsters (Hecht et al., 1986; Belinsky et al., 1986). Although in humans tobacco-induced cancers occur most commonly in the lung, alkylation studies demonstrated that in experimental animals the nose is the most sensitive organ for NNK-induced methylation. In the present study, an immunoperoxidase staining technique was applied to establish the cellular distribution of NNK-induced methylation in rat and hamster nasal cavity tissue.

Materials and Methods

Chemicals

NNK was obtained from Chemsyn Science Laboratories, Lenexa, Kansas, USA. The compound was > 99% pure according to HPLC. Tissues were fixed for one hour in freshly prepared chloroform:methanol:acetic acid (6:3:1 v/v, modified Carnoy). The methylmethacrylate for the embedding of nasal tissues was K-plast from MEDIM, Medizinische Diagnostik-Methoden GmbH, D-6300 Giessen, W-Germany. RNase A from bovine pancreas (Sigma Chemical Co., St. Louis, MO, USA), RNase T_1 from *Aspergillus oryzae* (*Boehringer, Mannheim, FRG*), peroxidase-(rabbit)antiperoxidase (PAP; American Qualex International Inc., La Mirada, CA, USA), goat-antirabbit IgG (GαR; against whole IgG; Campro Benelux G102, Elst, The Netherlands) and 3,3-diaminobenzidine-4-HCl (DAB; Sigma Chemical Co., St. Louis, MO, USA) were purchased from the sources indicated.

Antibodies

Rabbit antisera raised against an O^6-methylguanosine-hemocyanin conjugate and an imidazole ring-opened 7-methylguanosine-hemocyanin conjugate were obtained from Dr P. Kleihues (cf. Lüdeke and Kleihues, 1988) and Dr. R. Montesano (cf. Degan et al., 1988), respectively. The anti-7-meGua antibody has its highest affinity for imidazole ring-opened 7-meGua but recognizes to a lesser extent the ring-closed form of this adduct (Degan et al., 1988).

Animals

Adult, male Sprague-Dawley rats from the SPF breeding colony of the Netherlands Cancer Institute were kept on softwood bedding, 2 per polycarbonate cage, and fed standard food pellets (Hope Farms, Woerden, The Netherland). Outbred male golden hamsters (5-6 weeks old, Charles River Canada Inc., St-Constant, Quebec) were housed, 5 per polycarbonate cage, on hardwood bedding and fed with CIVO stock diet. The animals were kept under 12-12 light/dark cycles at 22° C and a relative humidity of 40-70%. Food and water were available *ad libitum*.

Immunocytochemical staining procedure

Sections of 2 µm were cut and mounted under pressure on chromalum-gelatin coated slides. The plastic was removed with chloroform and the sections were stained for O^6- and 7-meGua DNA adducts according to Scherer et al. (1988). The technique was slightly modified for use on plastic sections. Incubation with the first antibody was performed overnight at 4° C. Antibodies against O^6- and 7-meGua were diluted 1:15000. The sensitivity of the immunocytochemical staining method was estimated at 1-2 µmol O^6-meGua and about 25 µmol 7-meGua per mol

DNA-P. Loss of DNA during the staining procedure was negligible as judged by Feulgen staining performed after the complete immunocytochemical procedure.

Experiment 1

Rats received a single i.p. dose of NNK (30 mg/kg body weight, dissolved in saline). Control rats were treated with saline. The animals were bled 6, 12, 25 or 52 hours after NNK treatment. Immediately after exsanguination the nasal, maxillary and ethmoid turbinates of the nasal cavity were dissected and processed for embedding in K-plast.

Experiment 2

Rats and hamsters were treated twice weekly over a period of 8 weeks with 3.5 mg NNK/kg i.p. or 10 mg NNK/kg s.c., respectively. The animals (2 or 3 per time point) were killed by exsanguination 6, 24 and 72 hours after the first application, 72 hours after the 2nd, 4th, 8th and 16th application, and 1, 4 and 8 weeks after the last dose. Nasal tissues were immediately dissected and processed for embedding in K-plast. The time point of 72 hours was selected since we were interested in adduct accumulation rather than in the extent of adduct formation by each dose.

Results

Strong staining for both O^6- and 7-meGua was observed in nasal tissues of rats and hamsters. In experiment 1 most intensive DNA damage was found six hours after application in the Bowman glands of the submucosa of the olfactory epithelium (Figs. 1A and B). Twelve hours after application more nuclei were stained, but the staining intensity was weaker. At 25 and 50 hours after NNK only a few nuclei were stained. The sustentacular cells lining the olfactory epithelium showed weak nuclear staining only at six hours after NNK injection, whereas DNA adducts in basal cells could be demonstrated at both 6 (Fig. 1A) and 12 hours after NNK treatment.

In the respiratory epithelium (Fig. 1C) the highest staining intensity was found 12 hours after treatment with NNK. Both O^6- and 7-meGua specific staining were still rather intense after 50 hours. Only relatively few cells of the respiratory epithelium were stained. The distribution of the stained cells within the tissue points to Goblet cells. In addition, some secretory duct cells of serous glands in the respiratory submucosa, showed nuclear staining.

Accumulation of adducts after multiple low doses was studied in experiment 2. In rats and hamsters nuclear staining was restricted to the olfactory submucosa (Bowman glands), the respiratory epithelium and the secretory ducts of the serous glands of the respiratory submucosa. Exclusively in the hamster, the nuclei of sensory cells of the olfactory epithelium became methylated (Fig. 2). In the olfactory submucosa of both species, nuclear staining was significant 24 hours after the first dose, but the staining intensity at 72 hours was lower. Thereafter, staining intensity gradually increased in the course of the experiment up to 8 weeks. In rats, both O^6- and 7-MeGua levels had decreased one week after the last dose; after 4 weeks nuclear staining was no longer observed. In the respiratory epithelium the pattern of accumulation and loss of damage was similar.

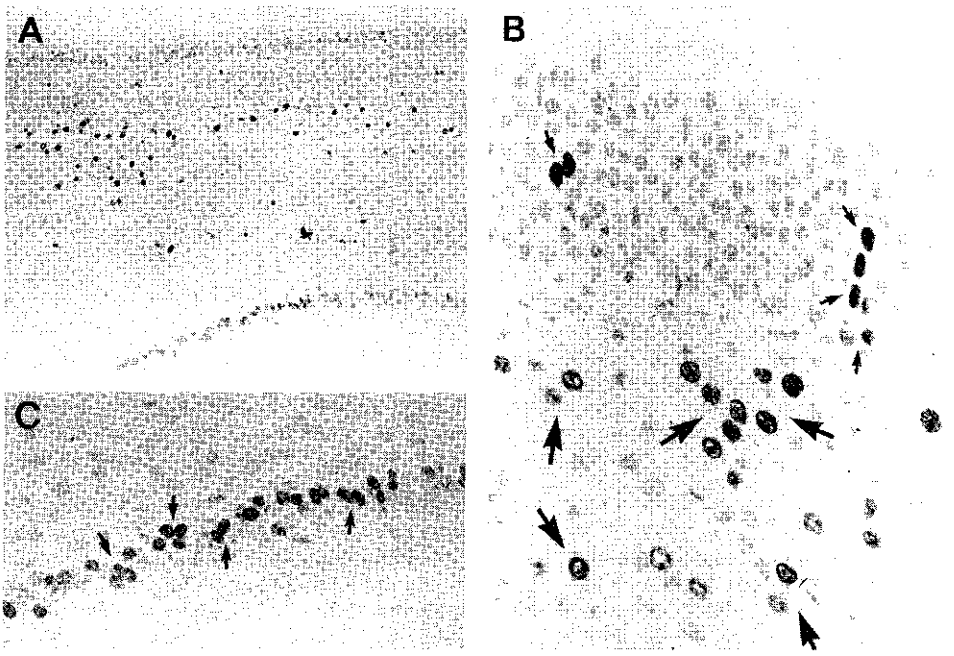


Fig. 1. Immunocytochemical staining of 7-meGua (arrows) in rat nasal epithelium 6 hours after treatment with 30 mg NNK/kg. A: Overview of the ethmoid turbinate. x 130. B: Olfactory epithelium: Bowman glands (heavy arrows), duct cells (small arrows). x 510. C: Respiratory epithelium. x 320. (Sections slightly counterstained with haematoxylin)

Discussion

The present results show that the immunocytochemical approach makes it possible to visualize DNA adducts in individual nuclei of the rodent nose. O^6- and 7-methylguanine were demonstrated, indicating that NNK is metabolized in this organ. This observation is consistent with the high α-hydroxylase activity (Brittebo et al., 1983) and relatively high levels of cytochrome P450 which can catalyze the α-hydroxylation (Hadley et al., 1983: Yang et al., 1985).

In both rat and hamster the highest levels of DNA modification were observed in the nuclei of Bowman glands and their efferent ducts (Figs 1 and 2). It has been reported that these cell types are most susceptible to the toxic effects of NNK (Belinsky et al., 1987). Relatively low DNA alkylation was observed in the epithelia. In the olfactory epithelium of the rat the DNA of the sustentacular cells as well as the basal cells was modified (Fig. 1A), whereas in the hamster the strongest epithelial staining was found in the sensory cells (Fig. 2). In the rat O^6- and 7-meGua-specific staining was only observed at 6 and 12 hours after the single, high dose of NNK. The subsequent decrease of O^6- and 7-meGua-specific staining not necessarily means repair since both cell types have a rather high cell turnover. Our findings are in agreement with metabolism data obtained

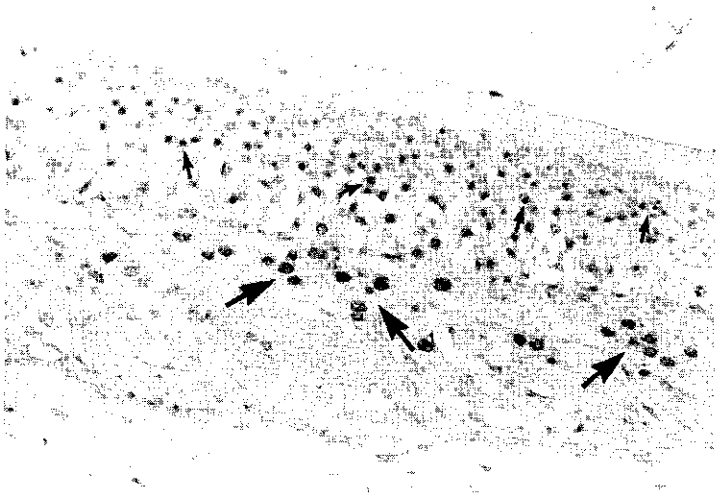


Fig. 2. Immunocytochemical staining of 7-meGua in hamster olfactory epithelium after 4 applications of 10 mg NNK/kg (s.c.). Bowman glands: heavy arrows; duct cells: small arrows. x 430. (Sections slightly counterstained with haematoxylin)

by autoradiography (Belinsky et al., 1987; Tjälve et al., 1985). The former authors also reported metabolic activity in the serous glands of the rat nose whereas we found DNA methylation to be limited to a small (not yet identified) subfraction of the secretory ducts of these glands.

Immunocytochemistry enabled us to demonstrate that accumulation occurred in the Bowman gland cells and in the respiratory epithelial cells. No accumulation was observed in either the sustentacular or basal cells of the olfactory epithelium. These data are in agreement with the findings of Belinsky et al. (1986; 1987). Apparently, the dose applied to rats and hamsters in the present experiments is high enough to saturate the repair enzymes. Belinsky et al. (1986) reported that the capacity for O^6-methylguanine repair in nasal tissue decreased with the number of carcinogen applications. DNA methylation, however, did not persist since 1 week after the last treatment the number adducts had already decreased, and no staining was seen anymore after 4 weeks.

As compared to other currently used techniques only immunocytochemistry shows the exact localization of cells involved in the metabolic activation of and damaged by carcinogenic agents. It thus permits the study of cell specific distribution, accumulation and repair of DNA adducts in the organ of interest. The introduction of thin plastic sections makes it possible to combine on serial sections, and for the same nuclei, DNA adduct-specific staining with other histochemical reactions. This permits the identification of damaged cells and will contribute to the elucidation of the relation between initial DNA damage and cancer induction.

Acknowledgements

Our study on NNK was financially supported by grant no. NKI 88-19 from The Dutch Cancer Foundation. The technical assistance of E. Vermeulen and C. Schippers-Gillissen is greatly appreciated. Thanks are also due to Drs. P. Kleihues and R. Montesano for their generous gift of the antisera against O^6- and 7-methylguanine, respectively.

References

Bartsch, H. & R. Montesano, 1984. Relevance of nitrosamines to human cancer. Carcinogenesis 5:1381-1393.

Belinsky, S.A., V.E. Walker, R.R. Maronpot, J.A. Swenberg & M.W. Anderson, 1987. Molecular dosimetry of DNA adduct formation and cell toxicity in rat nasal mucosa following exposure to the tobacco specific nitrosamine 4-(N-methyl-N-nitrosamino)-1-(3-pyridyl)-1-butanone and their relationship to induction of neoplasia. Cancer Res. 47:6058-6065.

Belinsky, S.A., C.M. White, J.A. Bucheron, F.C. Richardson, J.A. Swenberg & M. Anderson, 1986. Accumulation and persistence of DNA adducts in respiratory tissues of rats following multiple administrations of the tobacco specific carcinogen 4-(N-methyl-N-nitrosamino)-1-(3-pyridyl)-1-butanone. Cancer Res. 46:1280-1284.

Brittebo, E.B., A. Castonguay, K. Furuya & S.S. Hecht, 1983. Metabolism of tobacco-specific nitrosamines by cultured rat nasal mucosa. Cancer Res. 43:4343-4348.

Degan, P., R. Montesano & C.P. Wild, 1988. Antibodies against 7-methyldeoxyguanosine: Its detection in rat peripheral blood lymphocyte DNA and potential applications to molecular epidemiology. Cancer Res. 48:5065-5070.

Druckrey, H., R. Preussmann, S. Ivankovic & D. Schmähl, 1967. Organotrope carcinogene Wirkungen bei 65 verschiedenen N-Nitroso-Verbindungen an BD-Ratten. Z. Krebsforsch. 69:103-201.

Hadley, W.M. & A.R. Dahl, 1983. Cytochrome P-450-dependent monoxygenase activity in nasal membranes of six species. Drug Metab. Disp. 11:275-276.

Hecht, S.S., C.B. Chen, T. Ohmori & D. Hoffmann, 1980a. Comparative carcinogenicity in F344 rats of the tobacco-specific nitrosamines, N'-nitrosonornicotine and 4-(N-Methyl-N-nitrosamino)-1-(3-pyridyl)-1-butanone. Cancer Res. 40:298-302.

Hecht, S.S. & D. Hoffmann, 1988. Tobacco-specific nitrosamines, an important group of carcinogens in tobacco and tobacco smoke. Carcinogenesis 9:875-884.

Hecht, S.S., N. Trushin, A. Castonguay & A. Rivenson, 1986. Comparative tumorigenicity and DNA methylation in F344 rats by 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone and N-nitrosodimethylamine. Cancer Res. 46:498-502.

Hecht, S.S., R. Young & C.B. Chen, 1980b. Metabolism in the F344 rat of 4-(N-methyl-N-nitrosamino)-1-(3-pyridyl)-1-butanone, a tobacco-specific carcinogen. Cancer Res. 40:4144-4150.

Hoffmann, D., A. Castonguay, A. Rivenson & S.S. Hecht, 1981. Comparative carcinogenicity and metabolism of 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone and N'-nitrosonornicotine in Syrian golden hamsters. Cancer Res. 41:2386-2393.

Lijinsky, W.1987. Structure-activity relations in carcinogenesis by N-nitroso compounds. Cancer Metastasis Review 6:301-356.

Lüdeke, B.I. & P. Kleihues, 1988. Formation and persistence of O^6-(2-hydroxyethyl)-2'-deoxyguanosine in DNA of various rat tissues following a single dose of N-nitroso-N-(2-hydroxyethyl)urea. An immuno-slot-blot study. Carcinogenesis 9:147-151.

Scherer, E., J. Van Benthem, P.M.A.B. Terheggen, E. Vermeulen, H.H.K. Winterwerp & L. Den Engelse, 1988. Immunocytochemical analysis of DNA adducts at the single cell level: A novel tool for experimental carcinogenesis, chemotherapy and molecular epidemiology. In: H. Bartsch, K. Hemminki & I.K. O'Neill (Ed.): Detection Methods for DNA-Damaging Agents in Man: Applications in Cancer Epidemiology and Prevention. IARC Scientific Publ., 89, Lyon. pp. 286-291.

Tjälve, H., A. Castonguay & A. Rivenson, 1985. Microautoradiographic localization of bound metabolites in the nasal cavities of F344 rats treated with the tobacco-specific carcinogen 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone. J. Natl. Cancer Inst. 74:185-189.

Yang, C.S., Y.Y. Tu, D.R. Koop & M.J. Coon, 1985. Metabolism of nitrosamines by purified rabbit liver cytochrome P-450 isozymes. Cancer Res. 45:1140-1145.

FORMALDEHYDE TOXICITY - DNA-PROTEIN CROSS-LINKING STUDIES IN RATS AND NONHUMAN PRIMATES

H. d'A. Heck, M. Casanova, W. H. Steinhagen, J. I. Everitt, K. T. Morgan, and J. A. Popp

Chemical Industry Institute of Toxicology, Research Triangle Park, N.C., U.S.A.

Summary

DNA-protein cross-links induced by a single 6-hr exposure to formaldehyde (HCHO) were measured in the nasal mucosa of F-344 rats and in several regions of the upper respiratory tract of Rhesus monkeys. The concentration of cross-links increased nonlinearly with the airborne concentration in both species. Concentrations of DNA-protein cross-links in the turbinates and anterior nasal mucosa were significantly lower in monkeys than in rats. Cross-links were also formed in the nasopharynx and trachea of monkeys, but they were not detected in the sinus, proximal lung, or bone marrow. The species differences with respect to DNA-protein cross-link formation may be due to differences in nasal cavity deposition and in the elimination of absorbed HCHO. The results provide a strong case for the use of *delivered* doses in estimating cancer risk in different tissues and across different species.
Keywords: formaldehyde, delivered dose, DNA-protein cross-links, nasal mucosa, rat, monkey, upper respiratory tract

Introduction

Inhaled HCHO forms DNA-protein cross-links in the nasal respiratory mucosa of rats (Casanova et al., 1989). Formaldehyde has been shown to be genotoxic to human cells in vitro, causing mutations (Goldmacher & Thilly, 1983), deletions (Crosby et al., 1988), chromosomal aberrations (Dresp & Bauchinger, 1988), as well as inhibition of DNA synthesis (Snyder & Van Houten, 1986). The mechanism of HCHO mutagenesis is not understood in detail, but it is plausible that the formation of DNA-protein cross-links may be a critical step in many of these events (Craft et al., 1987; Benyajati et al., 1983; Natarajan et al., 1983).

Inasmuch as DNA-protein cross-links are potentially mutagenic, these reaction products may be important in the neoplastic transformation of rat nasal epithelial cells. Therefore, it is of interest to determine to what extent these products can be formed in species more closely related to man. The present experiments were undertaken to investigate the formation of DNA-protein cross-links in several regions of the respiratory tract of nonhuman primates and to compare the results with those obtained in rats.

Methods

Exposure procedures

Groups of four male F-344 rats (approximately 280 g) were exposed (6 hr) to selected concentrations (0.3, 0.7, 2, 6, or 10 ppm) of [^{14}C]formaldehyde (H^{14}CHO) in a

nose-only inhalation chamber. The procedure used for exposure of rats is described elsewhere (Casanova et al., 1989). The rats were sacrificed by decapitation immediately after the exposure, and samples of respiratory mucosal tissue were collected from the nasal turbinates and anterior walls of the nose including the nasal septum. Tissue samples from four rats were combined for the isolation of DNA and the analysis of DNA-protein cross-links. All experiments were carried out in triplicate.

Individual male Rhesus monkeys (*Macaca mulatta*) (approximately 7 kg) were exposed (6 hr) in a head-only inhalation chamber to $H^{14}CHO$ (0.7, 2, or 6 ppm) (Fig. 1). Immediately after the exposure, the monkeys were sedated with sodium pentobarbital and were sacrificed by exsanguination from the femoral artery. The head was opened and samples of respiratory mucosa were collected from the middle turbinates, anterior nasal walls and anterior septum, nasopharynx, maxillary sinuses, larynx-trachea-carina, major intrapulmonary airways, and proximal lung, as well as the bone marrow. The tissue samples were analyzed individually after pooling corresponding tissue samples from each side of the nose. Each experiment was carried out in triplicate.

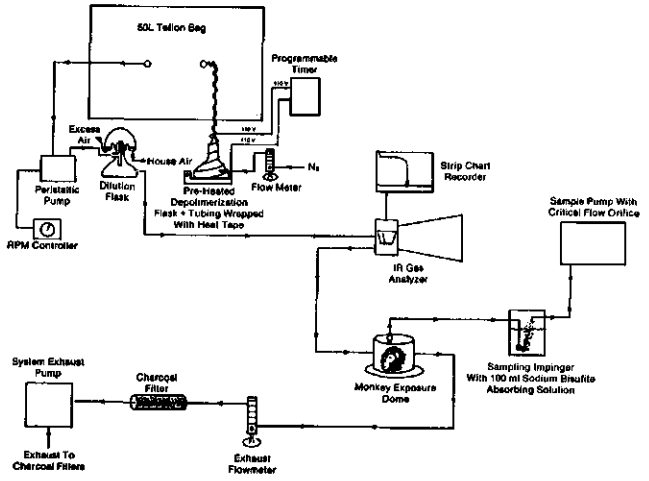


Fig. 1. Schematic diagram of primate [^{14}C]formaldehyde exposure system

Analysis of DNA-protein cross-links

The method used for the isolation of DNA and for the analysis of cross-links is described in detail elsewhere (Casanova et al., 1989). Briefly, tissue samples were homogenized in phosphate buffer then were suspended in a denaturing solution containing concentrated urea and 1% sodium dodecyl sulfate. The DNA was sheared, and proteins were digested with proteinase K. The solutions were extracted with chloroform/isoamyl alcohol/phenol, and DNA was isolated by hydroxyapatite chromatography and washed by ultrafiltration. Final purification of the DNA involved digestion with ribonuclease A.

The purified DNA was enzymatically hydrolyzed to deoxyribonucleosides, which resulted in the dissociation of DNA-protein cross-links and in the quantitative release of $H^{14}CHO$ (Casanova et al., 1989). The samples were analyzed by high-performance liquid chromatography. Released $H^{14}CHO$ was collected from the column and was derivatized with dimedone. The amount of $H^{14}CHO$ in the dimedone precipitate was

determined by liquid scintillation counting. Quantitation of DNA was performed by integration of nucleoside peak areas at 260 nm.

Results

In order to compare results obtained in the rat and monkey, a similar region of the respiratory tract of each species was selected. Since the region sampled in the rat included both the turbinates and anterior nose, the mean concentration of cross-links in the turbinates and anterior nose of the monkey was calculated, weighted according to the amount of DNA in each tissue. The calculated concentration of cross-links is the same as that which would have been measured if the two tissue samples had been combined prior to analysis.

The formation of DNA-protein cross-links in the turbinates and anterior nose of rats and monkeys exposed to $H^{14}CHO$ at concentrations ≤ 6 ppm is depicted in Fig. 2. (Results obtained at 10 ppm in the rat are reported elsewhere (Casanova et al., 1989).) The curves drawn in the figure were calculated by computer after fitting a pharmacokinetic model (Casanova & Heck, 1987) to the data of each species. Cross-linking results are expressed in Fig. 2 as B/*t*, where B is the concentration of cross-links in the DNA and *t* is the exposure time (6 hr).

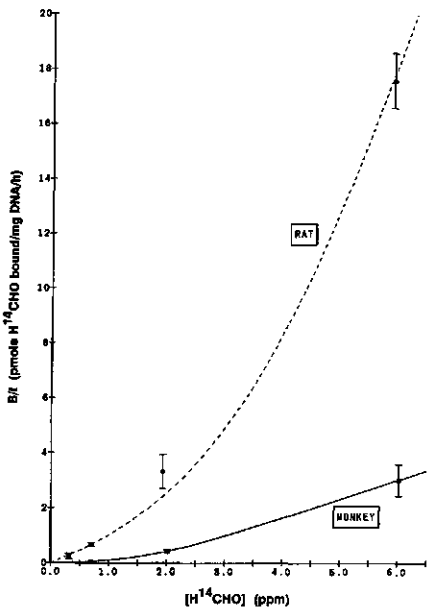


Fig. 2. Formation of DNA-protein cross-links in the turbinates and anterior nose of F-344 rats and Rhesus monkeys exposed for 6 hr to $H^{14}CHO$. Values shown are mean ± S.E., n = 3 exposure groups or animals per concentration. (Data in the rat are taken from Casanova et al., 1989.)

The data shown in Fig. 2 indicate that the formation of DNA-protein cross-links is a nonlinear function of the administered HCHO concentration in both species. In addition, it is clear that monkeys had much lower concentrations of cross-links in the

turbinates and anterior nose than did rats at a given HCHO concentration. The results can be explained in part by differences in nasal cavity deposition (see Discussion). Regional and cellular defense mechanisms may also play an important role in modulating the amount of HCHO that reacts with DNA.

Several other tissues were also examined with respect to the formation of cross-links. A summary of data obtained following a single 6-hr exposure of monkeys to $H^{14}CHO$ is presented in Table 1.

Table 1. Concentration of DNA-protein cross-links in the respiratory tract and bone marrow (femur) of Rhesus monkeys exposed to $H^{14}CHO$.

Tissue	Concentration of DNA-protein cross-links (pmol/mg DNA)*		
	0.7 ppm	2.0 ppm	6.0 ppm
Turbinates + anterior nose	0.36 ± 0.10	2.56 ± 0.31	18.2 ± 3.4
Sinus	ND	ND	ND
Nasopharynx	(0.09 ± 0.09)	(0.47 ± 0.09)	5.8 ± 2.4
Larynx/trachea/carina	ND	(0.4)	9.4 ± 5.4
Airways◇	ND	(0.70 ± 0.15)	5.7 ± 5.7
Proximal lung	ND	ND	ND
Bone marrow	ND	ND	ND

*Mean ± S.E.; ND = not detectable; values in parentheses are uncertain, as dpm for these samples were less than twice the background.
◇Major intrapulmonary airways greater than 2 mm diameter.

The data in Table 1 suggest that DNA-protein cross-links were formed in other regions of the respiratory tract of the monkey, at least at the higher airborne concentrations. The formation of cross-links in these tissues is consistent with the results of histologic examinations of the respiratory tract of monkeys subchronically exposed to 6 ppm of HCHO, which showed that HCHO can penetrate to deeper regions of the respiratory tract of monkeys than of rats (Monticello & Morgan, 1989). Three tissues, the maxillary sinuses, proximal lung, and bone marrow, had no detectable cross-links at any HCHO concentration. The sinus also showed no evidence of tissue damage by HCHO (Monticello et al., 1989).

Discussion

These experiments demonstrate that HCHO forms DNA-protein cross-links in the monkey and in the rat, but that the concentration of cross-links is approximately ten-fold lower in the primate than in the rodent. The difference in species susceptibility to cross-linking may be due in part to differences in nasal cavity deposition. Analogous to the formulation of Chang et al. (1983), the normalized "dose" of HCHO deposited per cm^2 of nasal mucosal surface area can be expressed as:

$$\text{"Dose"} = \frac{\text{Minute volume (liter/min)}}{\text{Respiratory mucosal surface area } (cm^2)} \times \text{Absorption efficiency}$$

where the *absorption efficiency* is defined as the fraction of inhaled gas absorbed by the sampled tissue. The absorption efficiency is a function of the airflow characteristics, the exposure concentration, and the solubility and reactivity of the gas.

The minute volumes of F-344 rats (Mauderly, 1986) and Rhesus monkeys (Bourne, 1975) have been reported, and surface areas of the respiratory mucosa in the nose of F-344 rats and Rhesus monkeys have also been determined (Gross et al., 1982; Gross et al., 1987). The absorption efficiency of HCHO by the turbinates and anterior nose of monkeys was estimated from measurements of the total $H^{14}CHO$ bound to DNA in different regions of the respiratory tract. The efficiency of absorption by the turbinates and anterior nose of rats is reported to be >93% (Patterson et al., 1986). A summary of the values obtained is presented in Table 2.

Table 2. Estimated minute volume and surface area of respiratory mucosa of male F-344 rats and Rhesus monkeys

"Dose" parameter	Male monkey (7 kg)	Male rat (280 g)
Minute volume (liter min^{-1})	2.4	0.28
Respiratory mucosal surface area (cm^2)	47.3	6.2
Minute volume ÷ surface area (liter min^{-1} cm^{-2})	0.05	0.045
Absorption efficiency (turbinates + anterior nose)	0.7-0.9	>0.93

Based on the values shown in Table 2, monkeys would be expected to receive a slightly lower "dose" of HCHO per cm^2 of nasal respiratory mucosal surface area than rats, which may account in part for the lower concentration of cross-links in the turbinates and anterior nose of primates than of rodents. However, the nearly ten-fold difference between rats and monkeys with respect to the concentration of cross-links in these tissues suggests that the monkey may be capable of eliminating absorbed HCHO or of repairing DNA-protein cross-links more efficiently than the rat. Identified pathways of elimination include covalent binding to albumin molecules present in nasal mucus (Bogdanffy et al., 1987) and detoxication catalyzed by formaldehyde dehydrogenase (Casanova & Heck, 1987). Saturation of these defense mechanisms could explain the nonlinear dependence of DNA-protein cross-link formation on the concentration of inhaled HCHO.

Although the monkey had a much lower concentration of cross-links in the turbinates and anterior nose than did the rat, the distribution of cross-links and respiratory tract lesions (Monticello & Morgan, 1989) was more widespread in monkeys than in rats. The concentration of cross-links and the severity of lesions (Monticello et al., 1989) in distal regions of the monkey respiratory tract were generally much less than in the nasal turbinates, suggesting that the dose of HCHO delivered to these tissues was much lower than that delivered to the nasal mucosa. It has been proposed that risk estimates

should be based on *delivered*, rather than *administered*, doses whenever possible (Starr & Buck, 1984). The results obtained in the monkey provide a very strong case that the use of delivered doses for risk assessment is essential to provide a plausible estimate of risk in different tissues and across different species.

References

Benyajati, C., A.R. Place & W. Sofer, 1983. Formaldehyde mutagenesis in Drosophila. Molecular analysis of ADH-negative mutants. Mutat. Res. 111:1-7.

Bogdanffy, M.S., P.H. Morgan, T.B. Starr & K.T. Morgan, 1987. Binding of formaldehyde to human and rat nasal mucus and bovine serum albumin. Toxicol. Lett. 38:145-154.

Bourne, G.H., 1975. Collected anatomical and physiological data from the Rhesus monkey. In: G.H. Bourne (Ed.): The Rhesus monkey. Academic Press, New York. p. 38.

Casanova, M., D.F. Deyo & H.d'A. Heck, 1989. Covalent binding of inhaled formaldehyde to DNA in the nasal mucosa of Fischer 344 rats: analysis of formaldehyde and DNA by high-performance liquid chromatography and provisional pharmacokinetic interpretation. Fundam. Appl. Toxicol. 12, in press.

Casanova, M. & H.d'A. Heck, 1987. Further studies of the metabolic incorporation and covalent binding of inhaled [^{3}H]- and [^{14}C]formaldehyde in Fischer-344 rats: effects of glutathione depletion. Toxicol. Appl. Pharmacol. 89:105-121.

Chang, J.C.F., E.A. Gross, J.A. Swenberg & C.S. Barrow, 1983. Nasal cavity deposition, histopathology, and cell proliferation after single or repeated formaldehyde exposures in B6C3F1 mice and F-344 rats. Toxicol. Appl. Pharmacol. 68:161-176.

Craft, T.R., E. Bermudez & T.R. Skopek, 1987. Formaldehyde mutagenesis and formation of DNA-protein crosslinks in human lymphoblasts in vitro. Mutat. Res. 176:147-155.

Crosby, R.M., K.K. Richardson, T.R. Craft, K.B. Benforado, H.L. Liber & T.R. Skopek, 1988. Molecular analysis of formaldehyde-induced mutations in human lymphoblasts and *E. coli*. Environ. Molec. Mutagenesis 12:155-166.

Dresp, J. & M. Bauchinger, 1988. Direct analysis of the clastogenic effect of formaldehyde in unstimulated human lymphocytes by means of the premature chromosome condensation technique. Mutat. Res. 204:349-352.

Goldmacher, V.S. & W.G. Thilly, 1983. Formaldehyde is mutagenic for cultured human cells. Mutat. Res. 116:417-422.

Gross, E.A., T.B. Starr, H.W. Randall & K.T. Morgan, 1987. Morphometric analysis of the primate nasal cavity. Toxicologist 7:193 (Abst. 770).

Gross, E.A., J.A. Swenberg, S. Fields & J.A. Popp, 1982. Comparative morphometry of the nasal cavity in rats and mice. J. Anat. 135:83-88.

Mauderly, J.L., 1986. Respiration of F344 rats in nose-only inhalation exposure tubes. J. Appl. Toxicol. 6:25-30.

Monticello, T.M. & K.T. Morgan, 1989. Formaldehyde-induced lesions in the respiratory tract: comparison of F-344 rats with Rhesus monkeys. Toxicologist 9, in press.

Monticello, T.M., K.T. Morgan, J.I. Everitt & J.A. Popp, 1989. Effects of formaldehyde gas on the respiratory tract of Rhesus monkeys: pathology and cell proliferation. Am. J. Pathol., in press.

Natarajan, A.T., F. Darroudi, C.J.M. Bussman & A.C. van Kesteren-van Leeuwen, 1983. Evaluation of the mutagenicity of formaldehyde in mammalian cytogenetic assays in vivo and vitro. Mutat. Res. 122:355-360.

Patterson, D.L., E.A. Gross, M.S. Bogdanffy & K.T. Morgan, 1986. Retention of formaldehyde gas by the nasal passages of F-344 rats. Toxicologist 6:55 (Abst. 217).

Snyder, R.D. & B. Van Houten, 1986. Genotoxicity of formaldehyde and an evaluation of its effects on the DNA repair process in human diploid fibroblasts. Mutat. Res. 165:21-30.

Starr, T.B. & R.D. Buck, 1984. The importance of delivered dose in estimating low-dose cancer risk from inhalation exposure to formaldehyde. Fundam. Appl. Toxicol. 4:740-753.

ONCOGENE ACTIVATION IN CHEMICALLY INDUCED RAT NASAL TUMORS

S.J. Garte

Institute of Environmental Medicine, New York University Medical Center, New York, New York

Summary

Squamous cell carcinomas of the rat nasal epithelium induced by inhalation of three direct-acting alkylating agents were examined for activation of cellular oncogenes. Tumors induced by methylmethane sulfonate and beta-propiolactone contained oncogenes capable of transforming NIH3T3 cells. The active genes were not members of the *ras* family nor other known oncogenes. The two carcinogens reproducibly induced two carcinogen specific distinct genes. The gene from beta-propiolactone induced tumors is between 6 and 9 kb. The acylating agent dimethylcarbamyl chloride produced tumors that were consistently negative in the focus and nude mouse assays. The results show that the rat nasal mucosa is an excellent model for understanding the importance of carcinogen specificity in activation of cellular oncogenes. Furthermore, it appears that *ras* genes are not involved in neoplasia in this tissue.
Key Words: alkylating agents, inhalation, methylmethane sulfonate, beta-propiolactone, dimethylcarbamyl chloride, transfection

Introduction

The induction of carcinomas in the rat nasal epithelium by inhalation of direct-acting chemical carcinogens is an excellent model system to study molecular parameters of carcinogenesis (Snyder et al., 1986). The same tumor type is induced by a number of agents, with disparate chemical reactivities. The tumors are visible before death of the animal, allowing for rapid recovery of tumor tissue at an early stage of malignant progression. Since the spontaneous incidence of rat nasal tumors is very low, the issue of etiological uncertainty is not a problem in relating molecular findings to carcinogen identity (Sellakumar et al., 1987).

Our laboratory has been studying the activation of cellular oncogenes in carcinomas of the rat nasal mucosa for several years. Our results strongly suggests that quite different carcinogen specific mechanisms are involved in the carcinogenic activity of three direct-acting alkylating agents that produce nasal tumors of identical histologic type (Hochwalt et al., 1988). We have also found a surprising tissue specific lack of involvement of the *ras* family of oncogenes in neoplasia of rat nasal epithelium. Instead, we have discovered at least one novel oncogene activated in these tumors whose properties may provide important insights into mechanisms of carcinogenesis in this tissue.

Earlier work in experimental tumor virology demonstrating the existence of transforming oncogenes (Bishop, 1983) has been extended to discoveries of cellular oncogenes and their activation by environmental carcinogens, such as radiation and chemicals (Barbacid, 1987). The concept that all cancers induced by viral, chemical, or physical agents, as well as those due to genetic susceptibility, or of "spontaneous" origin share a common

pathway involving activation of a specific set of cellular oncogenes is an attractive idea for the unification of formerly diverse mechanistic theories. Verification of this hypothesis requires that data from research into cellular oncogene activation be correlated with biological and biochemical data from tumor model systems.

Such experimental animal tumor models have been useful in the study of oncogene activation as a function of certain parameters of carcinogenesis. Issues such as the relative roles of carcinogen and species specificity (Garte et al., 1985; Guerrero, et al. 1984), the correspondence of the initiating event to a specific mutation in a ras oncogene (Zarbl et al., 1985; Quintanilla et al., 1986) and the role of multiple oncogene activation in experimental tumors (Sawey et al., 1987) have been addressed using a variety of well defined model systems. The activation of ras genes in animal tumors has been found in several systems, although the frequency and specific mutagenic mechanism of activation, as well as the identity of the active gene has proved to depend on carcinogenic etiology (Barbacid, 1987; Guerrero et al., 1984; Wiseman et al., 1986) and/or the tissue type of tumor (Zarbl et al., 1985). It is noteworthy that activation of ras genes in any given model is not always uniformly consistent. Furthermore, several investigators have reported the discovery of NIH3T3 transforming activities from animal tumor DNAs that bear no homology to any of the three ras genes (Garte et al., 1985; Reynolds et al., 1987; Schechter et al., 1985).

Materials and Methods

Male Sprague-Dawley rats (8 weeks old) from Charles River were exposed to 50 ppm methylmethane sulfonate (MMS), 10 ppm beta-propiolactone (BPL), or 4 ppm dimethylcarbamyl chloride (DMCC) for 6 h/day for 30 days in stainless steel and plexiglass dynamic exposure chambers. Carcinogen vapor was generated by passing a measured stream of air over the surface of the heated liquid. Chamber atmospheres were analyzed by MIRAN 1A i.r. gas analyzers at 0.5 h intervals during exposures. Animals were killed at time of tumor appearance, tumor tissue was asceptically dissected free of adjoining tissue, and frozen in liquid nitrogen. Sections of each tumor were examined for histologic diagnosis.

DNA Mediated Gene Transfer.

Tumors were ground to a fine powder in a stainless steel mortar and pestle in liquid nitrogen. High molecular weight DNA was prepared by protease digestion of powered tumor tissue or primary transfectant cells, followed by phenol-chloroform extraction and ethanol precipitation. Cells of the NIH3T3 fibroblast line were seeded from frozen stocks and grown in Dulbecco's Modified Eagle's Medium supplemented with 10% fetal bovine serum. The calcium phosphate-DNA co-precipitate method of Wigler et al. was used in transfection assays. From 20-30 μg DNA was used in each of three to six 25 cm^2 flasks (Corning). Two days after transfection, cells were trypsinized and subcultured 1:3. After three weeks, foci were picked and/or flasks were stained with Giemsa and scored. All experiments were scored double-blind with prior randomization of flasks at the time of subculture.

Southern Blot Analysis.

Transformed NIH3T3 foci were grown to mass culture, and DNA purified as

described above. The DNA was digested to completion with restriction enzymes (New England Biolabs), subjected to gel electrophoresis through 0.8% agarose, and transferred to a nitrocellulose filter according to Southern (1975). Repetitive DNA or oncogene probes (1 μg) were nick translated to $1x10^8$ c.p.m./μg with CTP^{32} (New England Nuclear) and hybridized to the filter in 2 x Denhardts solution, 6 x SSC, 0.1% SDS at 65°C for 18 h. When rat repetitive DNA was used as the probe, 100 μg sheared mouse DNA was added to prevent cross-hybridization. The filter was washed to a final stringency of 0.1 x SSC (for rat probes) or 0.5 x SSC (for oncogene probes) and 0.1% SDS at 65°C.

Results and Discussion

The transforming activities of DNA purified from 24 rat nasal squamous cell carcinomas are shown in Table 1. Each of the eight tumors induced by MMS were positive, as were 5 of the 10 BPL-induced tumors. None of the 6 rat nasal carcinomas induced by DMCC produced any foci. Furthermore, DNAs from 2 of these DMCC tumors were negative in the nude mouse co-transfection assay (Fasano et al., 1984). We have found similar consistently negative results using 17 mouse skin tumor DNAs induced by DMCC (Hochwalt et al., 1988).

Table 1. Transfection of NIH3T3 cells by Rat Nasal Squamous Cell Carcinoma DNA.

Tumor	Agent	No. Foci/ No. Plates	Transfection Efficiency Foci/μg DNA	+/-[a]
1	MMS	12/14	0.063	+
2	MMS	10/15	0.055	+
3	MMS	17/14	0.088	+
4	MMS	6/6	0.059	+
5	MMS	15/18	0.083	+
6	MMS	13/18	0.071	+
7	MMS	5/18	0.026	+
8	MMS	11/15	0.056	+
9	BPL	5/27	0.014	+
10	BPL	2/18	0.008	-
11	BPL	3/9	0.026	+
12	BPL	0/18	<0.004	-
13	BPL	5/27	0.014	+
14	BPL	10/27	0.029	+
15	BPL	5/27	0.014	+
16	BPL	1/18	0.004	-
17	BPL	0/18	<0.004	-
18	BPL	1/18	0.004	-
19	DMCC	4/27	0.011	-
20	DMCC	2/18	0.008	-
21	DMCC	3/27	0.008	-
22	DMCC	0/18	<0.004	-
23	DMCC	0/9	<0.008	-
24	DMCC	1/9	0.008	-

[a] For those marked '-', foci that were originally scored as positive failed to produce tumors in nude mice, or were negative in secondary transfection.

Transformed NIH3T3 foci induced by MMS or BPL induced tumor DNAs were smaller and somewhat more diffuse in morphology than those normally seen after transfection with ras oncogenes. Primary transfectant cells were tumorigenic in nude mice, and formed colonies in soft agar. DNA from primary, secondary, and tertiary transfectants contained rat specific sequences when subjected to Southern blot analysis (Southern, 1975) using a rat repetitive sequence probe 3B5 (Whitney & Furano, 1984) provided by Dr. A. Furano. However, probes specific for H-, K-, or N-ras genes failed to hybridize with any exogenous DNA restriction fragments. Hybridization using all available oncogene probes including neu, met, and raf were also negative. So that we could compare the non-ras oncogenes from different tumors induced by BPL and MMS, four primary transfectant DNAs derived from two tumors induced by each agent were digested with a panel of six restriction enzymes and the digested DNA was tested in a transfection experiment. The results in Table 2 show a common pattern of restriction enzyme sensitivity between the two BPL induced tumors, which is distinct from the pattern exhibited by the MMS induced tumors. These results strongly suggests that the oncogenes activated in rat nasal squamous cell carcinomas by MMS and BPL are distinct from each other as well as from the ras family.

Further characterization of the BPL induced gene was done by preparative electrophoresis of Pst I (a non-cutter) digested secondary transfectant DNA, followed by transfection assay of gel fractions of varying molecular weight ranges. This experiment shows the size of the novel oncogene activated in BPL induced rat nasal carcinomas to be between 6 and 9 kb. Molecular cloning by marker ligation and selection and further characterization of this novel oncogene is in progress.

Table 2. Transfection of Restriction Endonuclease Digested Primary Transfectant DNA's From Rat Nasal Carcinomas

Original Tumor	Xba I	Pst I	Eco RI	Hind III	Bam HI	Smc I
BPL-Induced						
carcinoma #1	+	+	+	-	-	-
carcinoma #2	+	+	+	-	-	-
MMS-Induced						
carcinoma #1	-	-	+	+	-	-
carcinoma #2	-	-	+	+	-	-

BPL = beta-propiolactone, MMS = methylmethane sulfonate

Activation of ras oncogenes in animal tumor models has been found to be a frequent event in several tissues of rats and mice. The absence of ras gene activation in over 20 rat nasal squamous cell carcinomas induced by 3 alkylating agents is unusual, and leads to a hypothesis that ras genes are not involved in transformation pathways in this cell type. If this hypothesis receives further experimental verification, the role of oncogene activation in carcinogenesis would be clearly dependent on very specific parameters of tissue and histologic origin. Rat nasal adenocarcinomas induced by NMU injection exhibited a 50% (2/4) activation of K-ras (J. Rice, personal communication) suggesting that even within the

same organ, different patterns of oncogene activation might be detected depending on the cell type from which the tumor originates. We have found evidence for a similar phenomenon in rat skin (Sawey et al., 1987). We have also found that a mouse skin carcinoma induced by BPL contains an H-ras oncogene activated by a codon 61 A to T transversion point mutation (Hochwalt et al., 1988), the same genetic lesion reported by others in the same tumor type induced by aromatic hydrocarbons (Quintanilla et al., 1986; Bizub et al., 1986). This finding, coupled with the activation of a non-ras oncogene in rat nasal squamous cell carcinomas induced by the same agent is further evidence supporting the importance of tissue specific mechanisms of oncogene activation.

The fact that histologically identical rat nasal tumors induced by 2 carcinogens, MMS and BPL, reproducibly contain different oncogenes, strengthens the argument for carcinogen specificity in oncogene activation previously found in the rat mammary (Zarbl et al., 1985) and mouse thymoma (Guerrero et al., 1984) models. The results using DMCC are particularly striking in this regard. This agent, which is known to produce O^6 alkyl guanine adducts upon reaction with DNA in vitro (Segal et al., 1982) might be expected to yield G ---> A mutations at the 12th codon of a ras gene. However, in 23 rodent tumors, including 6 rat nasal squamous cell carcinomas, 7 mouse fibrosarcomas and 10 mouse skin squamous cell carcinomas (Garte et al., 1985; Hochwalt et al., 1988) induced by DMCC no ras or any other functionally activated transforming oncogenes have been detected.

The finding of novel NIH3T3 transforming oncogenes in MMS and BPL induced nasal tumors is not unique to this animal model. Tumor derived oncogenes without homology to ras have been detected in several systems (Reynolds et al., 1984; Schechter et al., 1985; Park et al., 1986; Koda et al., 1987; Martin-Zanca et al., 1986). The existence of such genes implies that the currently known set of cellular oncogenes, as defined either by homology with genes transduced by retroviruses, or by their functional activity in transforming established rodent cell lines such as NIH3T3, is far from complete. The data presented here add support to the concept that the precise determination of the genetic components of neoplasia must be a function of cell specific as well as carcinogen specific events (Garte, 1987).

References

Barbacid, M. 1987. Ras genes. Ann. Rev. Biochem. 56:779-827.

Bishop, J.M. 1983. Cellular oncogenes and retroviruses. Ann. Rev. Biochem. 52:301-354.

Bizub, D., Wood, A.W., and Skalka, A-M. 1986. Mutagenesis of the H-ras oncogene in mouse skin tumors induced by polycyclic aromatic hydrocarbons. Proc. Natl. Acad Sci. 83: 6048-6052.

Fasano, O, Birnbaum, D., Edlund, L., Fogh, J., and Wigler, M. 1984. New human transforming genes detected by a tumorigenicity assay. Mol. Cell. Biol. 4(9):1695-1705.

Garte, S.J. 1987. Activation of multiple oncogene pathways: A model for experimental carcinogenesis. J. Theor. Biol. 129:177-188.

Garte, S.J., A.T. Hood, A.E. Hochwalt, P. D'Eustachio, C.A. Snyder, A. Segal, and R.E. Albert. 1985. Carcinogen specificity in activation of transforming genes by direct-acting alkylating agents. Carcinogenesis 6: 1709-1712.

Guerrero, I., P. Calzava, A. Mayer, and A. Pellicer. 1984. A molecular

approach to leukemogenesis; mouse lymphomas contain an activated c-ras oncogene. Proc. Natl. Acad. Sci. 81: 202-205.

Hochwalt, A.E., Solomon, J.J., and Garte, S.J. 1988. Mechanism of H-ras oncogene activation in mouse squamous carcinoma induced by an alkylating agent. Cancer Res. 48:556-558.

Hochwalt, A.E., Wirgin, I., Felber, M., Currie, D.D., and Garte, S.J. 1988. Detection of novel non-ras oncogenes in rat nasal squamous cell carcinomas. Molec. Carcin., 1:4-6.

Koda, T., Sasaki, A., Matsushima, S., and Kakinuma, M. 1987. A transforming gene, hst, found in NIH3T3 cells transformed with DNA from three stomach cancers and a colon cancer. Jpn J. Cancer Res. 78:325-328.

Martin-Zanca, D., Hughes, S.H., and Barbacid, M. 1986. A human oncogene formed by the fusion of truncated tropomyosin and protein tyrosine kinase sequences. Nature 319:743-748.

Park, M., Dean, M., Cooper, C.S., Schmidt, M., Obrien, S.J., Blair, D.B. and Van de Woude, G.F. 1986. Mechanism of met oncogene activation. Cell 45:895-904.

Quintanilla, M., Brown, K., Ramsden, M., and Balmain, A. 1986. Carcinogen-specific mutation and amplification of Ha-ras during mouse skin carcinogenesis. Nature (London), 322: 78-80.

Reynolds, S.H., Stowers, S.J., Patterson, R.M., Maronpot, R.R., Aaronson, S.A., and Anderson, M.W. 1987. Activated oncogenes in B6C3F1 mouse liver tumors: implications for risk assessment. Science, 237: 1309-1316.

Sawey, M.J., Hood, A.T., Burns, F.J., and Garte, S.J. 1987. Activation of c-myc and c-K-ras oncogenes in primary rat tumors induced by ionizing radiation. Mol. Cell. Biol. 7:932-935.

Schechter, A.L., Tern, D.V., Vaidyanathan, L., Decker, S.J., Drebin, J.A, Green, M.I., and Weinberg, R.A. 1985. The neu oncogene: an erbb-related gene encoding an 185,000-M tumor antigen. Nature 312:513-515.

Segal, A., Solomon, J.J., Mate, U., VanDuuren, B.L. 1982. Formation of 6-dimethylcarbamyloxy-dGuo, 6-dimethylamino-dGuo, and 4-dimethylamino-d-Thd following in vitro reaction of dimethylcarbamyl chloride with calf thymus DNA, and 6-diethylcarbamyloxy-dGuo following in vitro reaction of diethylcarbamyl chloride with calf thymus DNA. Chem. Biol. Interact. 40:209-231.

Sellakumar, A., Snyder, C.A., and Albert, R.E. 1987. Inhalation carcinogenesis of various alkylating agents. J. Natl. Cancer Inst. 79:285-289.

Southern, E.M. 1975. Detection of specific sequences among DNA fragments separated by gel electrophoresis. J. Mol. Biol. 98: 503-517.

Snyder, C.A., Garte, S.J., Sellakumar, A., and Albert, R.E. 1986. Relationships between the levels of binding to DNA and the carcinogenic potencies of rat nasal mucosa for three alkylating agents. Cancer Lett. 33:175-181.

Whitney, R.R. and Furano, A.V. 1984. Highly repeated DNA families in the rat. J. Biol. Chem. 259(16):10481-10482.

Wiseman, R.W., Stomers, S.J., Miller, E.C., Anderson, M.W., and Miller, J.A. 1986. Activating mutations of the c-Ha-ras protooncogene in chemically induced hepatomas of the male B6C3F1 mouse. Proc. Natl Acad. Sci., USA 83: 5825-5829.

Zarbl, H., S. Sukumar, A.V. Arthur, D. Martin-Zanca, and M. Barbacid. 1985. Direct mutagenesis of Ha-ras-1 oncogenes by N-nitroso-N-methylurea during initiation of mammary carcinogenesis in rats. Nature 315: 382-385.

Part 4

Extrapolation, risk assessment and regulation

NASAL CARCINOGENESIS IN RODENTS: IMPLICATIONS FOR CANCER RISK ASSESSMENT

Murray S. Cohn

U.S. Consumer Product Safety Commission, Washington, D.C., U.S.A.[1]

Summary

Although risk assessment based on the response of nasal carcinogenesis in animals is approached much like that for other responses, there are some attributes of the general class of compounds causing nasal cancer which focus risk assessment in certain directions. Some of the expectations as a result of this focus include linearity of the dose-response curve at low dose, interspecies extrapolation on a parts per million basis, and an unlikelihood that if a compound causes nasal cancer after inhalation, it would also cause this response after administration by other routes, such as ingestion. These expectations derive from the reactivity of the general class of compounds causing nasal cancer, and the observation in animals of nasal cancer usually appearing after compound inhalation, but not ingestion.
Keywords: nasal cancer, risk assessment

Introduction

Carcinogenic risk assessment is an evolving field, with the goal of replacing assumptions with scientific data and insights to the extent practical so that more accurate predictions of risk are possible. In recent years several such changes have been incorporated which, while increasing the sophistication and complexity of the process, have led to a higher quality of analysis when compared to first attempts at risk assessment back in the early 1970's. These changes include computing a "best" fit to non-linear high dose data prior to extrapolation to low doses, rather than simply fitting a straight line through the entire dose-response curve, and using pharmacokinetics to deduce effects on the shape of the dose-response curve as a result of phenomena such as metabolic saturation at high, but not low, doses which affect "target site" dose. However, even with these improvements, there is still a considerable dependence upon the use of theory and assumption for the estimation of carcinogenic risks resulting from the use of various chemicals and mixtures.

The purpose of this paper is to examine the general methodologies of human carcinogenic risk assessment, focusing on how they might be applied in the case of substances which, under certain circumstances of exposure,

1. "The material found within this paper contains the view of the author, who is an employee of the U.S. Consumer Product Safety Commission, and does not necessary reflect official opinions or policies of the U.S. Consumer Product Safety Commission."

cause nasal cancer in animals. Some assumptions made in the case of exposure to certain nasal carcinogens may be somewhat different than those made for carcinogens in general since nasal carcinogenesis may be unique from a risk assessment perspective. Such differences may come into play, for example, when considering interspecies extrapolation, an area where the concept of "target site" dose is receiving increasing attention.

Discussion

The Carcinogenic Risk Assessment Process

Risk assessment for carcinogens falls into three general stages: unit risk assessment, exposure assessment, and an integration of these two assessments to produce estimates of individual or population risk. Although this paper will concentrate on unit risk assessment, the route of human exposure is critically important to the assumptions made. Thus, the discussion on the unit risk assessment will include consideration of exposure via ingestion as well as inhalation.

Unit risk assessment for carcinogens is the process of estimating human risk per unit of exposure, for example, risk per mg/kg/day. The assessment based on animal data can be broken down into three major extrapolations. High to low dose extrapolation projects risks at low doses based on observed risks to animals exposed to relatively high doses in long term bioassays. Some factors considered include proposed mechanism of action, shape of the high dose data and the goodness of fit of dose-response models to these data, and pharmacokinetic effects (i.e. relationship between administered dose of compound and "target" dose of active carcinogen).

Not much is known about the relative sensitivities of humans and animals to the effects of carcinogens, leading of necessity to the use of broad assumptions to perform species to species extrapolation. Some common assumptions made include equal sensitivity to the same dose in mg/kg/day or in ppm, and the use of the so-called surface area correction. The latter assumes that humans are more sensitive than animals by a factor of human weight divided by animal weight, taken to the one-third power, as compared to the mg/kg/day basis. As more information is gathered on mechanism of action and target site doses of the active species, these assumptions hopefully will be replaced.

The third extrapolation relates conditions of exposure of the test animals in long term bioassays to those expected to be experienced by a target human population. One problem is how to use experimental data obtained via one route of administration (e.g. ingestion) to generate risk estimates for another route (e.g. inhalation). Until recently, route to route extrapolation has been largely avoided. Another problem is to relate different regimens of exposure, e.g. use of cancer incidence reported in lifetime dosing tests to predict risk from short term or intermittant exposure. Although in the past adjustments have been made on a seemingly obvious basis (e.g. risks are related to proportion of lifetime exposed), the use of pharmacokinetic and metabolic information and modeling is allowing the refinement of these adjustments. Furthermore, by relating time vs concentration curves for a compound in various tissues (such as blood) after administration by different routes,

pharmacokinetic modeling is now leading to increased use of route to route extrapolation.

Properties of Nasal Carcinogens Important to Carcinogenic Risk Assessment

A review of chemicals associated with nasal cancer in animals leads to some observations which suggest or support the use of certain assumptions for the extrapolations referred to in the previous section. The pertinent properties of these chemicals, as derived from data gathered by the International Agency for Research on Cancer (IARC, 1987), are presented in Table I.

Table 1. Certain properties of compounds causing nasal cancer in animals.

Compound	Geno tox	Metab Activ Req	Species	Nasal Canc Route of Administ	Other Site Inhal	Other Site Oral
Acetaldehyde	Yes	No	Rat	Inhalation	None	
Bis(chloromethyl)-ether	Yes	No	Rat	Inhalation	Lung	
1,2 Dibromo-3-chloropropane	Yes	No	Rat	Inhalation	Adrenal	Mammary
			Mouse	Inhalation	None	Stomach
Dimethylcarbonoyl chloride	Yes	No	Rat	Inhalation	None	
			Hamster	Inhalation	None	
1,4 Dioxane	No		Rat	Oral	None	Liver
Epichlorohydrin	Yes	No	Rat	Inhalation	None	Stomach
Ethylene Dibromide	Yes	No	Rat	Inhalation	Spleen	Liver
			Mouse	Inhalation	Spleen	Lung
Formaldehyde	Yes	No	Rat	Inhalation	None	
Hexamethyl phosphoramide	Yes	Yes	Rat	Inhalation	None	Lung
Hydrazine	Yes	No	Rat	Inhalation	None	Liver
			Hamster	Inhalation	Thyroid	
Nitrosamines (over 10 within this general class)	Yes	Yes	Mouse, Hamster, Rat, etc.	Inhalation, oral, others	Liver, Kidney, etc.	Liver, Kidney, etc.
Para-cresidine	Yes	Yes	Rat	Oral		Bladder
			Mouse	Oral	None	Bladder
Propylene oxide	Yes	No	Rat	Inhalation	Adrenal	Stomach
			Mouse	Inhalation	None	
2,3,7,8 Tetra-chlorodibenzo-p-dioxin (TCDD)	No		Rat	Oral		Liver

Genotox = Is the compound genotoxic; Metab Activ Req = Is metabolic activation required for a positive response seen in short term tests; Nasal Canc Route of Administ = Route of administration by which nasal cancer was observed; Other Site Inhal = Other major site of interest responding after administration by inhalation; Other Site Oral = Other major site of interest responding after oral administration. [If space is blank then there were no data reported; if only "Inhalation" is indicated under "Nasal Canc Route of Administ", the indication of a site of response under "Other Site Oral" means that no nasal cancer was seen by oral administration (nasal cancer would be indicated if present)].

Observations that can be derived from the data in Table 1 are:

1) Most nasal carcinogenic responses resulting from chemical administration occur after inhalation exposure. Sometimes other systemic sites respond as well. In fact, the same chemicals administered by other routes do not lead to nasal carcinogenesis but rather to responses at other sites. The exception to this description is the general class nitrosamines, which are nasal carcinogens by inhalation, ingestion and other routes.

2) Nasal carcinogens as a group tend to be genotoxic. Furthermore, where such information is available, genotoxicity is almost always expressed without the need for metabolic activation (e.g. without use of a liver microsomal extract). The only exceptions found, in this review, of nasal carcinogens being non-genotoxic were 2,3,7,8 tetrachlorodibenzo-p-dioxin (TCDD) and 1,4 dioxane, which interestingly are nasal carcinogens after oral administration. Notably, the exception to the first observation, nitrosamines, requires metabolic activation for genotoxicity to be expressed.

3) Compared to responses at other sites (such as lung, liver, kidney) seen in the many bioassays conducted over the years, the observation of nasal cancer is relatively uncommon. However, since the number of ingestion bioassays far exceeds the number of inhalation bioassays performed in the past, it seems reasonable to expect that additional carcinogens would, upon testing by inhalation, cause a nasal carcinogenic response. Thus, the method of testing may be limiting the ability to detect nasal carcinogenesis.

The above three points lead to the hypothesis that in general, compounds causing nasal cancer tend to be reactive (not requiring metabolism to an active state) and thus exhibit their toxic effect after inhalation at the site of first contact, the nasal cavity, where the dose is relatively concentrated. Given by an alternative route such as ingestion, however, the dose to this tissue would be much lower due to dilution and reaction elsewhere, leading to response in other possibly more sensitive tissues as opposed to a nasal carcinogenic effect. Future work in metabolism and pharmacokinetics might provide insight into the effect of route of administration on a compound's ability to induce nasal carcinogenesis. The implications of this hypothesis on carcinogenic risk assessment, and the differences in assumptions made as compared to risk assessment for systemic carcinogens (or the exceptions such as nitrosamines and TCDD noted above) are discussed below.

Risk Assessment for Nasal Carcinogens

When estimating risks at low doses based on responses seen only at high doses, the most important consideration is the assumed shape of the dose-response curve in the low dose area. The most forceful argument that can be made in most cases is for linearity of the dose-response curve at low doses (Crump et al, 1976; Guess and Crump, 1976; Hoel, 1980). This theory depends upon the fact that cancer is a multistage process, in which a cell undergoes a series of alterations before attaining a cancerous state. Since there is a high background rate of cancer, including nasal cancer (0.8 cases/100,000/year in the United States; National Cancer Institute, 1981) relative to the magnitude of risks predicted at low doses of carcinogens, the shape of the dose-response

curve is expected to be linear if the effects of the carcinogen can add to or "interact" with this background. For example, if a compound can initiate the first step in the carcinogenic process, other background processes can further the development to a cancerous state. If the number of initiations doubles as a result of a doubled dose, the risk is expected to be doubled. Once the effect of the initiator overwhelms background (as in high dose bioassays), non-linearity can result.

Since carcinogens that exhibit genotoxicity are thought to most likely act by an initiative mechanism, nasal carcinogens as a group (i.e., those exhibiting the "pattern" of being nasal carcinogens only by inhalation and being genotoxic, generally without a requirement for metabolic activation) would be expected to interact with background, and be subject to low-dose linearity. For the few exceptions, e.g. systemic nasal carcinogens such as TCDD where the mechanism of action is largely unknown, non-linearity is certainly possible, especially if a threshold exists. Until the mechanism is elucidated, however, an assumption of background interaction and low-dose linearity is suggested.

In the above discussion, linearity at low dose refers to the "effective" dose vs. response curve. If experimentation demonstrates that the relationship between administered dose and "effective" dose (time vs. concentration profile at the target tissue) is non-linear with dose, such non-linearity is taken into account before fitting dose-response models to the experimental data. Casanova-Schmitz et al (1984) made an innovative first attempt at this in the case of formaldehyde-induced nasal cancer in rats, although more evidence is needed to show that such a non-linear relationship between administered and "effective" dose indeed exists in this case (Cohn et al, 1985; EPA, 1986).

Species extrapolation is approached systemically for most carcinogens. The dose, by any route of administration, is assumed to distribute internally. As stated previously, one commonly used method assumes equal sensitivity to this distributed dose by rodents or humans on a mg/kg/day basis; another, using the surface area correction, assumes humans are somewhat more sensitive, yet depends upon this systemic approach as well.

For compounds fitting a pattern of causing nasal cancer by inhalation, most likely acting directly with no metabolic activation requirement, and not causing nasal cancer by other routes of administration, this systemic approach does not seem to be appropriate. These compounds act directly at the site of exposure to cause the nasal carcinogenic response, and their distribution and metabolism are therefore not factors of great importance. What is important for this class of compounds is the concentration in air and the relative (between species) size of, and ability of the compound to reach, the target site. Since the latter is largely unknown, a suggestion is to assume animals and humans to be equally sensitive to the same airborne concentration of these compounds. The exceptions to the pattern, such as nitrosamines, TCDD and 1,4 dioxane would more likely fit the systemic approach. Continuing research, especially in the areas of pharmacokinetics and pharmacodynamics, should help to replace these assumptions with more factually based approaches.

An ability of nasal carcinogens to directly act at the site of exposure would affect extrapolation from one regimen of exposure to another. For systemic carcinogens, the processes of possible metabolic conversion to a toxic species and building up the active site concentration to a steady

state (from continuous inhalation) would impose a non-linearity on the system. For example, if the liver was the target site, a one hour exposure to a systemic carcinogen in which steady state was not achieved would lead to a certain integrated time vs. concentration value at that site. However, this integrated value would not be one-tenth of that from a ten hour exposure where steady state was achieved in four hours. Pharmacokinetic experimentation and modeling could allow such differences to be taken into account in the risk assessment process. For the majority of nasal carcinogens (those fitting the "pattern"), however, such differences would likely be trivial at best. Metabolism would not be required, and steady state should be achieved rapidly at a given concentration of the compound, due to the close proximity of the target tissue to the airborne carcinogen source.

The properties of the two types of nasal carcinogens would also be extended to extrapolation between routes. For systemic carcinogens, which would include exceptions to the "pattern" such as nitrosamines and TCDD, time vs. concentration profiles at a target tissue could be characterized from different routes of exposure using pharmacokinetic techniques. However, as compounds fitting the "pattern" are only nasal carcinogens by inhalation, risks cannot be estimated, based on the nasal response of such "pattern" compounds, for administration by other routes.

Thus, the properties of most nasal carcinogens, which seem to fit a pattern, result in somewhat different approaches being suggested for the risk assessment process. Although having these properties does not insure that a carcinogen will cause nasal cancer by inhalation (e.g. 2-nitropropene is genotoxic without requiring metabolic activation, but is a liver carcinogen and not a nasal carcinogen after inhalation), a larger number of nasal carcinogenic responses would be expected if more bioassays were to be conducted by the inhalation route.

References

Casanova-Schmitz, M., T.B. Starr & H. d'A. Heck, 1984. Differentiation between metabolic incorporation and covalent binding in the labeling of macromolecules in the rat nasal mucosa and bone marrow by inhaled [14C]- and [3H] formaldehyde. Toxicol. Appl. Pharmacol. 76:121-132.

Cohn, M.S., F.J. DiCarlo, A. Turturro & A.G. Ulsamer, 1985. Letter to the Editor. Toxicol. Appl. Pharmacol. 77:363-364.

Crump, K.S., D.G. Hoel, C.H. Langley & R. Peto, 1976. Fundamental carcinogenic processes and their implications for low dose risk assessment. Cancer Res. 36:2973-1979.

Guess, H.A. & K.S. Crump, 1976. Low-dose-rate extrapolation of data from animal carcinogenicity experiments - analysis of a new statistical technique. Math. Biosciences 32:15-36.

Hoel, D., 1980. Incorporation of background in dose-response models. Federation Proceedings 39:73-75.

International Agency for Research on Cancer, 1987. IARC monographs on the evaluation of carcinogenic risks to humans. Supplement 7. IARC, World Health Organization, Lyon, France.

National Cancer Institute, 1981. Surveillance epidemiology end results. Incidence and mortality data: 1973-77. NCI Monograph 57, National Cancer Institute, Bethesda, Maryland, United States.

U.S. Environmental Protection Agency, 1986. Expert review of pharmacokinetic data: formaldehyde. Final evaluation report. EPA Document Number TR-835-20, January 2, 1986.

QUANTITATIVE CANCER RISK ESTIMATION FOR FORMALDEHYDE

T.B. Starr

Chemical Industry Institute of Toxicology, Research Triangle Park NC, USA

Summary

An impressive array of data points to a significantly nonlinear relationship between administered and delivered doses, both for rodents and Rhesus monkeys, exposed to formaldehyde by inhalation. Disproportionately less formaldehyde binds covalently to the DNA of nasal respiratory epithelium at low than at high airborne concentrations. Use of this internal measure of delivered dose in analyses of rodent bioassay tumor response leads to multistage model estimates of low-dose risk, both point and upper bound, that are lower than equivalent estimates based upon airborne concentration. In addition, risk estimates obtained for Rhesus monkeys appear at least twenty-fold lower than corresponding estimates for identically exposed Fischer-344 rats.
Keywords: formaldehyde, delivered dose, DNA-binding, rat, monkey, risk assessment, nasal cancer

Introduction

It is now well-established that the typical chronic bioassay lacks sufficient power to discriminate among different mathematical dose-response models employed for low-dose extrapolation (Krewski et al., 1983). Generally, these models all provide adequate fits to tumor incidence in the observable response range, but their predicted risks at exposure levels even slightly below this range can differ by orders of magnitude (c.f., Starr & Buck, 1984). Optimization of the bioassay's experimental design (in terms of the number of treatment groups, their relative size, and their placement in relation to the maximum tolerated dose), to minimize the uncertainty in predicted risks at low exposure levels does not significantly improve the situation (Portier & Hoel, 1983). It thus appears that knowledge of risk for a given species at high exposure levels is by itself insufficient to predict accurately the risks expected at low exposure levels, even in the same species.

To further compound this difficulty, mechanism-derived dose-response models such as the multistage model lack the structure necessary to characterize physiologic and pharmacokinetic factors that govern the relationship between doses administered to whole animals and those ultimately reaching the target tissues. Yet the accuracy of predicted risks is critically dependent upon the use of valid measures of exposure in target tissues (c.f., Crump, 1979; Hoel et al., 1983).

Although data regarding delivered doses are rarely available, we are fortunate to have such information for formaldehyde. In this report, we first outline how these data may be utilized for quantitative risk estimation, and then explore the differences that their use makes in the actual quantitation of low-dose cancer risk.

Materials and Methods

Tumor Incidence

Incidence of nasal squamous cell carcinomas among Fischer-344 rats exposed to different airborne formaldehyde concentrations 6 hr/day, 5 days/week, for up to 24 months, is displayed in Table 1. These figures were abstracted from findings in the CIIT-sponsored inhalation bioassay (Kerns et al., 1983). Competing risks of death from causes other than a fatal tumor (including interim scheduled sacrifices) were taken into account as outlined previously (Starr & Buck, 1984). As noted therein, the various methods available to adjust for competing risks yield very similar estimates of tumor incidence.

Table 1. Nasal cavity squamous cell carcinoma incidence in formaldehyde-exposed rats.[a]

Administered dose, ppm[b]	Tumor incidence[c]
0	0/160
2.0 ± .01	0/160
5.6 ± .02	2/160
14.3 ± .04	87/160

[a] Taken from Starr & Buck (1984).
[b] Mean ± Standard Error over 24 months.
[c] Tumor-bearing animals/Number at risk.

Covalent binding of formaldehyde to respiratory mucosal DNA

Studies involving short-term inhalation exposures to single and dual radiolabeled formaldehyde vapor have provided strong evidence that the amount of formaldehyde bound covalently to replicating cell DNA in the respiratory epithelium of both rat and Rhesus monkey nasal cavities is nonlinearly related to airborne concentration (Casanova et al., 1989; Heck et al., 1989; Casanova-Schmitz & Heck, 1985; Casanova-Schmitz et al., 1984). Respiratory epithelium is the target tissue for nasal carcinogenesis in rats (Morgan et al., 1987), the site of extensive tissue damage in rats and Rhesus monkeys (Monticello & Morgan, 1989), and the site showing the highest levels of formaldehyde-DNA binding in these two species (Heck et al., 1989). A summary of findings from the DNA-binding studies is presented in Table 2.

Multistage model analyses of tumor response and low-dose risk

The Global82 computer program (ICF Clement Associates; Ruston, Louisiana) was used to obtain multistage model point and upper 95% confidence bound estimates of the lifetime squamous cell carcinoma risk from exposure 6 hours/day, 5 days/week to airborne concentrations of

1.0, 0.5, and 0.1 ppm formaldehyde. Analysis of rat tumor response versus airborne formaldehyde concentration employed the data directly from Table 1. Extrapolation of resulting risk estimates to monkeys then requires the assumption that monkeys and rats identically exposed to the same airborne concentration experience the same lifetime risk of squamous cell carcinoma. However, use of covalent binding as the index of exposure necessitated making certain additional assumptions and interpolations.

Table 2. Concentrations of covalently bound [^{14}C]formaldehyde (CVB) in respiratory mucosal DNA of Fischer-344 rats and Rhesus monkeys immediately following exposure for six hours.

Air Conc ppm	Old[a] CVB/Rat pmol/mg DNA	New[b] CVB/Rat pmol/mg DNA	New[b] CVB/Monkey pmol/mg DNA
0.3	---	1.4 ± 0.6	---
0.7	---	3.9 ± 0.4	0.36 ± 0.10
2.0	22 ± 0.6	19.9 ± 3.7	2.6 ± 0.3
6.0	233 ± 23	106. ± 6.	18.2 ± 3.4
10.0	406 ± 99	266. ± 30.	---
15.0	631 ± 64	---	---

[a]Taken from Casanova-Schmitz et al. (1984).
[b]Taken from Casanova et al. (1989).

As described previously by Starr & Buck (1984), the Casanova-Schmitz et al. (1984) measurement at 2 ppm was used directly, and estimates of covalent binding at lower concentrations were projected linearly from this point downward through zero. Estimates at 5.6 and 14.3 ppm were obtained by linear interpolation between the observations at 6 and 15 ppm, respectively, and zero. With the new data from Casanova et al. (1989), the binding estimate for rats at 14.3 ppm was obtained by linear extrapolation upwards from that observed at 10 ppm. Estimates for rats at 1.0 and 0.5 ppm and monkeys at 1.0 ppm were linearly interpolated from the respective measurements at 2.0, 0.7, and 0.3 ppm. Estimates for rats below 0.3 ppm and monkeys below 0.7 ppm were projected downward linearly from the binding observed at these points through zero. Finally, it was assumed that a given level of formaldehyde covalently bound to DNA would pose the same lifetime cancer risk to both rats and monkeys.

Results

Table 3 presents maximum likelihood estimates of the lifetime risk of squamous cell carcinoma in rats and Rhesus monkeys exposed for 6 hours/day, 5 days/week to 1.0, 0.5 and 0.1 ppm formaldehyde. The second and third columns reproduce results previously reported by Starr & Buck (1984), namely, that point estimates of risk for rats based on DNA-

binding are uniformly lower, by a factor of approximately fifty, than those based on airborne concentration. Data obtained with the new binding detection method yield risks for rats exposed to concentrations of 0.5 ppm or less that are lower still by a factor two or more. For Rhesus monkeys, the disparity between airborne concentration- and DNA binding-based risk estimates is much more dramatic. For example, at 1.0 ppm, the estimated risk is approximately 25,000-fold lower when based upon covalent binding, while at 0.1 ppm, it is less than 2 in 10^{12}.

Table 3. Maximum likelihood risk estimates per million, as derived with the 3-stage carcinogenesis model.

Air Conc ppm	Dose Measure/Species			
	Air/Rat	OCVB/Rat[a]	NCVB/Rat[b]	NCVB/Monkey[b]
1.0	251	4.6	6.2	0.0097
0.5	31	0.57	0.27	0.00025
0.1	0.25	0.0046	0.0015	0.0000019

[a]Using DNA-binding data from Casanova-Schmitz et al. (1984).
[b]Using DNA-binding data from Casanova et al. (1989).

Upper 95% confidence bounds on risk are presented similarly in Table 4. It is readily apparent that the new DNA-binding data for rats yielded bounds that are slightly larger than those reported previously by Starr & Buck (1984). Nevertheless, these bounds are still lower, by a factor of two or more, than the corresponding bounds based upon airborne formaldehyde concentration. Furthermore, the upper bounds obtained from the monkey measurements are nearly twenty-fold lower at concentrations of 0.5 ppm or less.

Table 4. Upper 95% confidence bound risk estimates per million, as derived with the 3-stage carcinogenesis model.

Air Conc ppm	Dose Measure/Species			
	Air/Rat	OCVB/Rat[a]	NCVB/Rat[b]	NCVB/Monkey[b]
1.0	1800	620	1200	140
0.5	810	310	420	41
0.1	160	62	74	8

[a]Using DNA-binding data from Casanova-Schmitz et al. (1984).
[b]Using DNA-binding data from Casanova et al. (1989).

Discussion

For formaldehyde, an impressive array of data points to a significantly nonlinear relationship between administered and delivered doses, with disproportionately less delivery occurring at low than at high exposures. It has been demonstrated that high level formaldehyde inhalation exposure induces the respiratory depression reflex (Chang et al., 1983), an inhibition of mucociliary clearance (Morgan, 1983; Morgan et al., 1983) and intracellular formaldehyde metabolism (Casanova & Heck, 1987), and stimulation of cell proliferation (Chang et al., 1983; Swenberg et al., 1983, 1986), all as nonlinear functions of the airborne formaldehyde concentration. Each of these phenomena appears to be an important controlling factor in the relationship between administered and delivered doses. Thus, a nonlinear dependence of covalent DNA-binding upon airborne formaldehyde concentration as seen in rats and Rhesus monkeys is not unexpected (Starr & Gibson, 1985).

As noted earlier, however, the mechanism-based dose-response models such as the multistage model have no structure with which to characterize these factors. This means that such information can only be incorporated in the quantitation of risk implicitly through the exposure measure being utilized, as is the case with the DNA-binding in respiratory epithelium. While a number of important issues remain unresolved (c.f., Starr, 1987), use of these data provides the best approach presently available for measuring exposure and incorporating detailed mechanistic information into the quantitative risk assessment process for formaldehyde.

References

Casanova, M. & H.d'A. Heck, 1987. Further studies on the metabolic incorporation and covalent binding of inhaled [^{3}H]- and [^{14}C]formaldehyde in Fischer-344 rats: effects of glutathione depletion. Toxicol. Appl. Pharmacol. 89:105-121.

Casanova, M., H.d'A. Heck, W.H. Steinhagen, J.I. Everitt, K.T. Morgan & J.A. Popp, 1989. Species and concentration dependence of DNA-protein cross-link (DPX) formation by formaldehyde (HCHO) in Rhesus monkeys and F-344 rats. The Toxicologist, in press.

Casanova-Schmitz, M., T.B. Starr & H.d'A. Heck, 1984. Differentiation between metabolic incorporation and covalent binding in the labeling of macromolecules in the rat nasal mucosa and bone marrow by inhaled [^{14}C]- and [^{3}H]-formaldehyde. Toxicol. Appl. Pharmacol. 76:26-44.

Chang, J.C.F., E.A. Gross, J.A. Swenberg & C.S. Barrow, 1983. Nasal cavity deposition, histopathology, and cell proliferation after single or repeated formaldehyde exposure in B6C3F1 mice and F-344 rats. Toxicol. Appl. Pharmacol. 68:161-176.

Crump, K.S., 1979. Dose-response problems in carcinogenesis. Biometrics 35:157-167.

Heck, H.d'A., M. Casanova, W.H. Steinhagen, J.I. Everitt, K.T. Morgan & J.A. Popp, 1989. Formaldehyde toxicity: DNA-protein cross-linking studies in rats and nonhuman primates. This volume.

Hoel, D.G., N.L. Kaplan & M.W. Anderson, 1983. Implication of nonlinear kinetics on risk estimation in carcinogenesis. Science 219:1032-1037.

Kerns, W.D., K.L. Pavkov, D.J. Donofrio & J.A. Swenberg, 1983. Carcinogenicity of formaldehyde in rats and mice after long-term inhalation exposure. Cancer Res. 43:4382-4392.

Krewski, D., K.S. Crump, J. Farmer, D.W. Gaylor, R. Howe, C. Portier, D. Salsburg, R.L. Sielken Jr. & J. Van Ryzin, 1983. A comparison of statistical methods for low-dose extrapolation utilizing time-to-tumor data. Fundam. Appl. Toxicol. 3:140-160.

Monticello, T.M. & K.T. Morgan, 1989. Formaldehyde-induced lesions in the respiratory tract: comparison of F-344 rats with Rhesus monkeys. The Toxicologist 9, in press.

Monticello, T.M., K.T. Morgan, J.I. Everitt & J.A. Popp, 1989. Effects of formaldehyde gas on the respiratory tract of Rhesus monkeys: pathology and cell proliferation. Am. J. Pathol., in press.

Morgan, K.T., 1983. Localization of areas of inhibition of nasal mucociliary function in rats following in vivo exposure to formaldehyde. Am. Rev. Respir. Dis. 127:166.

Morgan, K.T., D.L. Patterson & E.A. Gross, 1983. Formaldehyde and the nasal mucociliary apparatus. In: J.J.Clary, J.E. Gibson & R.S. Waritz (Ed.): Formaldehyde toxicology, epidemiology, and mechanisms. Dekker, New York. p. 193-210.

Morgan, K.T., X.-Z. Jiang, T.B. Starr, W.D. Kerns, 1986. More precise localization of nasal tumors associated with chronic exposure of F-344 rats to formaldehyde gas. Toxicol. Appl. Pharmacol. 82:264-271.

Starr, T.B., 1987. The delivered/administered dose relationship and its impact on formaldehyde risk estimates. In: Pharmacokinetics in risk assessment, Drinking water and health, Vol. 8. National Academy Press, Washington D.C. p. 327-333.

Starr, T.B. & R.D. Buck, 1984. The importance of delivered dose in estimating low-dose cancer risk from inhalation exposure to formaldehyde. Fundam. Appl. Toxicol. 4:740-753.

Starr, T.B. & J.E. Gibson, 1985. Mechanistic toxicology of formaldehyde and its implications for quantitative risk estimation. Ann. Rev. Pharmacol. Toxicol. 25:745-767.

Swenberg, J.A., E.A. Gross, H.W. Randall & C.S. Barrow, 1983. The effect of formaldehyde exposure on cytotoxicity and cell proliferation. In: J.J. Clary, J.E. Gibson & R.S. Waritz (Ed.): Formaldehyde toxicology, epidemiology, and mechanisms. Dekker, New York. p. 225-236.

Swenberg, J.A., E.A. Gross & H.W. Randall, 1986. Localization and quantitation of cell proliferation following exposure to nasal irritants. In: C.S. Barrow (Ed.): Toxicology of the nasal passages. Hemisphere, Washington D.C. p. 291-300.

THE U.S. ENVIRONMENTAL PROTECTION AGENCY'S METHODOLOGY FOR ASSESSING RISK OF EXPOSURE TO NASAL CARCINOGENS

William E. Pepelko

United States Environmental Protection Agency, Washington, DC, USA

Summary

The U.S. Environmental Protection Agency (EPA) has carried out risk assessments on a variety of chemicals that have been shown to induce nasal tumors in animals and/or humans. Human cancer potency estimates, however, were not always based on the induction of nasal tumors. For those that were, studies employing the inhalation route of exposure were used, with one exception. Extrapolation from the inhalation to the oral route has not been attempted for nasal carcinogens. A variety of uncertainties in assessing potency of nasal carcinogens is being addressed in "Methods for Determination of Inhalation RFDs" currently being developed by the EPA.
Keywords: risk assessment, methodology

Introduction

The EPA carries out risk assessment on potentially carcinogenic agents using recently developed risk assessment guidelines (EPA, 1987a). Since nasal carcinogens are evaluated with the aid of these guidelines, and since the methodology is similar irrespective of the target organ, these guidelines are briefly reviewed here. Chemicals assessed by EPA's Office of Health and Environmental Assessment (OHEA) for which there is some evidence of nasal tumor induction are then listed along with routes of exposure and the target organs used for cancer potency estimation. Finally, formaldehyde is discussed in more detail as an example of an EPA risk assessment for a nasal carcinogen.

EPA methods for assessing carcinogenic risk

Chemicals are selected for evaluation by determining (1) if the agent is present in the environment or proposed use will result in release into the environment, and (2) if the agent poses a potential carcinogenic hazard. The latter is determined by a review of physical-chemical properties, structure-activity relationships, metabolic and pharmacokinetic properties, toxicologic effects, and short-term and long-term tests.

A weight-of-evidence classification for potential carcinogenicity is then carried out. For agents considered to be either carcinogenic or potentially carcinogenic in humans, a potency estimate is made if adequate dose-response data are available. The EPA classification system for qualitatively assessing risk, which is an adaptation of the International Agency for Research on Cancer (IARC) approach, is summarized below.

When the weight of evidence for human studies is sufficient, indicating a causal relationship has been determined, the agent is classified as group A. These are considered to be known carcinogens.

When the weight of evidence for human studies is limited, indicating a causal relationship is credible, but other explanations such as chance, bias, or confounding cannot be adequately excluded, then the agent is classified as group B1.

When human evidence is inadequate or lacking and sufficient evidence of carcinogenicity in animals is available as shown by an increased incidence of malignant tumors or benign and malignant tumors combined: (a) in multiple species or strains; (b) in multiple experiments; or (c) to an unusual degree in a single experiment, then the agent is classified as group B2. Chemicals classified as either category B1 or B2 are considered to be probable human carcinogens.

When human evidence is inadequate or lacking and evidence for carcinogenicity in animals is limited due to a lack of more than one study, inadequate dosage levels, inadequate duration of exposure, etc., the agent is classified as group C. These are considered to be possible human carcinogens.

When human evidence is inadequate or lacking and animal evidence is also inadequate or lacking, the agent is classified as group D.

When there is evidence for noncarcinogenicity as shown by a lack of association between exposure and cancer in well-designed animal studies and either no data or evidence for noncarcinogenicity in human studies, the agent is classified as category E.

Agents that are judged to be in category A or B are regarded as suitable for quantitative risk assessments. Agents classified as group C are generally suitable for quantitative risk assessment, but judgments must be made on a case-by-case basis. Human epidemiologic studies are preferred if adequate exposure data exist. In the absence of appropriate human studies, data from animals responding most similarly to humans are used. If it is uncertain which species responds to the agent in question most similarly to humans, the most sensitive responding species is used because the human is considered to be as sensitive as the most responsive species.

Benign and malignant tumors are combined for risk estimation unless the benign tumors are not considered to have the potential to progress to the associated malignancies of the same histogenic origin. Tumor sites with statistically significant elevations are usually pooled for estimation of total risk. Risk estimates, however, may also be carried out for individual tumor sites or types to determine risk for that site.

When the exposure route in the species from which the dose-response information is obtained differs from the route occurring in environmental exposures, route extrapolation may be carried out if it is feasible based upon metabolic and pharmacokinetic considerations. All assumptions regarding the extrapolation must be presented along with a consideration of uncertainties.

In the absence of adequate information to the contrary, mathematical extrapolation to low doses is carried out using a linearized multistage procedure. When pharmacokinetic or metabolic data or other substantial evidence on the mechanistic aspects of carcinogenesis exists, a low-dose extrapolation model, other than the linearized multistage procedure, might be more appropriate on biologic grounds. If adequate data exist, a time-to-tumor model may be used.

In the absence of comparative toxicologic, physiologic, and pharmacokinetic data for a given suspect carcinogen, the EPA takes the position that species extrapolation of effective dose on the basis of body surface area is most appropriate.

Nasal carcinogens assessed by OHEA

Evidence for induction of nasal tumors has been shown for 26 of the chemicals assessed by OHEA. Cancer potency, however, either was not estimated, or was based on other tumor sites or a combination of tumor sites for 17 of these chemicals. These 17 include acrylonitrile, benzo(a)pyrene, 1,2-dibromomethane, 1,2-dichloro-2-butene, dimethylsulfate, 2,3,7,8-tetrachlorodibenzo-p-dioxin, 1,1-dimethylhydrazine, nickel, n-nitrosodiethanolamine, n-nitrosodiethylamine n-nitroso-n-propylamine, n-nitrosodimethylamine, n-nitrosomethylvinylamine, n-nitrosopiperidene, n-nitrosopyrrolidene, phenacetin, and vinyl chloride. Nasal tumors were not used for potency determination because the study selected was of better quality than the one inducing nasal tumors, other sites appeared to be more sensitive to tumor induction, or the route of exposure by which nasal tumors were induced was not considered to be appropriate for human exposure.

Data were considered to be inadequate for potency estimation in four of the chemicals. The study selected for potency estimation used the oral route of exposure (gavage, drinking water, or food) for 10 of the chemicals and inhalation for the other three. Liver and stomach tumors were used to estimate potency for most of the chemicals, although tumors of the brain, bladder, and lungs were selected for acrylonitrile, nickel, and phenacetin, respectively. While inhalation is considered to be the usual route of exposure for induction of nasal tumors, they were induced by other routes in six of these 17 chemicals.

It should be pointed out that the EPA does not assume site concordance for cancer when extrapolating from animals to humans. Thus, although nasal tumors were not used for cancer potency estimates in any of these chemicals, this does not rule out the possibility, or even the likelihood, that the nasal region may be the critical site for tumor induction in humans.

Chemicals for which OHEA has estimated carcinogenic potency based on nasal tumor induction are listed in Table I. While adequate animal evidence was available for all of these chemicals, human evidence was considered to be adequate only for bis(chloromethyl)ether and limited for formaldehyde. The potency values for nasal tumor induction were all based on animal studies since human exposure data were inadequate for the two chemicals classified in categories A and B1. Additional potency estimates were developed using other tumor sites for three of these chemicals; kidney, liver, and stomach tumors induced via the diet for 1,2-dibromo-3-chloropropane; liver tumors induced via gavage for epichlorohydrin; and stomach tumors induced via drinking water for hydrazine.

All of the potency determinations for the nasal carcinogens listed in Table I, except one, were derived on the basis of air concentrations using inhalation exposure studies. The only exception was 1,4-dioxane where the potency was estimated for oral intake only. It was converted to an equivalent air concentration, assuming that route extrapolation is valid, only to compare it with the other values listed in Table I.

There is no direct proof as to why the nasal region is the critical target site for oral exposure to 1,4-dioxane. The nasal region is not uniquely susceptible since tumors can also be induced at other sites (NCI, 1978). The high vapor pressure of this chemical, however, suggests that exposure occurs by exhalation through the lungs. If this is true, in order to accurately extrapolate a drinking water dose to an inhaled concentration, it would be necessary to determine the relationship between the ingested dose and concentration in the nasal passages.

Table I. Chemicals assessed by the EPA for which the cancer potency is based on induction of nasal tumors.

Chemical	q_1^*	Category	Reference
Acetaldehyde	2.2×10^{-6}	B2	Woutersen et al., 1984
Bis(chloromethyl)ether	2.7	A	Kuschner et al., 1975
1,2-Dibromo-3-chloropropane	2.4×10^{-3}	B2	Reznik et al., 1980
Dimethylcarbamoylchloride	1.9×10^{-2}	B2	Sellakumar et al., 1980
1,4-Dioxane	2.3×10^{-6}	B2	Hoch-Legeti et al., 1970
Epichlorohydrin	1.3×10^{-6}	B2	Laskin et al., 1980
Formaldehyde	1.3×10^{-5}	B1	Kerns et al., 1983
Hydrazine	4.9×10^{-3}	B2	MacEwen et al., 1981
Propylene oxide	3.7×10^{-6}	B2	NTP, 1985

The q_1^* represents the 95 percent upper-bound risk estimate for lifetime exposure to 1 ug/m^3.

Inhalation of 1,4-dioxane volatilizing from the treated water could further complicate dosimetry.

Formaldehyde can be taken as an example of a chemical recently assessed by the EPA (1987b). To determine the weight of evidence for carcinogenicity in humans, 28 epidemiologic studies were evaluated. In the three studies that were adequate in design to detect small to moderate risks associated with formaldehyde exposure, there was a statistically significant association between respiratory cancer and formaldehyde exposure. Because of confounding factors, especially exposure to other agents, the human evidence was considered to be limited. Nasal cancer was also induced in several animal studies, including that by Kerns et al. (1983). Based on the available evidence, formaldehyde best fit category B1, a probable human carcinogen.

Since adequate exposure data were not available for humans, the animal exposure study by Kerns et al. (1983), in which rats were exposed to 2, 5.6, and 14.3 ppm for 24 months, was used to estimate potency. The data were adjusted for interim sacrifices and early deaths. Parts per million were considered to be equivalent between humans and experimental animals. This assumes that respiration varies directly with metabolic rate. While respiration has been shown to be slightly inhibited in rats breathing high concentrations of formaldehyde, an adjustment would result in only a minor change in the potency estimate.

Since risk at low exposure cannot be measured directly by either animal or human epidemiologic studies, a number of low-dose extrapolation models were developed. Preliminary risk estimates were calculated based on 11 of these models using data from the Kerns et al. (1983) study. When information is limited, models incorporating linearity are recommended by the EPA guidelines. Since formaldehyde is known to be mutagenic, is structurally related to other carcinogens, is cytotoxic, and is clearly carcinogenic in the rat, the linearized multistage model, without restrictions on the order of the polynomial, is the model of choice. The recommended q_1^* value of 1.3×10^{-5} was derived using this model.

The argument can be made that there is a dose level below which the added risk of cancer from exposure to formaldehyde is zero. There is, however, no consensus in the scientific community on this topic.

Additionally, there is no way, through the use of mathematical models, to demonstrate either the existence or nonexistence of a threshold. If thresholds exist, they are likely to vary among members of the population at risk and may be modified by other environmental agents. Therefore, use of a dose-response model incorporating a single threshold would provide an estimate of average population threshold that would have little utility. Thus, it is assumed that there is no threshold.

Dosimetric considerations

A variety of errors is possible when extrapolating risk from animals to humans. Some of these errors, such as differences in metabolic pathways and target organ sensitivity, are common to any species extrapolation. Others are more specific to nasal tumor induction. For example, the surface area of the nasal passages will often differ among species; the efficiency of deposition, especially for inhaled particulate matter, can differ; clearance rates can vary, allowing for differing degrees of particle solubilization and thereby bioavailability; and finally, high experimental concentrations may result in an inhibition of respiration (Chang et al., 1981).

Unfortunately, adjustments to account for such factors are limited by lack of adequate data as well as sophisticated methodology. In order to improve dosimetry estimates for inhaled chemicals, the EPA is currently developing appropriate methodology which will be published as inhalation reference dose guidelines. The availability of adequate data, however, will still limit the application of these guidelines.

As noted previously, some chemicals have been shown to induce nasal cancer by routes other than inhalation. While not having done so yet, it is likely that the EPA will be called upon to carry out route extrapolation for cancer potency of nasal carcinogens in the future. In order to do so it will be necessary to account for several factors including volatility of the parent compound, solubility, bioaccumulation at the target site, as well as requirements for metabolic activation.

References

Chang, J.C.F., W.H. Steinhagen & C.S. Barrow, 1981. Effect of single or repeated formaldehyde exposure on minute volume of B6C3F1 mice and F-344 rats. Toxicol. Appl. Pharmacol. 6:451-459.

EPA, 1987a. The risk assessment guidelines of 1986. Office of Health and Environmental Assessment. EPA/600/8-87/045. Available from NTIS, Springfield, VA. PB88-123997/As.

EPA, 1987b. Health and environmental effects profile for formaldehyde. Prepared by the Office of Health and Environmental Assessment, Environmental Criteria and Assessment Office, Cincinnati, OH, for the Office of Solid Waste, Washington, DC. EPA/600/X-85/362.

Hoch-Legeti, C., M. Argus & J. Arcos, 1970. Induction of carcinomas in nasal cavity of rats by dioxane. Br. J. Cancer 24:164-167.

Kerns, W.D., K.L. Pavkov, D.J. Donofrio, E.J. Grallo & J.A. Swenberg, 1983. Carcinogenicity of formaldehyde in rats and mice after long-term inhalation exposure. Cancer Res. 43:4382-4392.

Kuschner, M., S. Laskin, R.T. Drew, V. Cappiello & N. Nelson, 1975. Inhalation carcinogenicity of alpha halo ethers. III. Lifetime and limited period inhalation studies with bis(chloromethyl)ether at 0.1 ppm. Arch. Environ. Health 30:73-77.

Laskin, S., A.R. Sellakumar, M. Kuschner, N. Nelson, S. LaMendola, G.M. Rusch, G.V. Katz, N.C. Dulak & R.E. Albert, 1980. Inhalation carcinogenicity of epichlorohydrin in non-inbred Sprague-Dawley rats. J. Natl. Cancer Inst. 65:751-757.

MacEwen, J.D., E.H. Vernot, C.C. Haun, E.R. Kinkead & A. Hall, III, 1981. Chronic inhalation toxicity of hydrazine: oncogenic effects. Wright-Patterson Air Force Base, Aerospace Medical Research Laboratory (AFAMRL-TR-81-56). Tech. Rep. Ser. No. PB-288-428. 64p.

NCI (National Cancer Institute), 1978. Bioassay of 1,4-dioxane for possible carcinogenicity. NCI Technical Report 80, 1-106.

NTP (National Toxicology Program), 1985. Toxicology and carcinogenesis studies of propylene oxide in F344/N rats and B6C3F_1 mice (inhalation studies). NTP technical report series no. 267.

Reznik, G., H. Reznik-Schuller, J.M. Ward & S.F. Stinson, 1980. Morphology of nasal cavity tumors in rats after chronic inhalation of 1,2-dibromo-3-chloropropane. Br. J. Cancer 42:772-781.

Sellakumar, A.R., S. Laskin, M. Kuschner, G.V. Katz, C.A. Snyder & R.E. Albert, 1980. Inhalation carcinogenesis of dimethyl carbamoyl chloride in Syrian Golden hamsters. J. Environ. Pathol. Toxicol. 4:107-115.

Woutersen, R.A. & L.M. Appleman, 1984. Lifespan carcinogenicity study of acetaldehyde in rats. III. Recovery after 52 weeks of exposure. Final Report No. V84.288/190172. CIVO-Institutes TNO, The Netherlands.

Regulations of Nasocarcinogens in Germany (FRG)

K. Norpoth

Institute of Hygiene & Occupational Medicine, University Medical Center, Essen, FRG

Under the aspects of regulations, nasocarcinogens do not constitute a special class of hazardous substances. So far as they are able to induce also other tumors inside or outside of the respiratory tract, organ specificity of the effect has a poor significance for protection measures. On the other hand these compounds are suitable paradigms for explicating basic principles of health protection from environmental carcinogens in the Federal Republic of Germany and to illustrate how they are realised by legislation and by related activities. The demand to minimize each cancer risk is included in the general regulations on food, water and air (Table 1) which are subjected to continuous control by means of sample analyses. Everybody who give rise to damage via contamination of food, water or air is regarded to be responsible for it. An important principle is to proceed by the way of precaution. For example not only the proven human carcinogens are thought to demand strong regulations but also compounds which are seriously suspected to cause cancer in man. Only for a part of these compounds including formaldehyde a threshold limit value (MAK) exists. Some nasocarcinogens are listed in the Technical Guidance "Air" as a number of chromium compounds (calcium-, chrom III -, strontium - and zinc-chromate) as well as dimethylsulfate, nickel and some nickel compounds. Their emissions are restricted to 1 mg/m^3 air at a mass stream of 5 g/h. The preferential area of differentiated single regulations for nasocarcinogens however is the work environment (Table 6). Production, transport and use of the compounds are concerned in the decree on hazardous substances (Gefahrstoff-Verordnung vom 26.8.1986). That means, that the decree covers mainly, but not exclusively, the health protection measures at working places

Regulations for different categories of carcinogens

Decisions on what compounds must be considered as proven or potential human carcinogens rest with a group of scientists, the "Commission of the Deutsche Forschungsgemeinschaft (DFG) for the investigation of Health Hazards of Chemicals in the working Area". In the opinion of this group which is used to make known all arguments the decisions are based on no save concentrations for carcinogenis substances can be established. The given list of about 160 compounds consists of three categories, the nasocarcinogens of

which are given in the tables 2 - 4. Category A 1 covers the proven human carcinogens. I have selected also the direct alkylating agents causing respiratory cancer. In category A 2 such compounds are noted which have proven so far to be unmistakably carcinogenic in animal experimentation only; namely under conditions which are comparable to those for possible exposure of a human being or from which such comparability can be deduced. It should be emphasized that no difference in regulations exists for the two categories mentioned. A third category B is given with compounds which are justifiably suspected of having carcinogenic potential.

On the base of the decisions made by the scientific comittee another group, the "Comittee on Hazardous Chemicals" (AGS) by order of the Federal Ministry of Labour assigns technical guiding concentrations (TRK) for carcinogenic substances (table 5). They are minimum possible concetrations with the current technology and they serve as a guidance for protective measures and monitoring at the workplace. Adherence to TRK values is intended to reduce the risk of a health hazard but cannot completely eliminate it. TRK-values are established for carcinogenic substances of the categories A_1 and A_2 and for those compounds listed in category B, for which no threshold value (MAK value) exists.

The legal base on which TRK-values are established, the already mentioned decree on hazardous substances, contains another classification of carcinogens, namely according to the assumend carcinogenic potency. In this way, an element of quantitative risk estimation is taken into considaration. Compounds of the category I are considered as extremely hazardous, those of category II as very hazardous and compounds of the category IIIas less hazardous.
The classification involves not only the pure substances but also all preparations containing the compounds at definite concentrations (table 6). The production as well as the use of any substance of the categories I and II require announcement to the governmental authorities, while the production and use of a substance from category I in addition requires licensing. A permission will not be issued if the necessity for the use of a substance is not clear. Any substance from any of the three categories must be labeled with the indication to the "Hazardous Substance Decree" and the tip-off "able to cause cancer". Any substance containing carcinogenic impurity must be labeled with the name of that impurity and with informations on its carcinogenicity. Employees are not allowed to work with substances classified to be in category I. Exceptions are made possible for security and control measures, as well as for the employees in

research laboratories. Such substances can also be used as controls for the analytical purposes. The employer is responsible for the reduction of air concentrations of these substances. No having meal, drinking or smoking is allowed at any place where such substances are used. These working areas must have special lavatories and bath. Obligatory, the air concentrations of the compounds must be checked routinely to ensure that the actual concentrations are kept as low as possible below the TRK-value.

Medical surveillance

The Hazardous Substance Decree prescribes that the employees exposed to a substance exceeding a so called "Auslöseschwelle" (action level) must routinely be examined for their health conditions. An exposure exceeding an "action level" is not to be assumed if the adherence to the "TRK-value" is well checked and controlled on time. One of the ways to have this controlled is to measure in order to know whether the mean exposure level is less than one-fourth of the "TRK-value" during a working day or whether the mean exposure level remains constant below the "TRK-value" for a long period of time. The medical examin-ations are to be performed at least every six months and maximum for a period of sixty months. Instructions for the performance of these medical examinations are given by the accident-insurance authorities ("Gesetzliche Unfallversicherungen"). Besides a general regulation for "Cancer Causing Industrial Agents" (G 40), there exist other special regulations for single carcinogens, e.g., for chromates, nickel and nickel compounds. The most important goal for such medical examinations is to diagnose the occurance of a cancer as early as possible in order to increase the possibility for a successful medical treatment. Wood dusts however, are not included in the general regulation.
According to an international agreement (ILO-Abkommen 131), the legal accident-insurance authorities have recently started to establish medical examinations for the retired workers. An organisation is established to have this examinations be performed on those whose job-category is classified to be among the "over action level exposed to industrial carcinogens". Included are the nasal carcinogens such as chromium and nickel compounds, but not the wood dusts. Workers have to decide wether they will take part in these examinations.

Suspected nasocarcinogens

There exist no uniform regulations for the group of suspected carcinogens which have not proven so far unmistakably carcinogenic under conditions comparable to those for possible exposure of a human being at the workplace. It is already mentioned that threshold values (MAK values) are given furthermore for some of these compounds. An example is formaldehyde (Table 7). The maximum allowable air concentration within the meaning of the MAK-value definition was recently lowered to 0.5 ppm. The reason was to avoid local irritation - not to avoid cancer. Further regulations concern the liberation of formaldehyde from chipboards and the limitation of formaldehyde concentrations in cosmetics. So far formaldehyde is subjected to strong regulations but it is excluded from the prescriptions for proven carcinogens. Really, however, there is a clear tendency, to replace the compound, wherever possible, by less harmfull alternatives.

Final remarks

The regulatory measures described may suggest to be the essentials of a perfect and highly effective protection policy. We are however at an early stage of a full developed regulatory system having poor knowledge of the real significance of many carcinogenic hazards. Nasocarcinogens again are a good example. Little is known about the contribution of nitrosamines to human nasocarcinogenesis. The role of aldehydes is not clear. Nasal cancers at working places of the leather industry are not involved in the regulations mentioned. We are far from understanding the mechanism of nasocarcinogenesis caused by wood dusts. Thus this major principle among the well known causes of nasal cancer must be subjected to strong regulations hoping that these would lower the risk. But only after decades it will become clear whether that expectation is justified. It is the progress in cancer research that makes our regulatory system convincing but there is also a lack of knowledge what makes this system somewhat dubious.

Tab. 1

Regulation matter	Main legal basis for regulation	General regulations	Special regulations for nasal carcinogens
Foodstuffs	Laws on foodstuffs & requisites of 1974-08-15 as well as other provisions	Production, use & transport of any foodstuff in a way that might hazard health is prohibited	None
Water	Drinking water decree (TVO) of 1975-01-31	No presence of any hazardous substance in drinking water is permitted	None
Air	Federal Immissions Protection Law of 1974-03-15 and Technical Guidance "Air" of 1974-08-28	Any installation which contributes to air pollution requires licence and control. Everybody giving rise to damage is responsible for it	In the Technical Guidance "Air"

Tab. 2

A 1) Working materials that have been unequivocally proved carcinogenic. Compounds capable of inducing malignant tumours as shown by experience with humans

- Zinc chromate
- Beech wood dust
- Oak wood dust
- Nickel (as inspirable dusts/aerosols from nickel, metal, nickel sulfide and sulfidic ores, nickel oxide and nickel carbonate arising in production and processing)
- bis-Chlorometyl ether
- 2,2'-Dichlorethyl sulfide
- Monochlorodimethyl ether

Tab. 3

A 2) Working materials which have been unequivocally proven carcinogenic. Compounds which in the Commission's opinion have proven so far to be unmistakably carcinogenic in animal experiments only; namely under conditions comparable to those for possible exposure of a human being at the workplace, or from which such comparability can be deduced:

Calcium chromate

Chromium(III) chromate

("Chromic chromate")

Chromium(VI) compounds

Dimethyl sulfate

Diazomethane

Dichloroacetylene

1,2-Epoxypropane

Hydrazine

N-Nitrosomethylethylamine

N-Nitrosodiethylamine

N-Nitrosomorpholine

N-Nitrosodiethanolamine

N-Nitrosodi-n-propylamine

N-Nitrosomethylphenylamine

N-Nitrosopiperidine

N-Nitrosopyrrolidine

Tab. 4

B) Compounds which are justifiably suspected of having carcinogenic potential. In addition, recent results of cancer research call for the consideration of additional materials for which a noteworthy carcinogenic potential is suspected and which urgently need further clarification. Where MAK values already exist for the compounds listed below, they are tentatively retained:

1,4-Dioxane

Formaldehyde

Isopropyl oil

Wood dust
(with the exception of beech and oak wood dust)

Tab. 5

Industrial cancer causing agents	Weight in working material (%) for groups		
	I Extremely hazardous	II Very hazardous	III Hazardous
Sym. dichlorodimethyl ether	≥ 0.05	< 0.05 - 0.005	< 0.005 - 0.0005
Monochlorodimethyl ether	≥ 1	< 1 - 0.1	< 0.01 - 0.01
Calcium chromate		≥ 1	< 1 - 0.1
Chromium(III) Chromate		≥ 1	< 1 - 0.1
Dichloroacetylene		≥ 1	< 1 - 0.1
Diethyl sulfate		≥ 1	< 1 - 0.1
Diazomethane		≥ 1	< 1 - 0.1
Nickel carbonyl		≥ 1	< 1 - 0.1
Strontium chromate		≥ 1	< 1 - 0.1
Zinc chromate		≥ 1	< 1 - 0.1
Nickel (as inspirable dusts from nickel metal, nickel sulfide, and sulfidic ore, nickel oxide and nickel carbonate arising in production and processing)		≥ 5	< 5 - 0.5

Tab. 7

Regulations and suggestions to limit the hazards caused by formaldehyde in West Germany

Atmospheric threshold value for the working place (MAK)	Emission limit for steady installations	Free atmosphere	Indoor air
ppm	mg/m^3	mg/m^3	mg/m^3
0,5	20 (exceptions possible)	short term: 0.07 long term : 0.03	0.12 (suggested)

Tab. 6

Substance	TRK ml/m³	TRK mg/m³	Remarks
Chromates (calcium chromate, chromium(III) chromate, strontium chromate, zinc chromate)			calculated as CrO_3 in total dust
- arc-welding by hand with coated electrodes	-	0,2	
- others	-	0,1	
Diethyl sulfate	0,03	0,2	
Dimethyl sulfate			
- production	0,02	0,1	
- use	0,04	0,2	
1,2-Epoxypropene	2,5	6,0	
Hydrazine	0,1	0,13	
Nickel as metallic nickel, nickel sulfide and sulfide-containing ores, nickel oxide and nickel carbonate	-	0,5	calculated as Ni in total dust
Nickel compounds in the form of inspirable droplets	-	0,05	calculated as Ni for the entire inspirable portion
Nickel carbonyl	0,1	0,7	
Wood dust			wood dust as a total dust*
- new installations		2	differentiation between dust samples from various kinds of wood is not at present possible in routine analyses
- others		5	

*
total dust refers to that portion of dust that can possibly be inhaled. It is collected by asampling device operating at an air velocity of 1,25 ms/s ± 10%

Part 5

Panel discussion and concluding remarks

SUMMARY PANEL DISCUSSION

M.C. Bosland

TNO-CIVO Toxicology and Nutrition Institute; New York University Medical Center, New York, USA

Panel Members: H.M. Bolt (Chairman), M.C. Bosland, M. Boysen, V.J. Feron, J. Higginson, H.A. Milman, K.T. Morgan, and A. Somogyi

In the panel discussion a few central themes were addressed, along with a number of minor questions, all focussing on the aims and scope of the symposium. A summary of the discussion and of the major recommendations and issues of consensus and controversy is given below.

Species differences in nasal morphology

The transitional zone of cuboidal epithelium in the anterior nose is quite extensive in the rat, and it also occurs in other rodents and sub-human primates. It is not clear whether it exists in man; it was suggested that cuboidal epithelium in the human anterior nose could be metaplasia rather than a normal mucosa. In rodents, it is a primary site of lesions, probably due to air flow patterns. Since very little is known about this apparently important epithelial zone, more research is warranted. There are many differences between species in the shape of the nasal cavity and its turbinates, as well as in the location and size of the various epithelial compartments of the nasal mucosa. Even in different rat strains there are distinct differences in this respect. These all probably can contribute to species- and stain differences in toxic and carcinogenic responses of nasal tissues.

Standardization of methods and classification of lesions

There was general agreement that the procedure of Young (1981) should be regarded as the basis for histologic examination of the rat nose. As the rat and, for that matter, other rodents have a very complex nose in terms of anatomy and epithelial topography, it was considered desirable to extend the method of Young, with its 4 standard sections of the rat nose, with additional sections, particularly of the anterior part of the nose, to include important areas such as the transitional zone of cuboidal epithelium, the organ of Masera and the vomeronasal organ. Standardization of histologic examination of the primate nose is currently lacking, but is urgently needed. The discussions in session II on classification of nasal tumours in animals, indicated the need for standardization and classification criteria. In man, the classification of tumours as adenocarcinoma, squamous cell carcinoma or papilloma has proven useful, but improvement of classification may help to clarify confusing terms such as "sinonasal cancer".

Wood dust

Although wood dust was unanimously regarded as a human nasal carcinogen, it appeared that very little is known about its constituents. K.-H. Norpoth indicated that mutagenic activity has been found in extracts of wood dusts, as well as the presence of α- and β-tannine and quercitine. He

also mentioned that wood dust extracts had been found carcinogenic to the mouse skin in a dose-dependent manner, and that a wide variety of skin tumours was induced, suggesting, in his opinion, the presence of a number of different carcinogens in the extracts. Although some aldehydes are definitely present in wood dusts, their concentration was indicated to be too low to account for the carcinogenic potential of wood dust. It was proposed to create standardized wood dust samples for research purposes in a similar fashion to what has been done for asbestos. Both analytical work and animal studies would greatly profit from the availability of such samples, standardized according to size, shape, chemical properties, mutagenic activity, et cetera.

It was suggested that specific chemical constituents and other properties of wood dust, such as physical irritation alone may not be sufficient to elicit the observed frequency of nasal cancer in exposed populations, but that combinations of such factors may play a critical role. Experiments with wood dust in hamsters by B. Wilhelmsson were briefly discussed. He found squamous metaplasia and at least one carcinoma in the nose of exposed animals. Combination of exposure to wood dust and to 12 ppm formaldehyde in rats was additive in inducing metaplasia but not in inducing tumourigenesis (one carcinoma was seen with formaldehyde alone). There are currently ongoing animal studies on wood dust carcinogenicity in the U.K. and Japan. There was general agreement that exposure to wood dust should be regulated, although it is not yet clear what should be regarded as a sufficiently safe level. A major problem will be that the results of such regulation will not become evident for decades because of the probably long latency time of the induced nasal tumours.

Nickel

There was no unanimous opinion about how to deal with the evidence for carcinogenicity of nickel and nickel compounds. One view was that if one compound belonging to a class of substances (i.e., inorganic nickel compounds) has been proven to be a human carcinogen, all other compounds in that class should be regarded as such, unless the opposite is shown. However, if a certain property of a compound, such as irritation, appears to be critical for the carcinogenic effect, then non-irritating other compounds of the same group should be regarded as probably without carcinogenic risk. The nickel compounds responsible for the carcinogenic effects seen in nickel refinery workers are unknown and we have only animal inhalation data on nickel subsulphide. However, the human findings cannot be ignored, thus all nickel compounds, unless proven otherwise, should be regarded carcinogenic, possibly only upon inhalation exposure.

Another opinion was as follows: most nickel industries, excluding refineries, show little evidence of increased risk for respiratory tract cancer. Most evidence for nickel carcinogenicity has been derived from studies on populations that had high exposures in the times before industrial hygiene improved, and soluble nickel seems to have been the most important carcinogenic factor. Thus individual nickel compounds should be evaluated separately. The IARC (1987) concluded that there is sufficient evidence from human studies for carcinogenicity of nickel compounds as a group, although this evidence was initially (IARC, 1976; 1982) regarded as limited.

Significance of rodent nasal tumours for human cancer risk

There seemed to be general agreement that if a substance induces nasal tumours in rodents, this cannot be ignored and should be regarded as indicator that this compound has carcinogenic properties. Since there are

species differences in deposition of inhaled toxicants, it may well be that if the nasal mucosa is the target in rodents, lower regions of the respiratory tract are targets in subhuman primates and in man. Bis(chloromethyl)ether is a classical example: this very potent carcinogen induces nasal tumours in rats and lung tumours in man. Weaker carcinogens, however, may present more difficulties for risk assessment. The induction of rodent forestomach tumours was mentioned as an example of evidence of carcinogenicity from rodent studies with implications for assessment of human cancer risk, although there is no such tissue in man. Compounds that clearly have mutagenic properties and cause (nasal) cancers usually do not present problems for risk assessment although there are exceptions. It was stressed that for risk assessment of non-mutagenic compounds that are carcinogenic at high doses, we need to know more about the mechanisms that underlie these effects.

Thresholds and cytotoxicity

Although thresholds are general phenomena in biology, it is subject of debate whether these exist in carcinogenesis. A threshold in cytotoxicity-induced cell proliferation plays a critical role in the carcinogenicity of formaldehyde in animal models. This has led to much controversy about the risk assessment of formaldehyde. It appeared clear during the discussion that we still do not know enough to arrive at a consensus in this regard; thus, more research in this area is warranted. It was stressed that a compound- and target tissue-specific approach are most suitable for dealing with risk assessment of carcinogens that have irritating properties and cause hyperproliferation.

Combination exposure to nasal carcinogens

There were divergent opinions about the possible additivity of (nasal) carcinogens. One view was that at least addition should occur in the case of exposure to two carcinogens that affect the same target tissue, if they are genotoxic. Another view was that we should not assume such additivity of two carcinogens, unless we have mechanistic information that suggests addition. It was stressed that general rules for risk assessment of exposure combination of carcinogens are not possible, and that a case-by-case approach is needed. Interestingly, it was mentioned that B. Wilhelmsson has found more dysplasia and metaplasia in wood workers exposed to formaldehyde than in those exposed to only wood dust. No data on nasal cancers are available from this study as yet. It was clear that more research is needed on nasal carcinogenesis by combination exposure to carcinogens and modifying factors. For example, ozone is not only cytotoxic to the lung but also to the nose in animal models; thus, it is of considerable interest to know more about combined exposure to ozone and nasal carcinogens such as aldehydes. Similarly, we need to know more about the relation between chronic sinusitis and nasal cancer risk in populations that are exposed to nasal carcinogens. This research should particularly also address the mechanisms that underlie combination effects. In this regard, it was indicated that the rat nose is very sensitive to carcinogenic insults, whereas the human nose is relatively insensitive, and that the opposite situation occurs for the lung. Thus, there may be species differences in mechanisms of nasal carcinogenesis. It was therefore suggested that species extrapolation should be carried out with great caution.

References

IARC 1987. Monographs on the Evaluation of the Carcinogenic Risk of Chemicals to Humans. Supplement 7. Overall Evaluations of Carcinogenicity: An updating of IARC Monographs Volumes 1 to 42. IARC, Lyon.

IARC 1982. Monographs on the Evaluation of the Carcinogenic Risk of Chemicals to Humans. Suplement 4. Chemicals, Industrial Processes and Industries Associated with Cancer in Humans. IARC, Lyon.

IARC 1976. Monographs on the Evaluation of the Carcinogenic Risk of Chemicals to Man. Vol 11. Cadmium, nickel, some epoxides, miscellaneous industrial chemicals and general considerations on volatile anaesthetics. IARC, Lyon.

Young, J.T. 1981. Histopathologic examination of the rat nasal cavity. Fundam. Apl. Toxicol. 1:309-312.

CONCLUDING REMARKS

J. Higginson[1], H.M. Bolt[2], and M.C. Bosland[3]

[1] Georgetown University Medical Center, Washington, USA
[2] Institut für Arbeitsphysiologie, Dortmund, FRG
[3] New York University Medical Center, New York, USA

Session I

This conference has demonstrated that the mammalian nose is a very complex tissue, regardless of species. There is considerable complexity in cell types and their location in the nasal mucosa, the structure of the nasal cavity and its turbinates, air-flow patterns, and metabolic capacities, both in terms of specific areas in the nose and of specific cell types. Thus, it is not surprising that there is great diversity in the responses of the nasal tissues to toxic insults, including carcinogenesis. Clearly, interdisciplinary research approaches are necessary as illustrated by this symposium. In addition to standardization of histological methods and classification of lesions, there is need for standard of sampling procedures for biochemical studies. Reference to samples of specific areas of the mucosal and submucosal compartments is preferable to samples from total nasal tissue ("nasal soups").

New observations on structure and function of the rodent nose were presented at the meeting, such as a new cell type (the M cell-like cell), details about mucosal innervation and submucosal glands, information about nasal immune function and nose-associated-lymphoid-tissue, and the description of a transitional zone of cuboidal epithelium between the squamous anterior portion of the nasal mucosa and the respiratory epithelial portion that is particularly sensitive to, for example, ozone. This abundance of new data indicates that we are just beginning to understand structure and function of the nasal passages.

Of particular interest with respect to toxic and carcinogenic responses of nasal tissues, were the discussions about reflex regulation of nasal airflow and about nasal blood flow being a major determinant of nasal deposition of certain volatile chemicals. Very little is known about the mechanisms of the regulation of nasal blood flow and nasal airflow under dynamic in vivo conditions, both in man and in rodents and subhuman primates. For example, exposure to inhaled toxicants in man can result in reflex regulated changes in the diameter of collapsible segments of the upper respiratory tract, and in changes in nasal blood flow. These changes can modify the dimensions of the nasal passages, and such changes may, in turn, influence airflow (Olson et al., 1988; Cole, 1988), and consequently reduce deposition of the toxicants, resulting in protection of the nasal mucosa. Experimental animals may differ from man in this respect, in addition to differences in nasal dimensions, air flow patterns, and size and location of specific mucosal areas. These factors may explain species differences in nasal toxicity and carcinogenicity. An unexplored area is the possible role of the amount and composition of the periciliary fluid of the respiratory epithelium in the nose in determining the toxic and carcinogenic responses of this epithelium. No information is available about the regulation of the amount and composition of this fluid or of the mucous blanket. Although the mucociliary apparatus in the rodent nose has been studied in detail, very little is known about the possible protective

functions of the mucous as such. Interestingly, the presence of potentially detoxifying enzymes, such as active esterase enzymes, in the nasal mucous was mentioned.

An exciting new area of research presented at this meeting was the use of human nasal tissues (biopsies) for morphological and biochemical studies, which were shown to be feasible. This avenue should be further explored, in addition to the use of human tissue for tissue culture experiments. Another promising approach for the purpose of interspecies extrapolation could be comparative studies using different types of subhuman primates, the nose of some of which may be more like the human nose, whereas others may have noses that are more comparable to those of rodents used in carcinogenicity testing. The potential of such studies was indicated by a poster presentation at this meeting about formaldehyde-induced morphologic lesions in Rhesus monkeys occurring as low in the respiratory tract as the carina.

The fact that wood dust is a human carcinogen may provide us with a tool to determine the usefulness of animal models for the study of nasal carcinogenicity by testing its effects on the rat and primate nose. Mucosal cell proliferation probably plays a key role in nasal carcinogenesis in many instances, either as a prerequisite or as modifying factor. Little detailed information, however, is available on temporal aspects of nasal epithelial cell proliferation responses to toxicants, on chronic responses, and on species- and site-specific differences in such responses. The trigger mechanisms of toxicant-induced cell proliferation in the nasal mucosa and the progenitor cells of resulting hyperplasia are unknown.

Session III

This session focused on two major aspects: nasal deposition and metabolism of xenobiotics and interactions of nasal carcinogens with nasal DNA. Recent progress made in these areas was highlighted and valuable information about mechanisms which may underlie the site-specific and species-specific action of nasal carcinogens was provided. However, the knowledge appeared still fragmentary, and further research efforts are required.

Application of pharmacokinetic principles and modelling revealed that inspiratory flow and blood perfusion rates as well as the local metabolic capacity are major determinants that influence disposition of volatile compounds. For example, the dissolution of acetone and ethanol from the gas phase into the blood was a limiting factor of absorption under given perfusion conditions. It was stressed that quantitative metabolism data *in vitro* versus *in vivo* may differ considerably.

Studies of the local covalent tissue binding of model compounds after systemic application indicated pronounced differences in metabolic competence between olfactory and respiratory epithelium. For example, bromobenzene metabolites were irreversibly bound to tissue macromolecules especially in Bowman's glands, whereas phenacetin which most probably requires a primary activation by cytochrome P-450 isozyme(s), was bound to the olfactory mucosa to a much higher degree than to respiratory mucosa. Both types of epithelium differ not only in amount of total cytochrome P-450, but display distinct P-450 iso-enzyme patterns. Moreover, the nasal epithelium contains glutathione-S-transferase, UDPG-transferase, epoxide hydrolase, carboxylesterase, aldehyde dehydrogenase, and other enzyme activities which are relevant for xenobiotic metabolism. It was stressed that in metabolism studies compounds must be individually assessed. The half-life of the relevant reactive metabolites, together with the local enzyme patterns, are factors that are of general importance.

That these considerations, in principle, also hold true for humans can be inferred from a poster that presented evidence for the presence of various cytochrome P-450-linked activities, as well as epoxide hydrolase and glutathione-S-transferase, but not of UDPG-transferase in nasal respiratory epithelium of 8 patients. The (mostly) detoxifying enzymes epoxide hydrolase and glutathione-S-transferase were present in much higher amounts in human than in the rat tissue. In the rat, glutathione-S-transferase activity is high in olfactory, lower in respiratory epithelium, and much lower in trachea and lung. This may have a bearing on the actions of compounds such as acrolein which is metabolized by both oxidative and glutathione-dependent pathways. Inhalation exposure to the highly irritant and chemically reactive acrolein leads to glutathione depletion and, even after intraperitoneal application, to changes in nasal enzymes related to glutathione metabolism. Several speakers throughout the symposium considered acrolein as a genotoxic, but non-carcinogenic compound. During the discussions it was stated that such an evaluation is questionable since the compound has been studied in a long term inhalation bioassay only in hamsters and not in rats which are probably more sensitive to its local tumourigenic action.

The "DNA adduct" part of the session started with a presentation of studies of a series of direct-acting "model" carcinogens ranging from the very reactive and short-lived dichloroacetyl chloride to the slowly reacting propylene oxide. A relation was demonstrated between the nasal tumourigenic potential and the hydrolysis time of these direct-acting carcinogens. There were some exceptions, i.e., those compounds which may be considered too short-lived to optimally reach the DNA target. DNA binding of both SN1 and SN2 reactants was linear with dose. The binding of SN1 agents (isopropyl methanesulfonate, dimethylcarbamyl chloride) was much lower than that of SN2 agents (methyl methanesulfonate, β-propiolactone). The half-lives of individual adducts in nasal tissue varied considerably.

Other presentations dealt with the nasal DNA adducts of the tobacco-specific nitroso-nicotine derived ketone (NNK). This compound can, in principle, cause DNA phenyloxobutylation and DNA methylation, specifically the formation of the promutagenic O^6-methylguanine. Apparently due to a more efficient repair in the lung, these lesions tended to accumulate in nasal mucosa more than in lung tissue. Malignant nasal tumours induced by NNK arose from the olfactory region, i.e., from the olfactory epithelium and from Bowman's glands. In the respiratory region, more benign tumours were seen. This tissue-specificity of tumour induction by NNK in the nose was shown to be mainly determined by two factors: DNA alkylation to form promutagenic adducts and cytotoxicity, resulting in cell proliferation. The use of O^6- and 7-N-methylguanine antibodies has allowed the immunocytochemical detection of DNA-lesions by NNK. This method was shown to be also suitable for quantitative evaluation. In rats and hamsters, especially Bowman's glands exhibited strong antibody binding which decreased with time. Based on this totally different methodology, results were obtained which were consistent with and complimentary to those achieved by analytical chemistry.

A sensitive method of determining formaldehyde-induced DNA-protein cross-links was used to compare previously published data from rat experiments with data obtained in Rhesus monkeys upon exposure to radiolabelled formaldehyde. The monkey showed relatively higher radiolabel incorporation into nasal DNA, but less covalent binding of formaldehyde to DNA than was observed in the rat. In the rat, formaldehyde-DNA binding was confined to the anterior portion of the nose, but in the monkey it reached also deeper portions of the respiratory system. This basic finding was supplemented by a poster that described morphologic lesions in Rhesus monkeys exposed to formaldehyde gas. The lesions concentrated, as expected, on the frontal

areas of the nose, but they appeared also in the nasopharynx, trachea, and as far down as the carina. Thus, somewhat different reactions of rats and a primate species to formaldehyde were established on two different biological levels, the molecular adduct level, and the light microscopic level.

A bridge between the primary effects at the DNA level and the later stages of tumour development is provided by recent insights in the field of oncogenes. There is distinct tissue- and species-specificity of oncogene activation by carcinogens, and multiple oncogene activation pathways may occur in many animal models of chemical carcinogenesis. Nasal carcinogenesis by direct-acting alkylating agents seems characteristically to involve not the ras gene but a novel oncogene. Further research in this area is clearly needed.

Sessions II and IV

In the last twenty five years an enormous change has taken place both in terms of our knowledge about carcinogenesis, and in terms of how we use that knowledge for informing the public about risks and for regulatory actions at the government level. In 1964, a meeting was held about nasopharyngeal carcinoma, a common cancer in Asia, which was then and still is of great interest. It is very interesting to note how little we knew at that time about nasal physiology and the deposition of particles and vapours or gases in the nose. In the meantime, we have greatly improved our insights about nasal physiology, toxicology, and carcinogenesis (see Barrow, 1986; Mathew and Sant'Ambrogio, 1988; Proctor and Anderson, 1982; Reznik and Stinson, 1983), which has culminated in this symposium.

The nose is subjected to an enormous number of environmental insults. A large number of people suffer inflammatory and metaplastic lesions and even papillomas in the nose. It is extraordinary, however, how rarely neoplasia follows all of these lesions. Thus, simple damage to the nasal mucosa is not a frequent prerequisite for cancer from a statistical point of view. This conclusion equally applies to chronic sinusitis, from which approximately 10% of the population suffer. Thus, one has to be careful to associate nasal metaplasia per se with neoplasia. The same situation occurs in many other tissues of the body: thus, not all metaplastic lesions are necessarily preneoplastic. As yet it is not very clear, either in animal models or in humans, how the preneoplastic potential of metaplasia can be distinguished. This will require much further research.

If one believes in concordance between topical sites (i.e. sites that are directly exposed), there may be a relation between the nose and the lungs; therefore, it may be perfectly justifiable to consider these sites together. Taking known carcinogens as example, cigarette smoke does, for practical purposes, not induce nasal cancer but it does cause lung cancer. Wood dust, on the other hand, induces nasal cancer but not lung cancer. Asbestos does not cause nasal cancer but induces cancer of the larynx, where it is locally deposited on the surface. These findings are very difficult to rationally explain with what has been presented at this conference. Nickel, on the other hand, seems to fit in well with both topical and non-topical sites (i.e., sites that are not directly exposed), where we should regard the nose as a topical site.

Quantitative risk assessment may, notwithstanding its appearance of exactness as to the estimation of degree of risk, has no generally acceptable biological base due to the use of improven assumptions, as in the case of formaldehyde. On the other hand, for wood dust and compounds such as nickel hard data are available from epidemiological studies, from which the real risks which can be determined. Good exposure data are missing from most if not all human studies, and this is particularly true for

formaldehyde, where assumptions on formaldehyde exposure are usually extrapolated. Further, exposures to other factors, such as wood dust may also have been present. On the basis of very weak exposure data estimates were made about exposure levels, both in terms of particulate and gaseous formaldehyde. Recently, the 30-40 existing epidemiological studies on formaldehyde were reviewed (Ad Hoc Panel on Health Aspects of Formaldehyde, 1987), and the deficiences in determining exact formaldehyde exposures in the past were emphasized. The three major recommendations of the report by the Ad Hoc Panel on Health Aspects of Formaldehyde (1987) were that 1) follow-up of existing cohorts should be continued, 2) more studies should be conducted to improve the methodology of formaldehyde exposure assessment, particularly of particulate formaldehyde, and 3) the confounding of formaldehyde exposure data by other known risk factors should be explored.

Where should we go from here? Sufficient work has been done on nasal carcinogenesis to justify the establishment of a multidisciplinary committee that will review developments and current knowledge in nasal cancer research and risk assessment of nasal carcinogens, that will draft guidelines to standardize research methods and nomenclature and classification of lesions, and that will define research needs. This approach was very successful in the case of asbestos: standard samples of asbestos were made available; the classification of asbestosis was defined, and further research needs were identified. Such a committee would also provide some form of continuity for the field of nasal carcinogenesis research and risk assessment.

References

Ad Hoc Panel on Health Aspects of Formaldehyde, Epidemiology of Chronic Occupational Exposure to Formaldehyde 1987. Universities Associated for Research and Education in Pathology, Inc., Bethesda.

Barrow, C.S. (Ed.) 1986. Toxicology of the Nasal Passages. Hemisphere Publ., Washington.

Bertazzi, P.A., A.C. Pesatori, L. Radice, C. Zocchetti, & T. Vai, 1986. Exposure to formaldehyde and cancer mortality in a cohort of workers producing resins. J. Work Environ. Health 12:461-468.

Cole, P. 1988. Nasal Airflow Resistance. In: O.P. Mathew, G. Sant'Ambrogio (Ed.): Respiratory Function of the Upper Airway, p. 391-414. Dekker, New York.

Mathew, O.P. & G. Sant'Ambrogio (Ed.) 1988. Respiratory Function of the Upper Airway. Dekker, New York.

Olson, L.G., J.M. Pouke, P.L. Hoekje, & K.P. Strohl. A Biomechanical View of Upper Airway Function. In: O.P. Mathew and G. Sant'Ambrogio. (Ed.) 1988. Respiratory Function of the Upper Airway, p. 359-489. Dekker, New York.

Proctor, D. & I. Anderson (Ed.) 1982. The Nose: Upper Airway Physiology and the Atmospheric Environment. Elsevier, New York.

Reznik, G. & S.F. Stinson 1983. Nasal Tumors in Animals and Man. Vol. III. Experimental Nasal Carcinogenesis. CRC Press, Boca Raton.

Posters

FUNCTIONAL MORPHOLOGY OF M CELLS IN NOSE-ASSOCIATED LYMPHOID TISSUE (NALT) IN WISTAR RATS

E.G.J. Hendriksen, B.J. Spit and C.F. Kuper

TNO-CIVO Toxicology and Nutrition Institute, Zeist, Netherlands

Mucosa-associated lymphoid tissue (MALT) in rat is found in the gastro-intestinal tract (GALT) and lower respiratory tract (BALT). Similar tissue is present in the nose of rats denoted as nasal lymphoid tissue or NALT (1). The NALT exists of multiple domes; on top of these domes are non-ciliated cells, so-called M cells, surrounded by ciliated cells.

In accordance with studies concerning the GALT of mouse (2) and BALT of rabbit (3) and rat (4), studies were performed to investigate the functional morphology of M cells in NALT in Wistar rat. Immuno-electron microscopy was used to demonstrate the Ia-marker, a class II MHC antigen present on antigen-presenting cells, on the M cells. This was done by preincubation of dissected NALT tissue with monoclonal mouse-anti rat Ia-marker (clone Mas 029c, Seralab, England) and protein A-15 nm colloidal gold complex. The uptake of antigens by the M cells was demonstrated through in vivo exposure to horseradish peroxidase (HRP) for 45 minutes, developed by diaminobenzidine (DAB), and HRP-5 nm colloidal gold complex for 5 minutes.

The selective staining for Ia-marker on the non-ciliated M cells, the DAB staining in and around these cells and the presence of gold particles in the vesicles of the cells, are suggestive of M cells being the antigen uptake and presenting cells. HRP-gold particles were not found in connection with the intraepithelial lymphocytes, possibly due to the short time of exposure.

References

1. Spit, B.J., E.G.J. Hendriksen, J.P. Bruyntjes & C.F. Kuper, 1988. Cell Tissue Res., in press.
2. Owen, R.L., 1977. Gastroenterol. 72:440-451.
3. Tenner-Racz, K., P. Racz, Q.N. Myrvik, J.R. Ockers & R. Geister, 1979. Lab. Invest. 41:106-115.
4. Brugge-Gamelkoorn, G.J. v.d., M. v.d. Ende & T. Sminia, 1986. Cell Tissue Res. 245:439-444.

COMBINED CHOLINERGIC AND PEPTIDERGIC NERVE ENDINGS IN THE RESPIRATORY EPITHELIUM OF THE RAT NASAL SEPTUM

B.J. Spit and E.G.J. Hendriksen

TNO-CIVO Toxicology and Nutrition Institute, Zeist, Netherlands

In a previous abstract (1) we reported about nerve endings in the respiratory epithelium of the rat nasal septum. It was shown that clear vesicles 40 nm in diameter and dense core granules occur in both the terminal boutons ending just below the tight junction and in axon bulges (boutons en passant) in the plane of the basal lamina.

Now we report the results obtained with specific staining and immuno-gold labelling techniques in relation to the nature of the clear vesicles and dense core granules. First synaptic membrane densities could not be detected with the alcoholic phosphotungstic acid technique, in contrast to brain tissue which reacted positively with this technique.

Specific acetylcholinesterase activity could be demonstrated with the Kasa and Csillik technique (2). Activity of this enzyme was present in the endoplasmatic reticulum and nuclear envelope of the basal cell, in the apical endoplasmatic reticulum of the ciliated cell, focally in the space between basal axons', and in the space between terminal bouton and ciliated cell.

Substance P and vasoactive intestinal peptide (VIP) were tested with immuno-gold labelling on Lowicryl K4M embedded epithelium tissue. Granula in the basal axons were positive for substance P.

It is concluded that: 1. the basal cell in the respiratory epithelium is functionally more than a resting undifferentiated cell waiting for waking up to divide, 2. groups of ciliated and goblet cells seem to be innervated.

References

1. Spit, B.J. & E.G.J. Hendriksen, 1986. Ultramicroscopy 19:102-103.
2. Kasa, P. & B. Csillik, 1966. J. Neurochem. 13:1345-1349.

HISTOPATHOLOGICAL EFFECTS OBSERVED IN RAT NASAL EPITHELIUM IN TWO 3-DAY INHALATION STUDIES WITH FORMALDEHYDE, ACETALDEHYDE, ACROLEIN, AMMONIA, AND A MIXTURE OF FORMALDEHYDE, ACROLEIN, AND AMMONIA, RESPECTIVELY

A. Teredesai and W. Stinn

INBIFO Institut für biologische Forschung, Cologne, FRG

The histopathological effects on the nasal epithelium of male Sprague Dawley rats following 3 consecutive days of inhalation (7 hours/day) of formaldehyde (0.2, 0.8, and 3 ppm), acetaldehyde (1, 5, and 21 ppm), acrolein (0.1, 0.4, and 1.7 ppm), and ammonia (6, 26, and 102 ppm) as well as mixtures of formaldehyde, acrolein, and ammonia (0.4, 0.4, 34; 0.9, 0.8, 75; 1.9, 1.8, 158 ppm) were investigated. The medium and the high concentrations of acrolein alone corresponded to the low and the high concentrations of acrolein in the mixture. Alone, none of the concentrations of formaldehyde, acetaldehyde, and ammonia caused histopathological changes; however the high concentration of acrolein alone caused the ulceration of the respiratory epithelium in 4 of 10 rats as well as reserve-cell hyperplasia and squamous metaplasia without cornification of the respiratory epithelium and atrophy of the olfactory epithelium in all rats. The incidence of these findings was lower in the posterior than in the anterior nasal passage. Ulceration did not occur in the posterior nasal passage. Although the histopathological changes caused by the high concentration mixture were of the same type as those caused by the high concentration of acrolein alone, the incidence of ulceration was higher for the former. There was no difference with respect to the incidence of reserve-cell hyperplasia and squamous metaplasia. Furthermore, whereas the medium concentration (0.4 ppm) of acrolein alone did not cause any changes, at the same acrolein concentration (0.4 ppm) in combination with formaldehyde and ammonia reserve-cell hyperplasia and squamous metaplasia were observed.

PATHOLOGY AND CELL PROLIFERATION IN THE RESPIRATORY TRACT OF THE RHESUS MONKEY EXPOSED TO FORMALDEHYDE GAS

T.M. Monticello, K.T. Morgan, J.I. Everitt and J.A. Popp

Chemical Industry Institute of Toxicology, Research Triangle Park, NC, USA

Formaldehyde (HCHO) is a nasal carcinogen in rats but it remains to be determined what cancer risk this chemical poses in humans. The present study was designed to characterize formaldehyde injury in the respiratory tract of non-human primates. Nine rhesus monkeys (Macaca mulatta) were divided into 3 groups of 3 animals/group. Group 1 (control) was sham exposed to 0 ppm HCHO for 6 hrs/day, 5 days/wk for 6 weeks. Groups 2 and 3 were exposed to 6 ppm HCHO, 6 hrs/day for either 1 week (i.e. 5 days), or 6 weeks (5 days/wk), respectively. Following the last HCHO exposure, animals were pulse labeled with tritiated thymidine and labelling indices (LI) were determined from histoautoradiograms. Lesions in the respiratory epithelium (mild degeneration and early squamous metaplasia) were most extensive in the nasal passages, minimal in the trachea and major bronchi, while the maxillary sinuses and lungs had not response to HCHO exposure. The percent surface area of the nasal passages with HCHO-induced lesions increased significantly from 1-week to the 6-week exposure group (24% $\pm$ 3%, 43% $\pm$ 3%, respectively; $p < 0.01$). Following 1 week of exposure, the respiratory epithelium of the nasal passages had the greatest elevation in LI (> 8-fold over controls), which remained significantly elevated following six weeks of exposure (> 13-fold). In specific locations of the nasal passages (e.g. middle turbinate), HCHO-induced lesions were associated with increases in cell proliferation rates up to 18-fold over controls. The LI of the larynx, trachea and carina increased with increased time of exposure. No elevations in LI were found in the maxillary sinuses or respiratory bronchioles. The absence of any response in the maxillary sinuses suggests that combining tumors of the nasal cavity and sinuses in epidemiologic studies may not be appropriate for HCHO cancer risk assessment. This study demonstrates differences in the distribution of HCHO-induced lesions between the rat and monkey. Such differences should be considered when using data derived from inhalation studies in rats for estimation of risks to humans.

SYNERGISTIC AND ANTAGONISTIC INTERACTIONS OF OZONE AND FORMALDEHYDE ON THE CELL PROLIFERATION IN RAT NASAL EPITHELIUM

J.W.G.M. Wilmer[1], W.R. Leeman[1], A. Zwart[1], P.G.J. Reuzel[1], V.J. Feron[1] R.A. Woutersen[1] and P.J.A. Rombout[2]

[1]TNO-CIVO Toxicology and Nutrition Institute, Zeist, Netherlands
[2]National Institute of Public Health and Environmental Protection, Bilthoven, Netherlands

Interactions of ozone and formaldehyde vapour on the cell proliferation of rat nasal epithelium were studied by exposing male Wistar rats to different combinations of ozone and formaldehyde for 22 h/day during 3 days. Cell proliferation was determined in the maxillary and nasal turbinates, lateral wall and septum by labelling with [^{3}H-methyl]thymidine at two levels in the nose, the transitional zone from squamous to respiratory epithelium (level II) and at the cross level of the organ of Jacobson (level III). Two exposure schemes were used: 1) 0.4 ppm ozone with 0.3, 1.0 and 3.0 ppm formaldehyde and 2) 1.0 ppm formaldehyde with 0.2, 0.4 and 0.8 ppm ozone. At level II, 0.4 ppm ozone with 0.3 and 1.0 ppm formaldehyde showed a decrease in cell proliferation in the maxillary and nasal turbinates and the lateral wall, when compared with 0,4 ppm ozone exposure alone. At these concentrations no effects were seen in the septum. 0.4 ppm ozone with 3.0 ppm formaldehyde showed a more than additive cell proliferation in the maxillary and nasal turbinates and the lateral wall, but a decrease in cell proliferation in the septum, compared to 3.0 ppm formaldehyde alone (ozone alone does not effect the cell proliferation in the septum). At level III the same trends were observed. However, a more than additive increase in cell proliferation was now observed for 0.4 ppm ozone with 1.0 and 3.0 ppm formaldehyde and an increase was found in the septum. Exposure to 0.4 ppm ozone and 3 ppm formaldehyde resulted in an increase of histopathological changes at level III when compared to ozone or formaldehyde exposure alone.
Exposure scheme 2 confirmed the observations on cell proliferation.

NASAL TUMOURS IN CPB:WU (WISTAR RANDOM) RATS

R.A. Woutersen, A van Garderen-Hoetmer, H.C. Dreef-van der Meulen and V.J. Feron

TNO-CIVO Toxicology and Nutrition Institute, Zeist, Netherlands

An increasing number of chemicals appears to be capable of inducing nasal tumours in rodents. In our laboratory, the Cpb:WU (Wistar random) rat has been used in long-term inhalation studies carried out since 1972. For the present survey data were collected from 10 long-term (24- to 30-month) toxicity/carcinogenicity studies with 7 different compounds, and from one 12-month inhalation study with vinyl chloride monomer. The Cpb:WU rats are from a closed colony, bred at random under SPF-conditions.

The nose was processed at 4 or 6 standard cross levels, sectioned at 5 μm and stained with haematoxylin and eosin. A few tumours were examined by electron microscopy using ultrathin sections stained with uranyl acetate and lead citrate.

The incidence of nasal tumours in untreated male controls was 1.1% (7/661), the tumours invariably being squamous cell carcinomas. In untreated female controls no nasal tumour was found. Occasionally, squamous cell carcinomas were seen in association with necrotizing inflammation of an incisor tooth as part of the "malocclusion syndrome".

The following types of compound-induced nasal tumours were found: (i) squamous cell carcinoma induced by formaldehyde and acetaldehyde, (ii) carcinoma in situ induced by acetaldehyde, (iii) polypoid adenoma induced by formaldehyde, (iv) adenocarcinoma of the olfactory epithelium induced by vinyl chloride, trichlorobutene or acetaldehyde, (v) carcinosarcoma induced by vinylchloride, and (vi) Schwannoma induced by trichlorobutene. Electron microscopical studies performed on acetaldehyde-induced adenocarcinomas pointed to the basal cells of the olfactory epithelium as the cell of origin of these tumours. We support Rivenson's suggestion to classify all tumours originating from the olfactory epithelium as neuroepitheliomas. Further subclassification of the neuroepitheliomas could then be based on the prevailing type of tumour cells.

CHLOROACETANILIDE INDUCED ONCOGENICITY IN THE NASAL MUCOSA OF RATS

D.P. Ward, R.C. Dirks and T.W. Fuhremann

Monsanto Agricultural Company, St. Louis, MO, USA

A series of structurally similar chloroacetanilide herbicides has demonstrated the potential to produce neoplastic responses in the nasal mucosa of rats. These lesions are generally microscopic in size, usually benign and appear to arise de novo, predominantly from the respiratory epithelium of the dorsal turbinate. They are characterized by rows and swirls of crowded but typical appearing columnar epithelial cells and are often crowned with cilia. The appearance of these tumours in rats is observed after chronic, high level oral exposure to these chemicals. These tumours have not been observed in mice under similar conditions of exposure. None of the chloroacetanilides tested have demonstrated appreciable or consistent genotoxic activity; however the specific mechanism of oncogenicity is unknown. Human exposure to the commercially available preparations of these herbicides is low; thus adequate margins of safety exist. Lifetime excess cancer risk for exposed persons is predicted to be less than 1×10^{-6} (one in one million) by the linearized multistage quantitative risk assessment model. Recent investigations on species differences in the metabolism and elimination of one of these chemicals, suggest that nasal tumours are not relevant for predicting human cancer risk.

LONG-TERM INHALATION STUDY AT LOW DOSE LEVELS OF N-NITROSODIMETHYLAMINE AND SO_2/NO_x IN RATS

R.G. Klein, I. Janowsky, B.L. Pool, P. Schmezer and W.J. Zeller

German Cancer Research Center, Heidelberg, FRG

A long term inhalation study is being performed in order to assess the biological activities of the air pollutants SO_2 and NO_x and of the carcinogen N-nitrosodimethylamine (NDMA) in the rat. It is the aim of the studies to elucidate various local and systemic effects of the air pollutants in relevant concentrations, as well as the carcinogenic activity of low NDMA doses, following the inhalative mode of application. Finally the effects of SO_2 and NO_x on the NDMA induced carcinogenesis are being analysed.

In a preceding dose-response study investigating 3 dose levels of NDMA alone, at the highest concentration of 1 ppm (4 hours per day, 5 days per week) we observed nasal tumours in more than 50% of the animals. The first nasal tumour in this group was found 7 months after starting the experiment (total dose: 6 mg/animal, 20 mg/kg). All rats of this group have died already (18 months after starting inhalation), while more than 50% of the animals in the other groups are still alive. Until now 2 nasal tumours occurred in the group with 0.2 ppm NDMA in the breathing air (total dose 8 mg/kg). No tumours could be observed in the lowest dosage group with 0.04 ppm NDMA (total dose 2 mg/kg) until now.

The actual nitrosamine concentration was determined analytically throughout the administration period. The mean breathing volume of the animals during inhalation was measured within each group and was the basis for calculating the NDMA uptake.

It appears that with lower nitrosamine concentrations, the nasal tumours are localized more in the front regions of the nose. Also a change from the neurogenic type to the mucoepidermoid type is being observed on histopathological examination. Squamous cell carcinoma was not detected until now. Only one tumour of the lung (small cell carcinoma) and one adenoma of the trachea were found at the 1 ppm level.

Experiments investigating the combination of SO_2 or NO_x and NDMA were started after the occurrence of the first nasal tumour following inhalation of 1 ppm NDMA alone. The dosage of NDMA used for the combination is 0.2 ppm (SO_2, NO_x 6 ppm, respectively). Up to now no tumours were observed in this experiment (12 months of administration, mean total dose of NDMA: 6 mg/kg body weight).

NASAL EPITHELIAL CHANGES IN FORMALDEHYDE EXPOSED WORKERS

M. Boysen[1], E. Zadig[1], V. Digernes[2], V. Abeler[3] and A. Reith[4]

[1]The National Hospital, [2]Dyno Industries Ltd., [3]Institute for Cancer Research, [4]The Norwegian Radium Hospital, Oslo, Norway

Formaldehyde induces squamous cell carcinoma of the nasal cavity in rats, but the extent of the carcinogenic risk of formaldehyde exposure in humans has not yet been defined. In addition to the carcinomas the experimental studies showed a high incidence of epithelial metaplasia and dysplasia and these epithelial changes occurred at a level of exposure often reached in occupational settings. Nasal epithelial dysplasia has almost exclusively been demonstrated in occupational settings with a known increased incidence of nasal carcinomas and considerable evidence exists supporting the concept that nasal dysplasia represents a pre-cancerous lesion. The present study was undertaken in order to evaluate the histological changes, in particular the possible presence of dysplasia, in formaldehyde exposed workers.

The material included 37 workers exposed to formaldehyde for more than three years and 37 age matched referents without exposure to known or suspected nasal carcinogens. Histological evaluation showed that formaldehyde exposed workers had a higher degree of metaplasia when compared to the referents and that the severity of epithelial changes increased with duration of exposure. Three cases of dysplasia were seen among the formaldehyde exposed.

Presence of nasal epithelial dysplasia indicates that formaldehyde is carcinogenic to humans. Combination of this finding with the inconclusive evidence from epidemiological studies suggest that formaldehyde is a weak carcinogen and that occupational exposure to formaldehyde alone, is insufficient to induce nasal cancer. Formaldehyde may, however, enhance the effect of more potent carcinogens.

DEPOSITION OF ULTRAFINE AEROSOLS IN F-344 RAT NASAL MOLDS

Y.S. Cheng[1], G.K. Hansen[1], Y.F. Su[1], H.C. Yeh[1] and K.T. Morgan[2]

[1] Lovelace Inhalation Toxicology Research Institute, Albuquerque, NM, USA
[2] Chemical Industry Institute of Toxicology, Research Triangle Park, NC, USA

Determination of regional respiratory tract deposition of inhaled materials is critical for the evaluation of health effects of air pollutants. Information is available on deposition of larger particles (>0.02 μm) in the nasal passages of laboratory animals, with the deposition fraction increasing with increasing particle size. Little information is available for ultrafine particles. Molds (models) were prepared from replica casts of the nasal passages of F-344 rats, using clear casting plastic. Total deposition of ultrafine aerosols in these molds was then determined using a unidirectional flow system. Measured pressure drops in the molds were a function of flow rate to the power of 1.4 to 1.6, indicating that flow through the molds was not laminar. Deposition data was obtained from these molds, using monodisperse sodium chloride aerosols with particle sizes ranging from 0.2 to 0.005 μm, at inspiratory and expiratory airflow rates of 200 to 600 ml/min. Similar deposition data were obtained for the two molds studied. The deposition efficiency decreased with increasing aerosol diameter and airflow rate, indicating that diffusion was the dominant mechanism. At an inspiratory flow rate of 400 ml per minute, which is comparable to a respiratory minute volume of 200 ml per minute for mature male F-344 rats, deposition efficiences reached 25 and 60% for 0.01 and 0.005 μm particles, respectively. Turbulent diffusion was considered to be the dominant mechanism for deposition of ultrafine particles in the nasal passages. This information is important for understanding the toxicity and carcinogenicity of submicrometer particles, including diesel soot, radon progeny and vapours. (Research conducted for US DOE, Office of Health and Environmental Research under contract No. DE AC04 76EV01013, and CIIT).

STUDIES ON THE TISSUE DISTRIBUTION, METABOLISM AND ELIMINATION OF THE HERBICIDE ALACHLOR IN DIFFERENT SPECIES.

A.G.E. Wilson and J.M. Malik.

Monsanto Agricultural Company, St. Louis, MO, USA

Alachlor, the active ingredient in Lasso® herbicide, has been shown to produce nasal tumours in the Long Evans rat. In contrast, alachlor is not oncogenic in the CD-1 mouse. Monsanto has conducted an extensive program aimed at understanding this species difference in toxicological response and to better define the risk to humans under various conditions of exposure. The results of these studies, which were designed to investigate the metabolism and pharmacokinetics of alachlor in rats, mice and non-human primates, will be discussed along with the relevance of these findings to man. Significant differences in sites of tissue localization and pharmacokinetics were observed between the three species. For example, whole-body autoradiography studies demonstrated a high degree of localization of alachlor-derived material in the nasal turbinates of the rat, but not in mice or monkeys. Pronounced differences were also apparent in the routes and rates of elimination and the nature of the metabolic biotransformation of alachlor in the rat, mouse and rhesus monkey. The metabolism of alachlor in man appeared similar to the rhesus monkey and dramatically different from the rat or mouse. The results of *in vitro* metabolic studies with nasal tissue will also be discussed. These studies support the view that the metabolism of alachlor by the rat is highly tissue and species specific and is not relevant for extrapolation to man.

XENOBIOTICS-METABOLIZING ENZYMES IN NASAL MUCOSA OF HUMANS

P.G. Gervasi, R. Fiorio, V. Longo and P. Puccini

Istituto di Mutagenesi e Differenziamento C.N.R., Pisa, Italy

The presence in nose, both in olfactory and in respiratory regions, of xenobiotics metabolizing enzymes has been reported in many mammalian species but no data are available for nasal human enzymes. Therefore we have investigated the metabolic capacity of respiratory nasal tissue of several human specimens obtained by surgical operations from patients affected by hypertrophy of inferior turbinates. Human nasal microsomes and 100,000xg supernatant fractions were prepared and microsomal P-450 content, NADPH-cytochrome c-reductase activity, aminopyrine N-demethylase, ethoxycoumarin O-deethylase, ethoxyresorufin O-deethylase, pentoxyresorufin O-depenthylase, hexamethylphosphoramide N-demethylase, UDP-glucoronyltransferase, glutathione S-transferase, epoxide hydrase, and DT-diaphorase activities were determined.

The oxidative and the non-oxidative enzymic activities occur in all nasal respiratory human samples showing few differences among them. The only exception is the complete lack of UDP-glucoronyltransferase activity in all specimens.

Furthermore, human nasal microsomes metabolized very well the carcinogenic diethylnitrosamine with specific activities similar or higher to those reported for human liver.

These data point to the potential importance of human nose for the metabolic activation, and thereby the toxicity and carcinogenicity of inhaled xenobiotics.

METABOLISM OF AFLATOXIN B_1 IN THE BOVINE OLFACTORY MUCOSA

P. Larsson and H. Tjälve

Faculty of Veterinary Medicine, the Swedish University of Agricultural Sciences, Uppsala, Sweden.

Carcinomas of the ethmoidal region of the nose are observed relatively frequently in cattle in several countries of the Third World (1,2). Viruses have been implicated as causative agents. However, it has been observed that affected animals sometimes suffer from aflatoxicosis, and a role of aflatoxin B_1 (AFB_1) in the etiology has also been proposed (2). Nasal tumours have been observed in sheep which are given AFB_1 experimentally (3).

We have examined whether the bovine nasal olfactory mucosa has a capacity to metabolize AFB_1. The contents of cytochrome P-450 and cytochrome b_5, and the NADPH cytochrome c reductase activity in the nasal olfactory mucosa have also been determined. Comparative experiments have been performed with the liver.

Incubation with ^3H-labelled AFB_1 showed that the nasal olfactory mucosa has a much higher capacity than the liver to form lipid-soluble, water-soluble and tissue-bound AFB_1-metabolites.

High-resolution microautoradiography showed a preferential localization of tissue-bound metabolites in Bowman's glands in the olfactory lamina propria mucosa and in some cells in the apical portion of the olfactory surface epithelium. Bowman's glands have been considered to undergo the initial malignant transformations in the nasal tumourigenesis in cattle (1,2).

Liquid chromatography of chloroform extracts of the nasal olfactory mucosa and the liver incubated with ^3H-AFB_1 showed formation of several metabolites. The dominating peak in both tissues was aflatoxin M_1 (AFM_1). However, the amount of AFM_1 was higher in the nasal olfactory mucosa than in the liver, and the amounts and proportions of several other metabolites also differed markedly between the two tissues.

The level of cytochrome P-450 in the nasal olfactory mucosa was found to be about one fourth of that in the liver, but the NADPH-cytochrome c reductase activity was much higher in the nasal olfactory mucosa than in the liver. In addition, the cytochrome b_5:cytochrome P-450 ratio was higher in the nasal olfactory mucosa than in the liver.

The higher metabolism of AFB_1 in the nasal olfactory mucosa than in the liver in spite of a lower content of cytochrome P-450 in the former than in the latter tissue may be related to differences in the isoenzyme profile. In addition, the microsomal electron transport to cytochrome P-450 may be facilitated by the high reductase:cytochrome P-450 ratio and the high cytochrome b_5:cytochrome P-450 ratio in the nasal olfactory mucosa.

It is considered that the results of the present study strengthen the hypothesis that exposure to AFB_1-contaminated feed may be an important etiological factor in the development of nasal tumours in cattle.

References

1. Sreekumaran, T. & A. Rajan, 1983. Ind. J. Canc. 20:10-14.
2. Pospischil, A., T. Haenichen & H. Schaeffler, 1979. Vet. Pathol. 16:180-190.
3. Lewis, G., L.M. Markson, & R. Allcroft, 1967. Vet. Rec. 80:312-314.

EFFECTS OF METHYL BROMIDE (MeBr) ON NASAL TISSUE BIOTRANSFORMATION ENZYMES

D.A. Thomas, S.A. Lacy, J.I. Everitt and K.T. Morgan

Chemical Industry Institute of Toxicology, Research Triangle Park, NC, USA

The olfactory mucosa is a well known site of chemical-induced nasal cancer. Metabolic activation and detoxification of many olfactory epithelial toxicants and carcinogens involve cytochrome P-450 (P-450)- and glutathione (GSH)-dependent biotransformation pathways. Xenobiotic-associated olfactory toxicity may also be mediated by perturbations in arachidonic acid (AA) metabolism by prostaglandin H synthetase (PHS) or lipoxygenase (LPO) enzyme systems. MeBr, which produces a selective olfactory degeneration in rats, was used to study enzyme systems potentially involved in nasal toxicity and carcinogenesis. Male F-344 rats were exposed to either 125 ppm or 200 ppm MeBr for 6 hrs. The MeBr-induced histopathologic changes in the olfactory mucosa were associated with: [1] decreased P-450-dependent alkoxy-O-dealkylation (AOD) activities, [2] decreased GSH, GSH transferase and GSH reductase activities, and [3] lower amounts of AA metabolites by the predominant olfactory LPO pathway. Pretreatment of rats with the P-450 inhibitor 1-phenylimidazole significantly decreased olfactory AOD, but had no effect on MeBr-associated toxicity. Pretreatment with indomethacin, a PHS inhibitor, also failed to protect rats against MeBr-induced olfactory damage. However, pretreatmnet with BW755c, a dual PHS and LPO inhibitor, protected rats from MeBr-mediated olfactory toxicity, prevented the MeBr-induced inhibition of GSH transferase, and produced a decrease in olfactory LPO-dependent AA metabolism. BW755c-associated inhibition of MeBr-induced olfactory toxicity is being further investigated. Studies of olfactory epithelial toxins, such as MeBr, combined with use of selected enzyme inhibitors can provide insight into the role of metabolism in olfactory toxicity and carcinogenesis.

EFFECTS OF CYTOCHROME P-450 (P-450) INHIBITORS ADMINISTERED IN VIVO ON RAT OLFACTORY MICROSOMAL ALKOXY-O-DEALKYLATION (AOD) ACTIVITIES.

S.A. Lacy, D.A. Thomas, K.T. Morgan and J.I. Everitt
Chemical Industry Institute of Toxicology, Research Triangle Park, NC, USA

Tissue-specific inhibition of selective P-450 isozymes may affect in situ metabolic activation of nasal carcinogens. To further characterize the nasal tissue P-450 system, male F-344 rats were given P-450 inhibitors α-naphthoflavone (α-NF), piperonyl butoxide (PB), 1-phenylimidazole (1-PI), metyrapone (MET), or SKF-525A at 50 mg/kg body weight (except PB at 100 mg/kg) in DMSO as a single (0.5 ml) i.p. injection. Animals were sacrificed four hours after dosing, the livers and lungs immediately perfused, and microsomal preparations made from olfactory, pulmonary and hepatic tissues. AOD activities were measured using either ethoxy (EROD)-, benzyloxy (BROD)- and pentoxyresorufin (PROD) by a direct fluorescence assay employing optimal substrate and protein concentrations for each tissue preparation. Relative AOD activities in olfactory tissue (EROD > PROD > BROD) differed significantly from that in pulmonary (BROD > PROD >> EROD) and hepatic (BROD > EROD > PROD) tissues, respectively. Olfactory tissue possessed higher EROD specific activity than either liver or lung. DMSO alone produced increases in olfactory, pulmonary, and hepatic AOD compared to saline controls. Olfactory AOD was decreased by all inhibitor treatments and most significantly (>75%) by 1-PI. In contrast, 1-PI, PB, and α-NF induced pulmonary EROD whereas MET, α-NF and PB enhanced hepatic BROD and PROD. Only SKF-525A produced AOD inhibition in all tissues. Thus, P-450 inhibitors administered in vivo produced differential and organ-specific effects on P-450-dependent AOD activities.

A SELECTIVE TOXIN FOR OLFACTORY TISSUE: INTERACTION WITH CYTOCHROME P-448

M.S. Skinner, J. Jenner and G.H. Dodd

Olfaction Research Group, University of Warwick, Coventry, UK

It has recently been shown (1) that there are differences in the cytochrome P-450 populations between hepatic and olfactory tissue.

3-Trifluoromethyl pyridine (TF) has recently been shown to be selectively toxic to olfactory tissue (2).

We have found that TF inhibits olfactory cytochrome P-448-dependent metabolism (possibly by acting as a competitive substrate), but not the corresponding hepatic activity. TF does not appear to inhibit cytochrome P-450 dependent metabolism in either tissue.

The effects of TF analogs on olfactory cytochrome P-448- and P-450-dependent mono-oxygenase activities are reported.

The selective inhibition reported here constitutes a possible test for specific olfactory toxins and pre-toxins (3).

References

1. Jenner, J. & G.H. Dodd, 1988. Biochem. Pharmacol., 37:558-559.
2. Gaskell, B.A., P.M. Hext, G.H. Pigott, M.C.H. Hodge & D.J. Tinston, 1988. Toxicol., in press.
3. Patent Application Number 8816853.9 (July) 1988.

GLUTATHIONE-DEPENDENT PARAMETERS OF DETOXIFICATION AND THEIR MODIFICATION BY FORMALDEHYDE AND ACROLEIN IN THE NASAL EPITHELIA OF THE RAT

H.J. Haussmann and R.A. Walk

INFIBO Institut für biologische Forschung, Cologne, FRG

The protein-based content of glutathione (reduced plus oxidized) in the nasal respiratory and olfactory epithelia of unexposed rats was found to exceed that in the lungs and the trachea by a factor of 2. After a 4-hour inhalation of acrolein (1 to 15 ppm), a dose-dependent decrease was observed in these tissues and organs. A 50% decrease of glutathione in both nasal tissues was observed after the exposure of the rats to 4 ppm acrolein. At 3, 9 and 24 hours after a single i.p. administration of acrolein or formaldehyde in sublethal doses, no effect on the glutathione content in the tissues was observed.

Three enzyme activities were determined in the postmitochondrial supernatant fractions, i.e. glutathione peroxidase, glutathione reductase and glutathione-S-transferase. In the unexposed rats the glutathione-S-transferase activity was found to be 10-fold higher in the nasal tissues than in the trachea and the lungs. After the single inhalation of acrolein, there was a slight increase in the activities of the 3 enzymes in the olfactory epithelium, whereas in the nasal respiratory epithelium, enzyme activities tended to decrease as acrolein concentrations increased. After the i.p. administration of acrolein or formaldehyde, only minor changes of the nasal enzyme activities were found. In the lungs, a stimulation of glutathione-dependent enzyme activities was found to occur after either inhalation or i.p. administration.

ON THE MECHANISM OF FORMATION OF A DNA ADDUCT, N^6-HYDROXYMETHYLDEOXYADENOSINE (hm6dA), BY FORMALDEHYDE (HCHO)

M. Casanova and H. d'A. Heck

Chemical Industry Institute of Toxicology, Research Triangle Park, NC, USA

Although HCHO forms DNA-protein cross-links *in vitro* and *in vivo*, no hydroxymethyl adducts were detected in hydrolyzates of DNA from the rat nasal respiratory mucosa after exposure to $H^{14}CHO$. In contrast, an adduct, hm6dA, was detected in hydrolyzates of DNA from Chinese hamster ovary (Cho) cells incubated with ^3HCHO (1). Experiments in this laboratory have shown that DNA-protein cross-links formed by HCHO are unstable to enzymatic hydrolysis of the DNA and dissociate, releasing free HCHO. The released HCHO could react with deoxyribonucleosides in the hydrolyzate to form hydroxymethyl adducts. To investigate whether the latter mechanism can explain the detection of hm6dA, a mixture of the four major deoxyribonucleosides was incubated with $H^{14}CHO$ (1 μM) in the tertiary amine buffer, *bis*-Tris, which was used to hydrolyze the DNA from Cho cells. All of the deoxyribonucleosides formed hydroxymethyl adducts in this buffer, which were well-separated from the parent compounds by HPLC. No adducts were produced when $H^{14}CHO$ was incubated with the deoxyribonucleosides in the primary amine buffer, Tris, which was used to hydrolyze the DNA from the rat nasal mucosa, due to preferential reaction of $H^{14}CHO$ with the amino group of the buffer. The yield of hm6dA obtained in *bis*-Tris buffer, adjusted for small differences in reactant concentrations, was not significantly different from the quantity of hm6dA detected in DNA hydrolyzates from Cho cells. These results suggest strongly that the hm6dA detected in hydrolyzates of DNA from Cho cells was produced *in the hydrolyzates* by reaction of released HCHO with deoxyadenosine. Caution is warranted when interpreting evidence for adduct formation by HCHO, because hydroxymethyl adducts are bound reversibly to deoxyribonucleosides.

Reference

1. Beland, F.A., M.F. Fullerton & R.H. Heflich, 1984. J. Chromatogr. 308:121-131.

IMMUNOCYTOCHEMICAL IDENTIFICATION OF DNA METHYLATION BY 4-(METHYL-NITROSAMINO)-1-(3-PYRIDYL)-1-BUTANONE (NNK) IN NASAL TISSUES OF RAT AND HAMSTER

J. van Benthem[1], J.W.G.M. Wilmer[2], W.R. Leeman[2], H.H.K. Winterwerp[1], L. den Engelse[1] and E. Scherer[1]

[1] The Netherlands Cancer Institute, Amsterdam, Netherlands; [2] TNO-CIVO Toxicology and Nutrition Institute, Zeist, Netherlands

The tobacco-specific 4-(methylnitrosamino)-1-(3-pyridyl)-1- butanone (NNK), is a potent carcinogen in experimental animals. Metabolic activation is required for the alkylation of DNA bases by NNK.

The cellular heterogeneity of NNK-induced DNA methylation in rat and hamster nasal tissue was investigated using a sensitive immunoperoxidase staining technique. Rats received a single i.p. dose of 30 mg/kg NNK, golden hamsters 4 s.c. doses of 10 mg/kg NNK each. Nasal tissues (ethmoid, nasal and maxillary turbinate) were imbedded in plastic and sections stained for O^6- or 7-methylguanine.

In rats as well as in hamsters, nuclear staining, specific for O^6- and 7-methylguanine, was particularly strong in the cells of Bowman glands. In rats a weak DNA modification was found in the sustentacular and basal cells of the olfactory epithelium, whereas in hamsters these adducts could be demonstrated in the nuclei of sensory cells. Both adducts were also found in the respiratory epithelium. In hamsters, modified DNA was also seen in the cells of serous glands where staining was rarely found in the rat.

The immunocytochemical approach used in the present experiments permits to study DNA-adducts at single cell level. Detailed knowledge can be obtained on the adduct distribution and repair in different cell types of a tissue and among functionally different cells of a certain cell type.

(Thanks are due to Drs. P. Kleihues and R. Montesano for their generous gift of the antisera against O^6- and 7-methylguanine, respectively.)

DNA ALKYLATION IN RAT NASAL TISSUES BY A SERIES OF 12 N-METHYL-ALKYLNITROSAMINES

A.P.M. Wijnakker[1], B. Lüdeke[2], P. Kleihues[2], E. Scherer[1] and J. van Benthem[1]

[1] The Netherlands Cancer Institute, Amsterdam, Netherlands; [2] University of Zürich, Zürich, Switzerland

In rats, asymmetric N-nitrosamines show a high degree of organ-specificity. In the present experiment the effect of a series of 12 N-nitroso-N-methyl-alkylamines (NMA) ranging from N-nitroso-dimethylamine (C_1), N-nitroso-methyl-ethylamine (C_2), etc. to N-nitroso-methyl-dodecylamine (C_{12}) on rat nasal tissue was investigated. O^6- and 7-alkylguanine formation in individual cells has been studied immunocytochemically on cryostat tissue sections of C_1-C_{12} treated rats. The intensity of the immunoperoxidase staining reaction was determined by computer-assisted scanning microdensitometry.

Six hours after a single i.p. dose (0.1 and 0.5 mmol/kg) of any of the 12 compounds (C_1-C_{12}) dose-dependent nuclear staining specific for O^6- or 7-alkylguanine could be demonstrated. After NMA with a long alkyl chain (hexyl or longer), DNA adducts were only found in the Bowman glands located in the olfactory submucosa. However, the strongest nuclear staining of this cell type was found after treatment with C_2 and C_4. In the olfactory epithelium, particularly the sustentacular cells, the strongest DNA modification was also found after treatment with these compounds. DNA damage in the respiratory epithelial and glandular cells (serous glands) was especially strong after treatment with C_3, C_4 and C_5.

This study shows highly divergent capacities of individual cells of nasal tissues to activate NMA. The short chain NMA result in the strongest DNA damage, which roughly parallels the tumour formation as reported by Lijinsky (1). Due to the especially high damage, cells of Bowman glands and of respiratory epithelium seem particularly at risk as tumour precursor cells.

(Thanks are due to Dr. R. Montesano for the generous gift of the antiserum against 7-methylguanine.)

Reference

1. Lijinsky, W., 1987. Cancer Metastasis Rev. 6:301-356.

CHROMOSOME ABERRATION (CA) AND SISTER CHROMATID EXCHANGE (SCE) ANALYSIS IN CULTURED PRIMARY RAT NASAL AND TRACHEAL EPITHELIAL (RNE, RTE) CELLS

D. Bachmayer and W.K. Schlage

INBIFO Institut für biologische Forschung, Cologne, FRG

To investigate biological effects of inhalable materials, assays with the primary target cells derived from the respiratory epithelia are especially useful. Here, a new approach is presented to assess SCE and CA in RNE and RTE cells exposed *in vitro* or *in vivo*. Epithelial cells were detached from the nasoturbinates using a mixture of protease type XIV, collagenase, and hyaluronidase, and from the trachea using protease type XIV. Buffering the enzyme solution with Hepes was found to increase the survival of RNE cells *in vitro*. A short trypsinization of freshly isolated RNE and RTE cells to separate cell aggregates increased cell survival and proliferation *in vitro*. RNE as well as RTE cells proliferated on glass slides, the preferred substrate for *in situ* chromosome spreading. RNE cell growth was best supported by serum-free Ham's F12 medium supplemented with various growth factors, and RTE cell growth by a mixture of serum containing Ham's F12 and 3T3-conditioned medium. At an optimal seeding density there was a sufficient number of metaphases for CA analysis after a 30-hour *in vitro* culture following an attachement period of 24 hours for RNE and 20 hours for RTE cells. BrdU concentrations higher than 1.6 µmol BrdU/l were cytotoxic. However, a 50-hour labelling period with 1.6 µmol BrdU/l did enable sister chromatid differentiation and a sufficient number of metaphases for SCE analysis. Under these conditions approx. 50 percent of the metaphases exhibited sister chromatid differentiation. This method was applied to primary RNE and RTE cells exposed *in vitro* to formaldehyde and acrolein.

IN VIVO - IN VITRO MUTATION ASSAYS FOR INHALATION EXPOSURES WITH RAT NASAL EPITHELIAL CELLS

A. Timm, A. Poth and H.G. Miltenburger

Technische Hochschule Darmstadt, Darmstadt, FRG

Nasal epithelial cells (NEC) are very suited for investigating the pathogenic potential of toxic agents which might be present in the air. To evaluate the health risks produced by such agents e.g. formaldehyde (FA) quantitative data on the reaction of NEC are needed. The pervasive use in domestic products and industrial processes has generated considerable interest in the toxicology of FA. A number of investigators has demonstrated FA-induced mutagenesis and carcinogenesis in animals after exposure to relatively high inhalation concentrations in the breathing air. However, there are also reports showing no mutagenic effect after FA-treatment. Also with low concentrations the NEC are at first hand and always exposed to FA. A major damage exerted by FA is the inhibition of the nasal mucociliary function in exposed people and animals. Due to this damage the dysfunction of the cilia layer involves potentially a genotoxic damage to the epithelial cells. Therefore, we studied the genotoxic effect of FA with NEC by using and adjusting several mutagenicity assays in rats after inhalation exposures. The mutagenicity assays were the micronucleus assay (MN), the chromosome aberration assay (CA), and the sister chromatid exchange assay (SCE) and for gene mutations the HGPRT assay. In order to develop mutagenicity testing methods with NEC we started with standard mutagens. Several test procedures were employed to find out suitable test protocols.

Intraperitoneally administered MMS was used as mutagen in the MN assay. It increased significantly the frequency of micronuclei as compared to the negative controls. After treatment with 100.0 mg/kg b.w. (non-toxic dosage) 3.2% cells with micronucelei were found in comparison to 0.3% cells with miconucelei in the negative control group.

In the SCE assay the effect of an in vivo treatment of rats with ENU administered intraperitoneally was compared with that found after EMS in vitro treatment of explanted rat NEC. Both mutagens induced an increase of the SCE rate: ENU, 60.0 mg/kg b.w. and EMS 0.31 mg/ml caused a 2.1 and a 2.7-fold increase, respectively, as compared to the corresponding negative control group.

In the HGPRT assay ENU again induced mutations. After i.p. treatment of rats with 60.0 mg/kg b.w. the mutation frequency in primary rat NEC cultures was increased at least by a factor of 13 and up to a factor of 38 as compared to the negative controls.

After these studies for establishing the mutation scoring method in NEC an inhalation study was performed with FA. In order to quantify the effect of low FA concentrations (e.g. 1.0 ppm) we started with a high concentration of 20 ppm. Rats were exposed to 20 ppm FA for 6 hours on 5 days, Monday to Friday. Immediately after the end of the exposure period NEC were prepared and after a culture time of three days analysed for structural chromosome aberrations. We could detect an increase of the chromatid breaks and exchanges as compared to the negative controls: 14% cells with aberrations exclusive gaps and 11% cells with exchanges in FA-exposed NEC as compared to 1% and 0% in the negative control group. The values were obtained in two independent experiments each with 4 animals in the negative control and FA-exposed group. 100 metaphases of each animal

were scored.

In conclusion, the NEC test systems are now established for scoring chromosome and gene mutations.

LIST OF PARTICIPANTS

* = Speaker ** = Chairman

D. Alexander
Glaxo Group Research Ltd.
Park Road, Ware, N/A
WARE, Herts, SG12ODJ
UK

D. Backmayer
INBIFO Institut für biologische Forschung
Fuggerstrasse 3
5000 Cologne 90
FRG

S. Belinsky *
National Institute of Environmental Health Sciences
P.O. Box 12233, MD D4-04
RESEARCH TRIANGLE PARK, NC 27709
USA

J. van Benthem
Netherlands Cancer Institute
(Antoni van Leeuwenhoekhuis)
Plesmanlaan 121
1066 CX AMSTERDAM
Netherlands

T.J. van Bergen
International Flavor & Fragrances
P.O. Box 309
1200 AH HILVERSUM
Netherlands

B.H. Bibo
AKZO Research
P.O. Box 9300
6800 SB ARNHEM
Netherlands

E. Boelsma – van Houte
Shell Internationale Petroleum Maatschappij BV
P.O. Box 162
2501 AN 's GRAVENHAGE
Netherlands

M.S. Bogdanffy *
E.I. Du Pont de Nemours & Comp.
P.O. Box 50, Elkton Road
NEWARK, DE 19714
USA

H.M. Bolt **
Institut für Arbeitsphysiologie
Ardeystrasse 67
D-4600 DORTMUND-1
FRG

G.A. Boorman *
National Institute of Environmental Health Sciences
P.O. Box 12233
RESEARCH TRIANGLE PARK, NC 27709
USA

M.C. Bosland
Institute of Environmental Medicine
New York University Medical Center
550 First Avenue
NEW YORK, NY 10016
USA

M. Boysen
Department of Otolaryngology
Rikshospitalet
University of Oslo
0027 OSLO 1
Norway

P. Brandtzaeg *
LIIPAT, Institute for Pathology
Rikshospitalet
University of OSLO
0027 OSLO 1
Norway

C.L.J. Braun
AKZO N.V.
P.O. Box 9300
6800 SB ARNHEM
Netherlands

J.N. Bremmer 1)
Shell Internationale Petroleum Maatschappij BV
P.O. Box 162
2501 AN 's GRAVENHAGE
Netherlands

A. Brito-Babapulle
Wellcome Research Labs
Langley Court
BECKENHAM, KENT
LONDON, BR3 3BS
UK

E.B. Brittebo *
The Swedish University of
Agricultural Sciences
Biomedicon, Box 573
S-751 23 UPPSALA
Sweden

T.V. McCaffrey *
Mayo Clinic
200 First Street SW
ROCHESTER, MI 55905
USA

J. Carstensen
Nordisk Gentofe A/S
1 Niels Steensenvej
DK-2820 GENTOFE
Denmark

M. Casanova
Chemical Industry Institute of
Toxicology
P.O. Box 12137
RESEARCH TRIANGLE PARK, NC 27709
USA

M.S. Cohn *
U.S. Consumer Product Safety
Commission
5401 Westbard Avenue
WASHINGTON, DC 20207
USA

L.G. Costa
Department of Environmental Health
University of Washington
P.O. Box SC-34
SEATTLE, 98195
USA

A.R. Dahl *
Lovelace Inhalation Toxicology
Research Institute
P.O. Box 5890
ALBUQUERQUE, NM 87185
USA

F. Debets
AKZO Chemicals B.V.
P.O. Box 247
3800 AE AMERSFOORT
Netherlands

J.P.A. Derks
VNCI
P.O. Box 443
2260 AK LEIDSCHENDAM
Netherlands

M. De Smedt
Commission of the European Community
DG V E/2
Bâtiment Jean Monnet
Plateau du Kirchberg
L-2920 LUXEMBOURG
Luxembourg

A. Dipple
Frederick Cancer Research Facility
National Cancer Institute
P.O. Box B
FREDERICK, MA 21701
USA

R.C. Dirks
Monsanto Company
800 N Lindberg BLvd
St. LOUIS, MI 63167
USA

D.F. Easton *
Section of Epidemiology
Institute of Cancer Research
Block D
BELMOND, Surrey SM2 5NG
UK

L. den Engelse
Netherlands Cancer Institute
(Antoni van Leeuwenhoekhuis)
Plesmanlaan 121
1066 CX AMSTERDAM
Netherlands

V.J. Feron *
TNO-CIVO Toxicology and
Nutrition Institute
P.O. Box 360
3700 AJ ZEIST
Netherlands

S.J. Garte *
New York University Medical Center
550 First Avenue
NEW YORK, NY 10016
USA

B. Gaskell
ICI Central Toxicology Lab.
Alderley Park, Macclesfield
CHESHIRE, SK104TJ
UK

C.F. Gembardt
Department of Toxicology
BASF AG
D-6700 LUDWIGSHAFEN
FRG

P. Gervasi
Instituto di Mutagenesi e
Differenziamento C.N.R.
Via Svezia, 10
56100 PISA
Italy

U.F. Gruber
Kantonsspital
University of Basel
CH-4031 BASEL
Switzerland

O. Grundler
BASF AG
P.O. Box ZST-2470
D-6700 LUDWIGSHAFEN
FRG

J.R. Harkema *
Lovelace Inhalation Toxicology
Research Institute
P.O. Box 5890
ALBUQUERQUE, NM 87185
USA

H.-J. Haussmann
INBIFO Institut für biologische
Forschung
Fuggerstrasse 3
D-5000 COLOGNE 90
FRG

H. d'A Heck *
Chemical Industry Institute of
Toxicology
P.O. Box 12137
RESEACH TRIANGLE PARK, NC 27705
USA

E.G.J. Hendriksen
TNO-CIVO Toxicology and
Nutrition Institute
P.O. Box 360
3700 AJ ZEIST
Netherlands

J. Higginson **
Georgetown University Medical Center
2121 Wisconsin Avenue NW Suite 220
WASHINGTON, DC 20007
USA

R.K. Hjortkjaer
NOVO Industri A/S
Novo Alle
DK-2880 BAGSVAERD
Denmark

G. Hoffmann
BASF AG
DOA, Ambulanz Sud
D-6700 LUDWIGSHAFEN
FRG

P. Janssen
Duphar BV
P.O. Box 2
1380 AA WEESP
Netherlands

A. Jonsson 1)
AB ASTRA
S-151 85 SODERTALJE
Sweden

M. Kaegler
INBIFO Institut für biologische
Forschung
Fuggerstrasse 3
D-5000 COLOGNE 90
FRG

A. Klaassen
Department E.N.T.
University of Nijmegen
Philips van Leydenlaan 15
6500 HB NIJMEGEN
Netherlands

R.G. Klein
Institut für Toxikologie und
Chemotherapie
German Cancer Research Institute
Im Neuenhelmer Feld 280
D-6900 HEIDELBERG
FRG

H.J. Klimisch
Department of Toxicology
BASF AG
D-6700 LUDWIGSHAFEN
FRG

E. Krieger
Cassella AG
Hanauer Landstrasse 526
D-6000 FRANKFURT AM MAIN 61
FRG

E. Kristiansen
National Food Agency
19, Morkhoj Bygade
DK-2860 SOBORG
Denmark

R. Kroes **
National Institute of Public
Health and Environmental Protection
P.O. Box 1
3720 BA BILTHOVEN
Netherlands

C.F. Kuper *
TNO-CIVO Toxicology and
Nutrition Institute
P.O. Box 360
3700 AJ ZEIST
Netherlands

S.A. Lacy
Chemical Industry Institute of
Toxicology
P.O. Box 12137
RESEARCH TRIANGLE PARK, NC 27709
USA

P. Larsson
Department of Pharmacology and
Toxicology
The Swedish University of
Agricultural Sciences
Biomedicum, Box 573
S-751 23 UPPSALA
Sweden

C.G. van der Lee
AKZO NV
P.O. Box 9300
6800 SB ARNHEM
Netherlands

W.R. Leeman
TNO-CIVO Toxicology and
Nutrition Institute
P.O. Box 360
3700 AJ ZEIST
Netherlands

D.J. Lewis
Huntingdon Research Centre
CAMBS, PE 18 6ES
UK

J.M. Malik
Monsanto Agricultural Co
700 Chesterfield Village PKWY
St. LOUIS, MI 63198
USA

I. Mattern
BASF AG
DOA, Ambulanz Süd
D-6700 LUDWIGSHAFEN
FRG

Y.J.B. van Megen *
St. Radboud Hospital
University of Nijmegen
Philips van Leydenlaan 15
6500 HB NIJMEGEN
Netherlands

B.A. Melaas
Hoechst Celanese
1250 West Mockingbird Lane
P.O. Box 569320
DALLAS, TX 75356-9320
USA

R.J. Millischer
Atochem
La Défense 10
Cedex 42
92091 PARIS LA DEFENSE
France

H.A. Milman **
U.S. Environmental Protection Agency
14317 Bauer Drive
ROCKVILLE, MA 20853
USA

T.M. Monticello
Chemical Industry Institute of
Toxicology
P.O. Box 12137
RESEARCH TRIANGLE PARK, NC 27709
USA

K.T. Morgan *,**
Chemical Industry Institute of
Toxicology
P.O. Box 12137
RESEARCH TRIANGLE PARK, NC 27709
USA

J.B. Morris *
Schools of Pharmacy
University of Connecticut
STORRS, CT 06268
USA

P. Newton
BIO/DYNAMICS INC.
P.O. Box 2360
NEW JERSEY, NJ 08875
USA

K.-H. Norpoth *
Institut für Hygiene und
Arbeitsmedizin der
Universität Essen
Huflandstrasse 55
D-4300 ESSEN 1
FRG

B. van Ommen
TNO-CIVO Toxicology and
Nutrition Institute
P.O. Box 360
3700 AJ ZEIST
Netherlands

W.E. Pepelko *
U.S. Environmental
Protection Agency
RD-689, 401 M Stree
WASHINGTON, DC 2046
USA

N. Petri
BASF AG
P.O. Box CIK/F - T 410
D-6700 LUDWIGSHAFEN
FRG

C. Pick
Glaxo Group Research
Park Road
WARE SG12 ODP
UK

A. Poth
Petersenstrasse 22
D-1600 DARMSTADT
FRG

D.F. Proctor *
School of Hygiene and Public Health
The John Hopkins University
BALTIMORE, MD 21205
USA

P.G.J. Reuzel
TNO-CIVO Toxicology and
Nutrition Institute
P.O. Box 360
3700 AJ ZEIST
Netherlands

M. Richold
Unilever Research
Colworth Lab. (ESL)
Sharnbrook
BEDFORD MK 44 1 LQ
UK

H.-B. Richter Reichhelm **
Bundesgesundheitsamt
P.O. Box 330013
D-1000 BERLIN 33
FRG

H. Roelfzema
Ministry of Social Affairs and
Employment
P.O. Box 69t SW
2273 KH VOORBURG0
Netherlands

K. Perrey
Im Höchsten 29
D-6072 DREIEICH - GÖTZENHAIN
FRG

G.M. Rusch
Allied-Signal Inc.
P.O. Box 1139 R
MORRISTOWN, NJ 07960
USA

P. Schaetti
CIBA-GEIGY AG
R-1066.304
CH-4002 BASEL
Switzerland

C. Schippers-Gillesen
Netherlands Cancer Institute
(Anthoni van Leeuwenhoekhuis)
Plesmanlaan 121
1066 CX AMSTERDAM
Netherlands

W. Schlage
INBIFO Institut für biologische
Forschung
Fuggerstrasse 3
D-5000 COLOGNE 90
FRG

E. Schraven
Cassela AG
Hanauer Landstrasse 521
D-6000 FRANKFURT AM MAIN 61
FRG

H.M. Schuller *
College of Veterinary Medicine
University of Tenessee
KNOXVILLE, TN 37901-1071
USA

M.S. Skinner
Warwick Olfaction Research Group
University of Warwick
COVENTRY CV4 7AL
UK

E. Smith
CIBA-GEIGY PLC
Hurdsfield Industrial Estate
MACCLESFIELD, Cheshire, SK10 2LY
UK

J.J. Solomon *
New York University Medical Centre
550 First Avenue
NEW YORK, NY 10016
USA

A. Somogyi **
Bundesgesundheitsamt
P.O. Box 330013
D-1000 BERLIN 33
FRG

B.J. Spit
Valkenkamp 4
3972 ZD DRIEBERGEN
Netherlands

B. Staatsen
National Health Council
P.O. Box 90517
2509 LN 's GRAVENHAGE
Netherlands

T.B. Starr *
Chemical Industry Institute of
Toxicology
P.O. Box 12137
RESEARCH TRIANGLE PARK, NC 27709
USA

L. Stayner *
National Institute Occupational
Safety and Health
Robert Taft Labs
4676 Columbia Parkway
CINCINNATTI, OH 45226
USA

P.J. Slootweg
Pathology Institute
Academic Hospital Utrecht
Pasteurstraat 2
3511 HX UTRECHT
Netherlands

A. Teredesai
INBIFO Institut für biologische Forschung
Fuggerstrasse 3
D-5000 COLOGNE 90
FRG

D.A. Thomas
Chemical Industry Institute of Toxicology
P.O. Box 2137
RESEARCH TRIANGLE PARK, NC 87709
USA

D. Ward
Monsanto Company
800 N Lindberg BLvd
St. LOUIS, MI 63167
USA

A. Timm
Laboratorium für Mutagenitätsprüfung
Technische Hochschule Darmstadt
Schnittspahnstrasse 3
D-6100 DARMSTADT
FRG

P.W. Wester
National Institute of Public Health and Environmental Protection
P.O. Box 1
3720 BA BILTHOVEN
Netherlands

H. Tjälve
Department of Pharmacology and Toxicology
The Swedish University of Agricultural Sciences
Biomedicum, Box 573
S-751 23 UPPSALA
Sweden

A.W. van der Wielen
Ministry of Housing, Physical Planning and Environment
P.O. Box 450
2260 MB LEIDSCHENDAM
Netherlands

E. Vermeulen
Netherlands Cancer Institute
(Anthoni van Leeuwenhoekhuis)
Plesmanlaan 121
1066 CX AMSTERDAM
Netherlands

A.P.M. Wijnakker
Netherlands Cancer Institute
(Anthoni van Leeuwenhoekhuis)
Plesmanlaan 121
1066 CX AMSTERDAM
Netherlands

H. Verschuuren
Dow Europe
Bachtobelstrasse 3
CH-8810 HORGEN
Switzerland

B. Wilhelmsson *
ENT-Clinic
Huddinge University Hospital
S-14186 HUDDINGE
Sweden

T. Vossenaar
Wassenaarseweg 80
P.O. Box 90606
2509 LP 's GRAVENHAGE
Netherlands

J.W.G.M. Wilmer *
TNO-CIVO Toxicology and Nutrition Institute
P.O. Box 360
3700 AJ ZEIST
Netherlands

Fl. de Vrijer
TNO-CIVO Toxicology and Nutrition Institute
P.O. Box 360
3700 AJ ZEIST
Netherlands

A. Wilson
Monsanto Company
800 N Lindberg BLvd
St. LOUIS, MI 63167
USA

R.-A. Walk *
INBIFO Institut für biologische Forschung
Fuggerstrasse 3
D-5000 COLOGNE 90
FRG

K. Woodward
Hannibal House, R. 917s
Elephant Castle
LONDON, SE1 6TE
UK

R.A. Woutersen *
TNO-CIVO Toxicology and Nutrition Institute
P.O. Box 360
3700 AJ ZEIST
Netherlands

H.H.K. Winterwerp
Netherlands Cancer Institute
(Anthoni van Leeuwenhoekhuis)
Plesmanlaan 121
1066 CX AMSTERDAM
Netherlands

1) unable to attend