THE IMMUNE SYSTEM AS A TARGET FOR ANTIBIOTICS

Offset printed by Pudoc, Wageningen

Cover design and illustrations : W.J.A. Valen

This thesis has been accomplished at :
Department of Experimental Animal Morphology & Cell Biology and
Department of Animal Husbandry
Agricultural University
Wageningen
The Netherlands

Voor Christine,
Irene en Quirine

Promotores : dr. W.B. van Muiswinkel, hoogleraar in de zoölogische celbiologie

dr. C.C. Oosterlee, hoogleraar in de veeteeltwetenschap

John Louis Grondel

THE IMMUNE SYSTEM AS A TARGET FOR ANTIBIOTICS

Proefschrift
ter verkrijging van de graad van
doctor in de landbouwwetenschappen,
op gezag van de rector magnificus,
dr. C.C. Oosterlee,
in het openbaar te verdedigen
op woensdag 10 december 1986
des namiddags te vier uur in de aula
van de landbouwuniversiteit te Wageningen

STELLINGEN

1 Antibacteriële middelen beïnvloeden het immuunsysteem.

2 De farmacokinetiek van antibiotica bij vissen verschilt dermate van warmbloedige dieren, dat nader onderzoek noodzakelijk is.

3 Zonder vermelding van vissoort en temperatuur in de doseringsschema's, is de conclusie van Austin dat een verstandig gebruik van antimicrobiële middelen vele bacteriële visziekten zal kunnen beheersen, niet gerechtvaardigd.
Austin, B., The control of bacterial fish diseases by antimicrobial compounds. In: Antimicrobials in agriculture, Woodbine (ed.), Butterworths, London, 1984, 255-268.

4 De conclusie van Zatz dat translocatie van proteine kinase C in hippocampus-plakjes alleen optreedt na stimulering met forbol-esters is voorbarig.
Zatz, M., Translocation of protein kinase C in rat hippocampal slices. Brain Research, 1986, 385: 174-178.

5 Dat het aantal werklozen per 100 afgestudeerden ver boven de 100 kan uitstijgen behoeft extra uitleg.
Extra Uitleg. In: Weekblad van het Ministerie van Onderwijs en Wetenschappen, nr. 64, 1986.

6 De functie van hoogleraar dient er primair een te zijn van wetenschappelijk manager met een, in vergelijking tot de huidige benoemingstermijn, beperkte aanstellingsduur.

7 Het feit dat onderzoek en onderwijs verenigd zijn in de taakstelling van een universitair (hoofd)docent getuigt van een ernstige onderschatting van het specialistisch karakter van beide typen werk.

8 Automatisering is maatwerk en behoeft een intensieve begeleiding.

9 In het deltaplan stroomt geld met een onberekenbare stroomsnelheid.

J.L. Grondel
The immune system as a target for antibiotics.
Wageningen, 10 december 1986.

CONTENTS

ABBREVIATIONS

ABBREVIATIONS

Immunological study

AIO	additional inexpensive obedient
APC	antigen presenting cell
ASN	active supernatant
B-cell	bursa (equivalent) derived lymphocyte
BSA	bovine serum albumin
C_1-Cg	components 1-9 of the complement system
CSN	control supernatant
Con A	concanavalin A
DTH	delayed type hypersensitivity
Fc	crystallizable fragment of immunoglobulin
FCS	fetal calf serum
GALT	gut-associated lymphoid tissue
HA	hemagglutination
Ig	immunoglobulin
IL-1,2	interleukin-1,2
i.m.	intramuscular
i.p.	intraperitoneal
i.v.	intravenous
LPS	lipopolysaccharide
MIC	minimum inhibitory concentration
MIF	migration inhibition factor
MLC	mixed leucocyte culture
MLR	mixed leucocyte reaction
OxyTC	oxytetracycline
PBL	peripheral blood leucocytes
PBS	phosphate buffered saline
PCS	pooled carp serum
PFC	plaque forming cells
PGE	prostaglandin E
PHA	phytohemagglutinin
PMN	polymorphonuclear neutrophils
PPD	purified protein derivative of tuberculin
PVP	polyvinylpyrrolidone
PWM	pokeweed mitogen
sIg	surface immunoglobulin

SN	supernatant
SRBC	sheep red blood cells
T-cell	thymus derived lymphocyte
WC	white cells

Pharmacokinetic study

A°, B°, C°	Zero-time plasma drug concentration intercepts of triphasic intravenous disposition curve.
α, β, γ	Values of α, β and γ are related to the slopes of the different phases of a tri-exponential drug disposition curve.
$T\frac{1}{2}$ α, β, γ	Values of $T\frac{1}{2}$ α, β and γ represent half-lives of a drug and are related to the different phases of a tri-exponential drug disposition curve.
AUC	Area under the plasma drug concentration-time curve after administration of a single dose.
C_p°	Plasma drug concentration immediately following intravenous administration of a single dose.
Cl_b	Body clearance of a drug, which represents the sum of all clearance processes in the body.
C_{max}, T_{max}	Maximum drug concentration at "time T" observed after a single intramuscular injection of a drug.
Compartment	Mathematical entity which describes the pharmacokinetic behaviour of a particular drug. For instance, "the three-compartment open model" adequately describes the disposition of OxyTC in carp and trout (see diagram in "Introduction to the papers").
F	Fraction of the administered dose which reaches the systemic circulation intact.
$K_{12, 13}$	First-order transfer rate constants for drug distribution between the central and peripheral compartments.
K_{el}	First-order elimination rate constant for disappearance of a drug from the central compartment.
pK_a	Negative logarithm of (acid) dissociation constant of an organic electrolyte (used for both acids and bases).
V1	Apparent volume of the central compartment.
Vd area	Apparent volume of distribution of a drug: proportionality constant relating the plasma concentration of a drug to the amount of drug in the body.

IMMUNOLOGICAL DEFENCE MECHANISMS AS A TARGET FOR ANTIBIOTICS

J.L. GRONDEL AND W.B. VAN MUISWINKEL

Defence mechanisms

The barriers, which bacteria have to overcome upon invading a host, are either non-specific or specific. In both types of resistance, humoral factors and specialized cells play pivotal roles forming an elaborate network of physical, chemical and cellular defence mechanisms, including the immune system.

The skin with its low pH and bactericidal fatty acids, or mucous epithelial surfaces containing growth-decreasing factors or phagocytizing cells is an example of non-specific, external defence. In addition to this barrier, internal non-specific defence is mediated by serum factors (e.g. transferrin and alternative complement route) and phagocytizing cells (macrophages and granulocytes). Specific defensive responses against invading micro-organisms are effected by the immune system.

An immune response is a rather complex interaction between distinct leucocyte populations and humoral factors, and can be characterized by two phenomena: amplification and regulation. Foreign materials (antigens) are processed by macrophages and presented to antigen-sensitive lymphocytes in the correct physical configuration. The lymphocytes are required for antibody formation and cellular immune responses. Co-operation between macrophages, T-cells and B-cells, either by direct cell-to-cell contact or by soluble helper substances (monokines and lymphokines) produced by the interacting cells, are a prerequisite for an appropriate response. The antigen-sensitive lymphocyte populations expand by proliferation and differentiate subsequently into effector cells. These cells and their products contribute to a rapid elimination of antigen.

When defence mechanisms fail to prevent the establishment of an infective agent in its host, the consequence will be disease. Under these circumstances, antibiotics have proved to be of remarkable value for the therapy of bacterial infections. The antibiotic generally inhibits growth of the pathogen, allowing the immune system to eliminate it. In this way, the antibiotic co-operates with the immune system. However, the effect on the immune status of animal or man is rarely taken into consideration when chosing an antibiotic for therapeutic use.

The importance of antimicrobial agents for the maintenance of animal health is generally accepted in animal husbandry. Besides being used for prevention and control of bacterial diseases, antibiotics are sometimes used because of their

growth-promoting effect. However, it is also known that some of these drugs can lead to adverse immunotoxic effects, and that prolonged use will increase the risk for raising drug-resistant bacterial strains.

Several studies have been performed on the interaction between antibiotics and the immune system in mammals, birds and fish. Some antimicrobial agents have been shown to interfere with immunological defence mechanisms. Impairment of the immune system may have serious implications for the outcome of therapy, especially when a relatively long recovery period is required.

It is difficult to draw general conclusions on the effects of antibiotics upon the immune system. Many reports are describing either positive, or negative or even no effects on the defence system. However, the picture is based on effects of different antibiotics studied in all sorts of animals. Moreover, the parameters used reveal overall effects on the well-being of an animal, which is not exclusively dependent on the immune status. Only a limited number of studies are available concerning the effects of antibiotics upon real immunological processes such as antigen processing, proliferation and maturation of lymphocytes and the production of immunoregulatory factors.

The effects of a wide range of antimicrobial agents on the mammalian immune response *in vivo* as well as *in vitro* have been reviewed by Finch (13) and Hauser & Remington (25). In this paper, we selectively review antimicrobial agents used in veterinary and human medicine with regard to their effects on non-specific and specific defence mechanisms.

Non-specific defence.

Chemotaxis and phagocytosis.

Chemotaxis and subsequent phagocytosis, important neutrophil and macrophage functions in defence against bacterial infections, have been found to be suppressed by several antibiotics *in vitro* and *in vivo*. Especially tetracyclines have been shown to interfere with these leucocyte processes. Both spontaneous and induced migration of human leucocytes *in vitro* were severely depressed by lymecycline (7), doxycycline (7) and tetracycline (14,15,16). Corresponding results were obtained in experiments on healthy volunteers given recommended dosages of the antibiotics

and in patients treated with doxycycline for post-operative infections. Tetracycline also inhibited the migration of human leucocytes *in vitro* (14, 15). On the other hand, the chemotactic response of bovine polymorphonuclear leucocytes (PMN) was not inhibited in the presence of tetracyclines, streptomycin or penicillin at concentrations normally achieved in blood during systemic treatment. In local therapy such as intramammary injections and other topical applications higher concentrations will be achieved.

Ziv et al. (46) and Dulin et al. (12) examined intramammary injected antibiotic products and corresponding vegetable based vehicles for their effect on phagocytosis by bovine neutrophils. The concentrations used in the phagocytosis assay were similar to those found in milk immediately, 6 and 12 hours after injection. The results indicate that some antibiotics, including penicillins alone or in combinations, chloramphenicol, cephalosporin, tetracycline, erythromycin, gentamicin and nitrofurantoin as well as the vehicles in which they are suspended cause a reduction in phagocytic capability of bovine milk PMN.

Phagocytosis and humoral factors.

In the initial phase of phagocytosis a phagocyte has to recognize and attach firmly to the foreign particle. These processes are facilitated by a subcomponent of the complement system (C3b) and immunoglobulin after binding at the particle surface (opsonization). The phagocytic cells expose receptors for C3b and the Fc portion of certain immunoglobulins at their cell surface.

Athlin et al. (3) adapted a fluorescence assay for the study of yeastcell adherence and phagocytosis by blood monocytes. This method is characterized by the quencing of fluorescence in non-ingested yeast-cells by the addition of crystal violet. The assay thereby allows discrimination between phagocyte-adherent and engulfed yeast-cells. Furthermore, it was shown that doxycycline at therapeutical concentrations (5 µg/ml) did not affect the adherence of yeast-cells to human blood monocytes *in vitro* . However, the median value of engulfed yeast-cells by doxycycline-treated monocytes was 30 % lower than in control cultures, though statistically not significant.

Augmenting effects of cephalosporins on the host defence were reported by Lam and co-workers (27). In their studies micro-diffusion chambers containing

either *E. coli* alone or *E. coli* plus PMN were implanted intraperitoneally in mice receiving a single dose (3 mg/kg) of cefotaxime, moxalactam or the new product CPW 86-363. These compounds show a broad antibacterial spectrum against enteric Gram-negative bacteria. It was demonstrated that moxalactam and CPW 86-363 led to a significant reduction in viability of the micro-organisms in the chambers containing both leucocytes and bacteria.

Milatovic (29) has shown that pretreatment of *Pseudomonas aeruginosa* with 1/3 of the minimum inhibitory concentration (MIC) of azlocillin, carbenicillin, cefoperazone or piperacillin changed the opsonic requirements of these bacteria. *P. aeruginosa* exposed to the β-lactam antibiotics were opsonized and engulfed by human PMN without participation of the complement system. It was suggested that the filament formation induced by these antibiotics is accompanied by changes of bacterial surface characteristics. Antibiotic-mediated bacterial killing by serum factors has been reported by Lam et al. (27). The survival of a previously serum resistant *E. coli* strain was evaluated in media containing fresh human serum and a low concentration (MIC/4) of the cephalosporins moxalactam, cefotaxime or CPW 86-363. Cefotaxime and CPW 86-363 improved the bactericidal activity of serum.

Tetracycline, oxytetracycline, lymecycline and doxycycline have been found to suppress the bactericidal effect of serum on *E. coli* (14). This effect can be reversed by the addition of Mg^{++} ions. Lochmann et al. (28) reported a significant deficiency in the values of C3, the key-molecule in the complement system, in rabbits immunized with staphylococcal haemolysin and simultaneously administered antibiotics (chloramphenicol or oxytetracycline). Interference with the complement system by sulphonamides and penicillins has been reported by Von Zabern et al. (44).

Human PMN showed enhanced intracellular killing of untreated *Pseudomonas aeruginosa* after pretreatment of the leucocytes *in vitro* with nocardin A, a monocyclic β-lactam antibiotic, and subsequently wash. These augmenting effects occurred at much lower concentrations than those which will induce antibacterial effects without PMN (5).

Studies by Hawkey et al. (26) with 3 anti-pseudomonal antibiotics, gentamicin, azlocillin and carbenicillin, did not reveal significant effects on the phagocytic function of human PMN *in vitro*.

Cannon et al. (9) investigated the *in vitro* effect of several antibiotics including sulphonamides and trimethoprim on neutrophil function. Human PMN were pretreated with 10 µg/ml of each drug at 37°C for 60 minutes. Sulphamethoxazole, sulphanilamide, trimethoprim, brodimoprim and co-trimoxazole significantly increased neutrophil activity. Whereas, sulphamerizine, sulphadiazine and ceftriazone did not change the phagocytic process. Wolff & Stankova (45) investigated whether sulphamethoxazole and trimethoprim (1:5) improved alveolar mononuclear phagocyte oxygen metabolism and intracellular killing of *S. aureus*. Therefore, rats were given sulphamethoxazole/trimethoprim (10/50 mg) per day for 6 weeks. It was shown that neither the hexose-phosphate shunt activity, nor the oxygen metabolism or the intracellular killing properties were affected.

Clindamycin is a well established drug in the treatment of serious anaerobic infections. Sub-inhibitory concentrations of clindamycin did interfere with the adhesion of *E. coli* to buccal epithelial cells and did promote phagocytosis and killing by human PMN (6). Obviously, the adherence to phagocytes was not affected. Here too, it was suggested that the alteration in the bacterial cell wall may inhibit adherence to epithelial cells, necessary for initiation/establishment of infection, and render the organism more susceptible to phagocytosis.

Specific defence mechanisms.

Antibody-mediated immune response.

When antigen enters the body, it must be processed by macrophages and presented to T- and B-lymphocytes capable of a specific response. Depending on the nature of the antigen, the B-cell receives appropriate stimuli from T helper cells and macrophages, and it will start to divide. While the antigen specific B-cells expand, some of these cells will start to differentiate into plasma-cells, which are specialized in immunoglobulin synthesis and secretion.

One of the protective effects of antibodies is mediated by the constant region (Fc) of the antibody molecule. After combining with antigen, antibody acquires the ability to activate the complement cascade, to bind to phagocytes or to provoke degranulation of mast cells and basophils.

Thong & Ferrante (39) investigated the effect of doxycycline on antibody responses to sheep red blood cells (SRBC) in mice. According to the authors a daily dose of 100 mg/kg i.p. for 5 days was the usual therapeutic regime in mice. Under these experimental conditions, the anti-SRBC response of the antibiotic-treated animals was not significantly affected. In a detailed study in rats by Van den Bogert & Kroon (40) oxytetracycline was administered by continuous, intravenous infusion (20 mg/kg/day). It was shown that the primary response to SRBC was severely impaired when the drug was given for more than 48 hours after priming. The kinetics of the IgM response (e.g. peak day) were not changed, but the amount of antibodies produced was much lower. The anamnestic reponse to SRBC was completely depressed when oxytetracycline was given during the first 48 hours of the primary response. The authors conclude from these experiments that oxytetracycline interferes with T-cell proliferation and memory cell formation.

Gillissen (18) and Gillissen & Pusztai-Markos (19) examined several cephalosporins and cephamycins in respect to their effect on humoral immunity. The response was evaluated with the direct plaque forming cell assay (IgM-producing spleen cells). Mice were i.v. injected only once with antibiotics at the same day as immunization with SRBC. The results showed that 3 (viz. cefotetan 30 mg/kg, cefmenoxime 30 mg/kg and cefoxitin 20 mg/kg) out of 6 antibiotics were reducing the antibody response. On the other hand the remaining antibacterial agents (cefotaxime, cefsulodin and cefoperazone) had an enhancing effect. A totally different picture was obtained when the effects of 7 days' therapy on the primary humoral reaction to SRBC was investigated in mice (34). Cefotaxime, amikacin, mezlocillin and piperacillin inhibited the IgM response by 88, 55, 100 and 56 %, respectively. Clindamycin did not interfere with the response. The mezlocillin-induced suppression was long-lasting, being still present 20 days after completion of the treatment.

It is obvious that multiple injections of the same drug result in a different pharmacokinetic behaviour compared to a single dose. This may be an explanation for the contradictionary results presented by Gillissen & Pusztai- Markos (19) and Roszkowski et al. (34).

Adverse effects of antibiotics on the development of gut-associated lymphoid tissue (GALT) and serum immunoglobulin levels in chickens and turkeys were investigated by Naqi et al. (30) and Cook et al. (11), respectively. The treatment

started by dipping eggs in a gentamicin solution (500 mg/l) for 15 minutes before incubation. The newly hatched birds were each injected with 0.2 mg (chickens) or 1 mg (turkeys) of gentamicin subcutaneously and fed a commercial diet containing chlortetracycline at 200 mg/kg for the duration of the study (turkeys: 21 days; chickens: 28 days). In the antibiotic-treated chickens a lowered IgG level was observed at 21 days. Later on day 28, all serum Ig fractions (IgM, IgG, IgA) were below control values. Also the numbers of IgM-, IgG-, and IgA-bearing lymphocytes in cecal tonsils and large intestine were reduced as compared to controls. A general suppression of all Ig-bearing cells was observed in the antibiotic-treated turkeys at an age of 21 days. At this age, reduced numbers of IgM-bearing spleen cells and IgG- and IgA-bearing cells in the large intestine were observed. Furthermore, the growth-rate of the bursa of Fabricius was lower than normal.

Feeding antibiotic-containing diets as well as application of antimicrobial agents with drinking water, may change the enteric microflora. Consequently, the antigenic load and/or composition will be altered.

Oxytetracycline adminstered in therapeutic doses for 6 days starting one day before immunization, affected the primary and the secondary response to SRBC in chickens (unpublished data). The primary plaque forming spleen cell response was delayed or suppressed in animals which received the antibiotic by i.m. injection or by oral application (drinking water). Amazingly enough, the haemagglutination titre was enhanced. The secondary direct plaque forming cell response was not significantly changed by oxytetracycline, when it was administered orally during the development of the primary response. However, the peak response of the indirect plaque forming cells was delayed for 1 day. Both the total and 2-mercaptoethanol-resistant haemagglutination titres were enhanced.

Despite the fact that oxytetracycline affected the development of the antibody forming cells in the spleen, the anti-SRBC-titres increased. This indicates that other lymphoid tissues such as bone marrow may also be important sites for antibody synthesis.

It has been shown in fish that oxytetracycline administered as food additive or by i.p. injection severely reduced the *in vivo* immune response. It was observed that the plaque forming cell response against SRBC was depressed by 80-95%

after treatment of carp (32, 33). Furthermore, it was demonstrated that oxytetracycline caused a delay in the peak response rather than suppression (24). Clear immunosuppression was shown in rainbow trout after feeding with pellets containing oxytetracycline (1, 41).

Mitogenic stimulation.

Mitogens can stimulate cell division. Certain mitogens have the ability to activate T- or B-cells specifically. The plant lectins phytohaemagglutinin (PHA) and concanavalin A (con A) provoke T-cells to proliferate, whereas lipopolysaccharide (LPS), derived from Gram-negative bacteria, can function as B-cell mitogen. Lymphocyte mitogenesis can be measured by adding tritiated thymidine to the culture medium. The amount of radioactivity incorporated into newly synthesized DNA is regarded as an estimate for cell proliferation.

The effect of a wide range of antibiotics on human T and B lymphocytes was studied *in vitro* by Banck & Forsgren (4). In their study fourteen antibiotics (aminobenzylpenicillin, benzylpenicillin, carbenicillin, cefazolin, cephalothin, chloramphenicol, 5-fluorocytosine, gentamicin, kanamycin, lymecycline, nalidixic-acid, sulphamethoxazole, tetracycline chloride and trimethoprim) did not inhibit or stimualte the PHA response when 50 µg of each drug/ml was added for 3 days. Inhibitory effects on both T- and B-cell mitogenic responses were detected for erythromycin, clindamycin and rifampin at relatively high concentrations. However, doxycycline, nitrofurantoin and fusidic acid significantly depressed both mitogenic responses at low concentrations. These antibiotics had to be present in culture from day 1 or 2 onwards to exhibit a strong suppressive effect. Only a moderate impairment was shown when the drugs were added on day 3. No significant effect was observed after addition of the drugs for the last 24 hours combined with tritiated thymidine.

Lymecycline and tetracycline chloride did not influence the thymidine incorporation into PHA-activated cells in the presence of 25 µg drug/ml. Whereas, minocycline inhibited this process significantly.

Anderson et al. (2) investigated the effect of erythromycin on mitogenic stimulation of human peripheral blood leucocytes. Erythromycin base at concentrations of $1x10^{-6}$ M - $1x10^{-5}$ M did not affect the PHA-induced proliferation.

Only at higher concentrations a dose-dependent suppression was observed. Ingestion of a single dose of 500 mg erythromycin stearate by normal volunteers was not associated with a significant change in mitogenic responsiveness to PHA or Con A, measured 90 minutes and 4 days after drug intake. The authors observed a consistent slight, but statistically insignificant, increase in thymidine incorporation into Con A-activated leucocytes.

Sulphonamides and trimethoprims did not modify the mitogenic response of human PBL as was demonstrated by Cannon et al. (9). However, a highly significant increase in lymphocyte transformation was produced by cotrimoxazole.

In mice, the immuno-depressive effect of antibacterial agents was tested in a detailed study by Voiculescu et al. (42). It was demonstrated that erythromycin, colistin and chloramphenicol strongly inhibited the antigen-dependent B-cell blastogenesis *in vitro*, related to *in vivo* antibiotic treatment. A T helper-cell deficiency in the colistin- and chloramphenicol-treated animals was suggested by the authors, because the B-cell response could be restored by supplementation with autologous T helper-cells. Further research on this subject was done by the same group (43) to investigate the involvement of T helper and T suppressor activities on the antibiotic-induced immunosuppression. It was shown that the ability of T helper-cells to enhance the PHA response of nylon column non-adherent cells was lowered in the colistin-, chloramphenicol- and erythromycin-treated mice. An improved T suppressor-cell activity was observed in the chloramphenicol-treated group.

These elegant studies indicate clearly that certain antibiotics selectively interfere with the immune system.

Roszkowski et al. (34) and Borowski et al. (8) showed that the cephalosporin cefotaxime affected the mitogen-induced proliferation of mouse spleen cells *in vitro* only at high concentrations. Cephradine was inhibitory at therapeutic levels. Furthermore, Borowski demonstrated that mezlocillin and piperacillin severely reduced the mitogenic response to Con A and LPS. Amikacin and clindamycin did not influence the proliferation. When the animals were injected with different concentrations of antibiotics (cefotaxime, amikacin, mezlocillin, piperacillin or clindamycin) twice a day for 7 consecutive days, only clindamycin did not affect the lymphocyte stimulation induced by Con A or LPS. Cefotaxime and amikacin were effective only in the highest doses tested, 1.2 mg/day and 0.3 mg/day, respectively.

It has been reported for birds, that the tetracycline analogue oxytetracycline interferes with the mitogenic response of chicken leucocytes (24). A dose-dependent suppression of lectin stimulation of peripheral blood and spleen leucocytes was observed. The kinetics of the DNA-synthetic activity were seriously affected. The inhibitory effect was most pronounced during the early stages of the mitogen-induced proliferation.

To investigate whether fish leucocytes, obtained from different lymphoid organs, were sensitive to antibiotic treatment *in vitro*, both PHA and LPS mitogenic responses were evaluated in the presence of various concentrations of oxytetracycline in carp (20). It was demonstrated that this drug significantly inhibited the thymidine incorporation. The 50 % inhibition level was already reached at 4-6 µg/ml. Furthermore, oxytetracycline caused a dose-dependent delay in the leucocyte response rather than a real suppressive effect (22). Obviously, the impairment of cellular functions like DNA synthesis was not due to cytotoxicity as was suggested in previous investigations.

Sulphatroxazole/trimethoprim (5:1), sulphadimethoxine, sulphadimidine, lincomycin/spectinomycin (1:2) and ampicillin did not suppress the mitogenic response. On the contrary, at low concentrations an increased thymidine uptake was observed. Gentamicin and furaltadone showed a dose-dependent inhibition. Chloramphenicol was stimulatory at concentrations below 5 µg/ml, whereas higher quantities became suppressive.

Immunoregulatory factors.

Immune responses, both cell- and antibody-mediated, are under strict control, mediated by a number of different mechanisms. Today, many soluble, factors produced by immuno-competent cells, are known to exert a regulatory effect. In this paragraph we will describe the effect of several antimicrobial agents on immuno-regulation.

In 1974 Serrou (37) published a report about the suppressive influence of rifampicin on migration inhibition factor (MIF) secretion by human lymphocytes. Inhibition of protein and lymphokine synthesis was also observed when tetracycline was present in human leucocyte cultures (17).

The effects of erythromycin on the release of prostaglandin E2 (PGE2) by mitogen-stimulated mononuclear leucocytes were investigated by Anderson et al. (2). This drug was causing significant inhibition of PGE2 release by resting and mitogen-activated cells at relatively low concentrations. According to the literature PGE2 exerts immuno-suppressive activities. Therefore, Anderson et al. (2) concluded that the observed increased leucocyte transformation following erythromycin ingestion, can be explained by reduced PGE2 production.

The above mentioned modification of the PHA response of mouse T cells by erythromycin, colistin and chloramphenicol, was also observed in experiments with T helper or T suppressor soluble factors (43). These data confirmed the T helper-cell deficiency in antibiotic-treated animals as well as the T suppressor-cell enhancement in chloramphenicol-treated mice. Furthermore, a significant immuno-suppressive activity has been demonstrated using the migration inhibition assay, following *in vivo* treatment with the same antibiotics. Whereas, benzylpenicillin, streptomycin, kanamycin and tetracycline did not affect the inhibition of macrophage migration.

A T cell-dependent immune response is amplified by the action of interleukin 1 (IL-1) and interleukin 2 (IL-2). Both factors are proliferation and/or differentiation signals for T and B lymphocytes during the response (38, 31). The amplification process is dependent upon both the level of interleukin synthesis and induction of interleukin receptors. Interleukins have been isolated and characterized in many mammalian species. The existence of interleukin-like factors has also been demonstrated in birds (35, 36) and fish (21, 10), emphasizing the phylogenetic importance of amplifying/regulatory factors for a regular immune reaction.

In chickens, the early stages of the mitogen-induced T cell proliferation can be inhibited by oxytetracycline (23). In this study, supernatants of Con A-induced spleen cell cultures were harvested at different time intervals and tested for their growth-promoting activity on T cell blasts, in order to determine the IL-2 production in the presence or absence of oxytetracycline. The antibacterial agent does not seem to have any effect on IL-2 production, whereas the uptake of tritium labeled thymidine by growth-factor-dependent T cell blasts was severely reduced.

The delayed-type hypersensitivity (DTH) reaction is based upon the interaction between antigen and primed T cells. Several lymphokines are released which

account for the typical events during the DTH response. DTH is characterized by the appearance of an induration and erythematous reaction which reaches a maximum at 24-48 hours. During this process, macrophages and lymphocytes infiltrate and accumulate at the inflammation site. DTH responses can be studied by priming animals with SRBC and challenging with the same antigen a few days later depending on the species. The topical swelling as a result of the hypersensitive reaction can be measured 24 hours after the last exposure to the antigen.

Thong & Ferrante (39) have shown that mice treated *in vivo* with different tetracycline analogues have a reduced capacity to mount DTH responses to SRBC. A significant reduction (30-45 %) was observed in experimental groups treated once with doxycycline, rolitetracycline, and tetracycline. Oxytetracycline did not evoke a significant effect. The suppressive effect of doxycycline was more pronounced when the drug was administered on the day of challenge than 2 days prior to priming. This suggests an interference with macrophages and/or lymphocytes.

A severely depressed DTH response was observed in rats when oxytetracycline (20 mg/kg/day) was continuously administered starting just before the moment of priming (40). Furthermore, it was shown that oxytetracycline, only suppressed the response when the drug was present between 18 and 72 hours after priming. According to these authors, this implies that during this particular period the number of T cell divisions is large enough to reduce the mitochondrial ATP generating capacity in the presence of the drug. Consequently, inhibition of cell proliferation will occur.

In contrast to tetracyclines, several cephalosporins and cephamycins significantly enhanced the DTH response in mice (19). The antibiotics were given once (30 mg/kg) on the day of immunization or 3, 2 and 1 day before. Pretreatment of the animals with the drugs (cefotaxime, cefoxitin, cefsulodin, cefoperazone, cefotetan and cefmenoxime) resulted in a more pronounced effect. However, a 7 days' chemotherapy with cefotaxime suppressed the DTH response (34).

Conclusions

It is clear that some of the commonly used antimicrobial agents can interfere with non-specific and/or specific defence systems. Antibiotics may display suppressing as well as enhancing immunological side effects, depending on test models and animal species. Therefore, it is impossible to draw general conclusions on the effects of antibiotics on the immune system as such. We also cannot define the clinical relevance of antibiotic-mediated immunomodulation at this moment. Yet, it is very important to exclude any immuno-suppression by certain drugs in animal and man. This is obvious, because the defence mechanisms have to execute the final elimination of the pathogens.

To day, many *in vitro* and *in vivo* immunological assays are available and provide us with sensitive tools for monitoring drug effects. However, it is essential to standardize these assays and to incorporate carefully designed studies, which reflect the disease status.

There exists a general relation between the specific growth-rate of bacteria and the nutrient concentration available. In addition, temperature is also an important environmental factor, which determines the rate of all biochemical reactions. For instance, fish pathogens are psychrophilic, which means that their optimal growth-rate is far below 37 °C, in contrast to thermophilic organisms. The immune system of ectothermic animals has to be adapted in such a way, that it can mount an adequate response at relatively low temperatures. This biochemical adaptation (e.g. metabolic rate and membrane lipid composition) may have implications for the pharmacokinetics of the drugs and for the susceptibility of the immunological process to toxic damage. It is clear that antibiotics have been used over a wide range of species. For some species pharmacokinetic data are scarce or even absent. Kinetic data on tissue distribution, plasma disposition and biological half-life time can differ markedly between mammalian species. Moreover, extreme differences may be expected with respect to the pharmacokinetic behaviour of the drug in birds and fish.

The immuno-enhancing effects of antibiotics, caused by interference with the bacterial physiology and/or by stimulation of the host immune system are promising. The combined action of immune system and drug will increase the defensive potential. The ultimate goal of antibacterial therapy is to achieve the best action against pathogens with minimal adverse side effects. It can

be seen in the diagram that many factors determine the clinical efficacy of a selected antibiotic. One of these factors is the binding to plasma proteins, because only free material will pass to the tissues. Another factor is the amount of unbound drug at the site infection and the degree of interference with local and systemic defence systems.

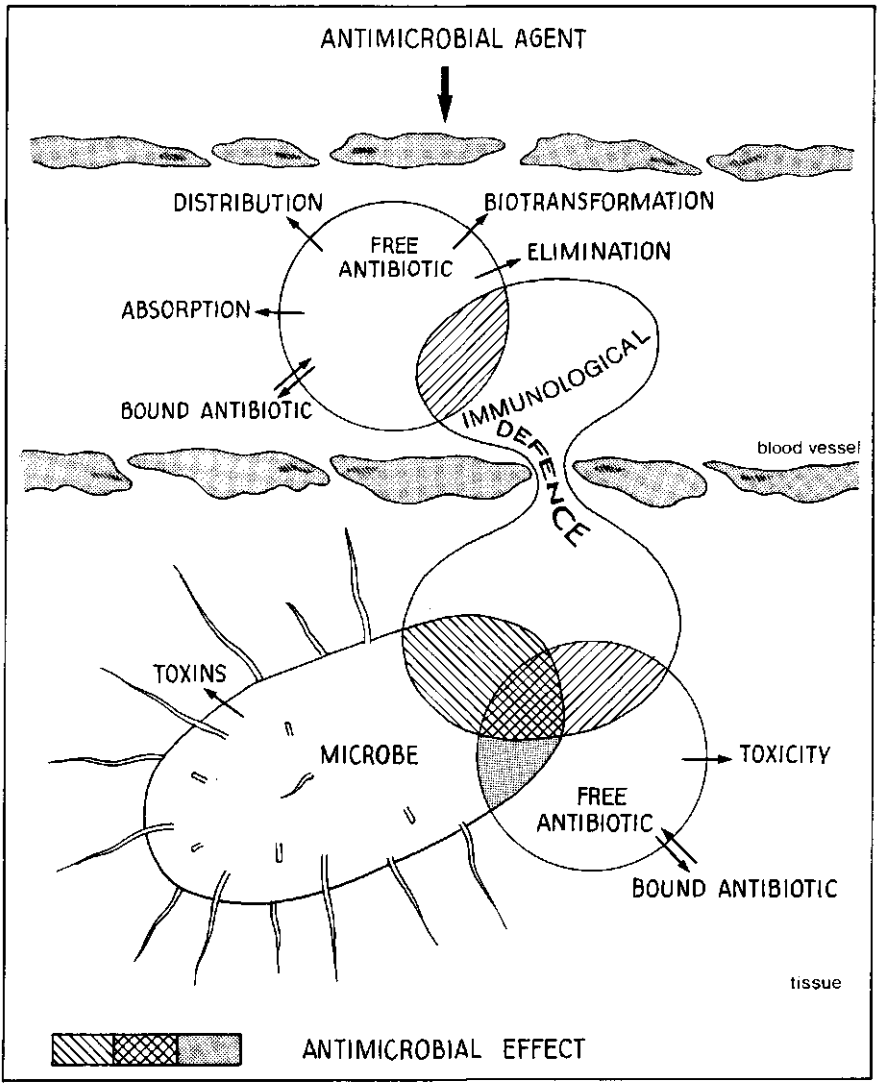


Diagram showing the various factors which can influence the outcome of antibiotic therapy

It is emphasized that a multidisciplinary approach, as is visualized in the diagram, is a necessity to tackle the problems efficiently. Immunological, pharmacological and microbiological research has to be extended over a wide range of animal species to support an effective management in animal husbandry and an optimal veterinary practice.

References

1. Anderson, D.P., Van Muiswinkel, W.B. and Roberson, B.S. (1984). Effects of chemical induced immune modulation on infectious diseases of fish. pp. 187-211 in Chemical regulation of immunity in veterinary medicine. M. Kende, J. Gainer and M. Chirigos, eds., Alan R. Liss, Inc., N.Y.

2. Anderson, R., Fernandes, A.C. and Eftychis, H.E. (1984). Studies on the effects of ingestion of a single 500 mg oral dose of erythromycin stearate on leucocyte motility and transformation and on release *in vitro* of prostagladin E2 by stimulated leucocytes. J. Antimicrob. Chemother. 14: 41-50.

3. Athlin, L., Domellof, L. and Norberg, B. (1984). Adherence and phagocytosis of yeast cells by blood monocytes: effects *in vitro* of a therapeutic doxycycline concentration. Acta Path. Microbiol. Immunol. Scand. Sect. 92: 227-230.

4. Banck, G. and Forsgren, A. (1979). Antibiotics and suppression of lymphocyte function *in vitro* . Antimicrob. Agents and Chemother. 16: 554-560.

5. Banks, R.M. and O'Grady, F. (1983). Therapeutic significance of nocardicin A stimulation of phagocyte function in experimental *Pseudomonas aeruginosa* infection. Br. J. Exp. Path 64: 231-237.

6. Bassaris, H.P., Lianou, P.E. and Papavassiliou, J.Th. (1984). Interaction of subminimal inhibitory concentrations of clindamycin and *Escherichia coli:* effects on adhesion and polymorphonuclear leukocytes function. J. Antimicr. Chemother. 13: 361-367.

7. Belsheim, J., Gnarpe, H. and Persson, S. (1979). Tetracyclines and host defense mechanisms: interference with leukocyte chemotaxis. Scand. J. Infect. Dis. 11: 141-145.

8. Borowski, J., Jakoniuk, P. and Talarczyk, J. (1985). The influence of some cephalosporins on immunological responses. Drugs Exp. Clin. Res. 11: 83-88.

9. Cannon, P., Climax, J., Darragh, A., Lambe, R. and Lenehan, T.J. (1983). The action of selected antimicrobial agents on certain functions of human leucocytes. Br. J. Pharmacol. 80 (suppl.): 596.

10. Caspi, R.R. and Avtalion, R.R. (1984). Evidence for the existence of an IL-2 like lymphocyte growth promoting factor in a bony fish, *Cyprinus carpio* L. Dev. Comp. Immunol. 8: 51-60.

11. Cook, J., Naqi, S.A., Sahin, N. and Wagner, G. (1984). Distribution of immunoglobulin-bearing cells in the gut-associated lymphoid tissues of the turkey: Effect of antibiotics. Am. J. Vet. Res. 45: 2189-2192.

12. Dulin, A.M., Paape, M.J. and Ziv, G. (1984). Effect of intramammary injection products on *in vitro* phagocytosis. J. Dairy Science. 67: Suppl. 1: 170.

13. Finch, R. (1980). Immunomodulating effects of antimicrobial agents. J. Antimicrob. Chemother. 6: 691-699.

14. Forsgren, A. and Gnarpe, H. (1973). Tetracycline interference with the bactericidal effect of serum. Nature New Biology. 244: 82-83.

15. Forsgren, A. and Schmeling, D. (1977). Effect of antibiotics on chemotaxis of human leukocytes. Antimicrob. Agents Chemother. 11: 580-584.

16. Forsgren, A., Schmeling, D. and Banck, G. (1978). Effect of antibiotics on chemotaxis of human polymorphonuclear leukocytes *in vitro*. Infection 6 (suppl. 1): S102-S106.

17. Ganguly, R., Pennock, D.G. and Kluge, R.M. (1984). Inhibition of protein synthesis and lymphokine production by tetracycline. Allergie u. Immunol. 30: 104-109.

18. Gillissen, G.J. (1982). Antibody production and cellular immunity. The influence of antibiotics on the host-parasite relationship. pp. 5-11. H. U. Eickenberg, Hahn, H. van Opferkuch, W. (Eds.) Springer-Verlag, Berlin.

19. Gillissen, G. and Pusztai-Markos, Zs. (1984). Influence of antibiotics on immunological parameters: significance in experimental infections. Drugs Exp. Clin. Res. 10: 813-819.

20. Grondel, J.L. and Boesten, H.J.A.M. (1982). The influence of antibiotics on the immune system I. Inhibition of the mitogenic leukocyte response *in vitro* by oxytetracycline. Dev. Comp. Immunol., Suppl. 2, pp. 211-216.

21. Grondel, J.L. and Harmsen, E.G.M. (1984). Phylogeny of interleukins: growth factors produced by leukocytes of the cyprinid fish, *Cyprinus carpio* L. Immunol. 52: 477-482.

22. Grondel. J.L., Gloudemans, A.G.M. and Van Muiswinkel, W.B. (1985). The influence of antibiotics on the immune system. II. Modulation of fish leukocytes responses in culture. Vet. Immunol. Immunopathol. 9: 251-260.

23. Grondel, J.L., Angenent, G.C. and Egberts, E. (1985). The influence of antibiotics on the immune system. III. Investigation on the cellular functions of chicken leukocytes *in vitro* . Vet. Immunol. Immunopathol. 10: 307-316.

24. Grondel, J.L., Nouws, J.F.M. and Van Muiswinkel, W.B. (1986). The influence of antibiotics on the immune system. IV. Immuno-Pharmacokinetic investigations on the primary anti-SRBC response in carp *(Cyprinus carpio L.)* after oxytetracycline injection. J. Fish Dis., in press.

25. Hauser, W.E. and Remington, J. (1982). Effects of antibiotics on the immune response. Am. J. Medicine. 72: 711-716.

26. Hawkey, P.M., Hawkey, C.A., Richardson, M.D. and Warnock, D.W. (1983). *In vitro* phagocytosis of *Candida albicans* by human polymorphonuclear phagocyte monolayers pretreated with anti-*Pseudomonal* antibiotics. Eur. J. Clin. Microbiol. 2: 358-359.

27. Lam, C., Laber, G., Hildebrandt, J., Wenzel, A., Turnowsky, F. and Schutze, E. (1984). Therapeutic relevance of antibiotic-induced augmentation of host defences in experimental infections. Drugs Exp. Clin. Res. 10: 703-711.

28. Lochmann, O., Janovska, D., Vymola, F. and Svandova, E. (1979). Effect of antibiotics on the formation of specific antibodies. J. Hyg. Epidem. Microbiol. and Immunol. 23: 220-225.

29. Milatovic, D. (1984). Influence of subinhibitory concentrations of antibiotics on opsonization and phagocytosis of *Pseudomonas aeruginosa* by human polymorphonuclear leukocytes. Eur. J. Clin. Microbiol. 3: 288-293.

30. Naqi, S.A., Sahin, N., Wagner, G. and Williams, J. (1984). Adverse effects of antibiotics on the development of gut-associated lymphoid tissues and serum immunoglobulins in chickens. Am. J. Vet. Res. 45: 1425-1429.

31. Oppenheim, J.J. and Gery, I. (1982). Interleukin 1 is more than an interleukin. Immunology Today. 3: 113-119.

32. Rijkers, G.T., Teunissen, A.G., van Oosterom, R. and van Muiswinkel, W.B. (1980). The immune system of cyprinid fish. The immunosuppressive effect of the antibiotic oxytetracycline in carp *(Cyprinus carpio L.)* . Aquaculture. 19: 177-189.

33. Rijkers, G.T., van Oosterom, R. and van Muiswinkel, W.B. (1981). The immune system of cyprinid fish. Oxytetracycline and the regulation of humoral immunity in carp *(Cyprinus carpio L.)* . Vet. Immunol. Immunopathol. 2: 281-290.

34. Roszkowski, w., Ko, H.L., Roszkowski, K., Jeljaszewicz, J. and Pulverer, G. (1985). Antibiotics and immunomodulation: Effects of cefotaxime, amikacin, mezlocillin, piperacillin and clindamycin. Med. Microbiol. Immunol. 173: 279-289.

35. Schauenstein, K., Globerson, A. and Wick, G. (1982). Avian lymphokines, I: Thymic cell growth factor in supernatants of mitogen-stimulated chicken spleen cells. Dev. Comp. Immunol. 6: 533-540.

36. Schnetzler, M., Oommen, A., Nowak, J.S. and Franklin, R.M. (1983). Characterization of chicken T cell growth factor. Eur. J. Immunol. 13: 560-566.

37. Serrou, B. (1974). Rifampicin and immunosuppression. Lancet II: 172.

38. Smith, K.A., Lachman, L.B., Oppenheim, J.J. and Favata, M.F. (1980). The functional relationship of the interleukins. J. Exp. Med. 151: 1551-1556.

39. Thong, Y.H. and Ferrante, A. (1979). Inhibition of mitogen-induced human lymphocyte proliferative responses by tetracycline analogues. Clin. Exp. Immunol. 35: 443-446.

40. Van den Bogert, C. and Kroon, A.M. (1982). Effects of oxytetracycline on *in vivo* proliferation and differentation of erythroid and lymphoid cells in the rat. Clin. exp. Immunol. 50: 327-335.

41. Van Muiswinkel, W.B., Anderson, D.P., Lamers, C.H.J., Egberts, E., van Loon, J.J.A. and IJssel, J.P. Fish immunology and fish health. Fish Immunology, M.J. Manning & M.F. Tatner, (Eds.), Academic Press, London 1985.

42. Voiculescu, C., Stanciu, L., Voiculescu, M., Rogoz, S. and Dumitriu, I. (1983a). Experimental study of antibiotic-induced immunosuppression in mice - I. Humoral and cell-mediated immune responsiveness related to *in vivo* antibiotic treatment. Comp. Immun. Microbiol. Infect. Dis. 6: 291-299.

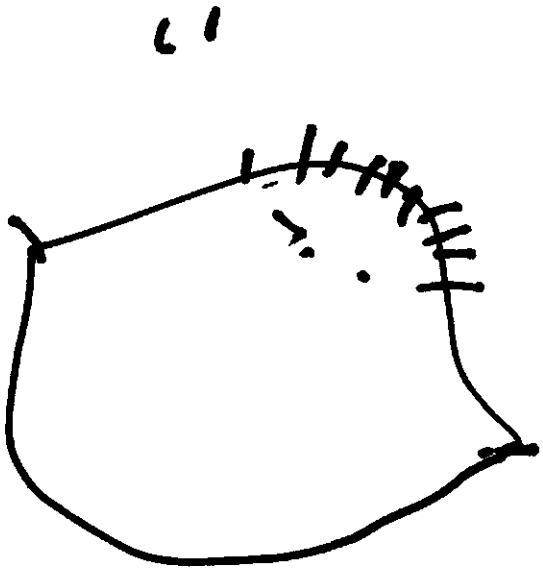
43. Voiculescu, C., Stanciu, L., Voiculescu, M., Rogoz, S., Dumitriu, I. and Nedelcu, C. (1983b). Experimental study of antibiotic-induced immunosuppression in mice - II. Th, Ts and NC cell involvement. Comp. Immun. Microbiol. Infect. Dis 6: 301-312.

44. Von Zabern, I., Przyklenk, H., Nolte, R. and Vogt, W. (1983). Effect of sulphonamides and penicillins on the complement system. Immunobiology, 165: (3-4) 378-379.

45. Wolff, L.J. and Stankova, L. (1983). Effect of sulfamethoxazole/trimethoprim on alveolar mononuclear phagocyte function. Clin. Res. 31: 123.

46. Ziv, G., Paape, M.J. and Dulin, A.M. (1983). Influence of antibiotics and intramammary products on phagocytosis of *Staphylococcus aureus* by bovine leukocytes. Am. J. Vet. Res. 44: 385-388.

INTRODUCTION TO THE PAPERS



INTRODUCTION TO THE PAPERS

There are many substances which are strongly growth inhibiting or detrimental to life of micro-organisms. Only relatively few of them can be featured by a selective toxicity towards bacteria without interfering with the host's biochemical pathways. Such compounds (antibiotics) are powerful instruments with which bacterial diseases can be treated effectively. According to the nature of the antibiotic, the micro-organisms are killed (bactericidal) or their growth is inhibited (bacteriostatic). In both cases the host defence mechanism has to eliminate the killed or growth-inhibited microbe properly to assure sterile conditions in the inner body tissues. Interference with these mechanisms by antibiotics may render the host more susceptible to super-infections or the initial infection may start again after stopping the drug treatment.

The aim of the studies presented in this thesis was to investigate the effect of antibiotics, particularly oxytetracycline (OxyTC), on the immune system of fish (carp) and birds (chickens) using immunological parameters.

A leukocyte stimulation assay was developed for carp to determine the in vitro effect of OxyTC on the mitogenic (phytohaemagglutinin and lipopolysaccharide) response (chapter 3). The in vitro experiments were extended by investigating the kinetics of the mitogenic response of carp leukocytes in the presence of OxyTC. Furthermore, attention was paid to other antimicrobial agents with respect to their interference with the phytohaemagglutinin-induced leukocyte activation (chapter 4).

Chapter 5 was an in vitro study on the existence of basic immunoregulatory factors in carp. This part was an experimental introduction to the studies in which the interference of OxyTC with cell cooperation (regulatory products) was investigated in chickens (chapter 6).

In chapter 7 the development of the anti-SRBC response was studied in carp during a short-term OxyTC treatment. Pharmacokinetic investigations were included to provide parameters for integration in the discussion on immuno-modulation.

The pharmacokinetic research in carp was extended in the work presented in chapter 8. Different routes of OxyTC administration were investigated and antibiotic tissue levels were determined. Furthermore, inter-species differences were studied with respect to pharmacokinetic behaviour of OxyTC

in rainbow trout and African catfish (chapter 9).

Analysis of the plasma drug concentration time curves following intravenous OxyTC administration revealed distinct phases. The two- and three-compartment open model adequately described the plasma drug levels in catfish and trout, respectively. A three-compartment model (see diagram) was also used for carp to derive pharmacokinetic parameters.

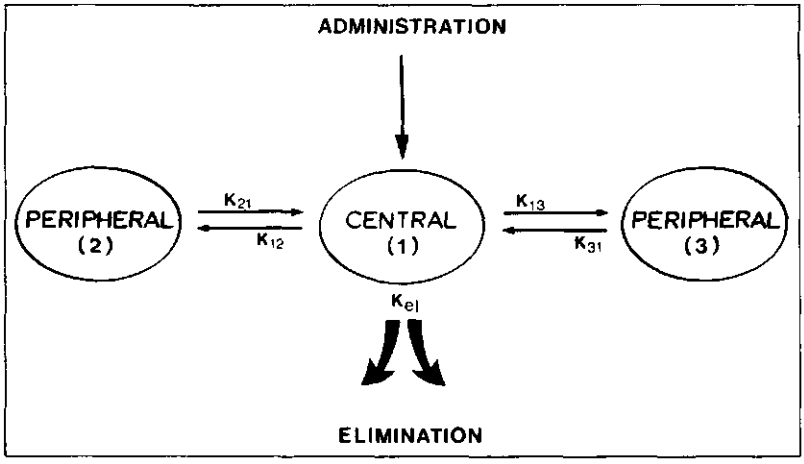
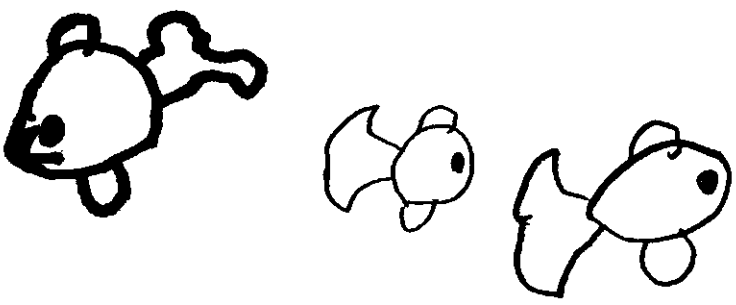


Diagram of the three-compartment open model. The drug is injected into the central compartment (1), where it distributes instantaneously. The drug distributes from the central compartment to the peripheral compartments (2, 3) and backwards with first-order rate constants K_{12}, K_{13} and K_{21}, K_{31}, respectively. Elimination, which comprises biotransformation and excretion, is assumed to occur exclusively from the central compartment. K_{el} is the first-order rate constant for drug elimination from the central compartment.

THE INFLUENCE OF ANTIBIOTICS ON THE IMMUNE SYSTEM. I. INHIBITION OF THE MITOGENIC LEUKOCYTE RESPONSE IN VITRO BY OXYTETRACYCLINE

J.L. GRONDEL AND H.J.A.M. BOESTEN



THE INFLUENCE OF ANTIBIOTICS ON THE IMMUNE SYSTEM. I. INHIBITION OF THE MITOGENIC LEUKOCYTE RESPONSE IN VITRO BY OXYTETRACYCLINE

John L. Grondel and Hubertus J.A.M. Boesten

Dept. of Experimental Animal Morphology and Cell Biology
and Dept. of Animal Husbandry
Agricultural University, P.O. Box 338,
6700 AH Wageningen, The Netherlands

INTRODUCTION

In large-scale fish culture, disease outbreaks can cause a substantial loss of animals. Vaccination programmes do prevent some of the infectious diseases, but not all. Antibiotics have proved to be of remarkable value for the therapeutic treatment and prevention (1, 2) of bacterial infections. Oxytetracycline (oxyTC), often used as food additive, is an effective drug against a wide range of bacterial diseases in fish. However, investigations on the occurrence of side effects of tetracyclines have been performed in medical research. Among these adverse effects gastro-intestinal disturbances, supra-infections of resistant bacteria, fungi or viruses and damage to liver and kidneys have been well described in clinical reports (3). Although rare, anaphylactoid reactions following the oral or intramuscular administration of tetracyclines in man have been reported (4).

It is known for some years that mitochondrial protein synthesis is impaired by oxyTC (5, 6, 7). As a consequence energy-dependent processes will be reduced. Especially rapidly dividing cells like intestinal epithelium (8) and activated lymphocytes are prone to these effects. Moreover, damage to the mucosa of the alimentary canal allows an easy entry of micro-organisms. Furthermore, the interference with the immune system will compromise the defense mechanisms.

In fish, only a few reports deal with drug-induced damage to blood and blood forming organs (9) or with impairment of the immune apparatus (10). Recently, it has been shown (11, 12) that oxyTC administered either by mixing with food or by intraperitoneal injection severely reduces the in

vivo immune response of carp. The allogeneic scale rejection was delayed and the humoral response to sheep red blood cells was inhibited. In order to obtain more information about the mode of action of oxyTC on the immune system of fish, we examined the effects of this agent on the mitogenic leukocyte response in vitro.

MATERIALS AND METHODS

Animals. Carp, Cyprinus carpio, were bred in our laboratory and kept in aquaria with running tap water at 20°C. Animals were fed pelleted dry food (K_{30}, Trouw & Co., Putten, The Netherlands), amounting 2.5% of their body weight/day, by means of a "Scharflinger" automatic feeder. Eight to ten months old animals, weighing 100-200 g were used.

Antibiotics and mitogens. Oxytetracycline (oxyTC) was purchased as Engemycine^R from Mycofarm, De Bilt, The Netherlands. Phytohaemagglutinin (PHA) grade P and Lipopolysaccharide (LPS) grade B, E. coli 055: B5 were obtained from Difco (Detroit, U.S.A.). Dilutions of oxyTC or the mitogens were made in culture medium without serum.

Cell culture. RPMI-1640 (Flow, Irvine, Scotland) buffered with 2.1 g/l $NaHCO_3$ (Merck, Darmstadt, F.R.G.) was supplemented with 100 IU/ml Penicillin-G (Serva, Heidelberg, F.R.G.), 100 µg/ml Streptomycine (Serva) and 5 IU/ml Heparine (Novo Industri, Copenhagen, Denmark). The culture medium contained 2 mM L-Glutamine (Merck) and was adjusted to pH 7.4. Single cell suspensions were prepared from thymus, pronephros, mesonephros and spleen. The organs were dissected out and suspensions were obtained by teasing the tissues in medium through a nylon mesh. The cells were washed twice in ice-cold medium. Cell viability was assessed using the trypan blue exclusion method and exceeded always 90%. The animals were bled before dissecting in order to reduce the contamination of the leukocyte suspension with erythrocytes. Bleeding of the caudal vein was performed under MS-222 (Sandoz, Basel, Switzerland) anaesthesia. Thymocyte suspensions contained less than 1% red blood cells while the erythrocyte content of other suspensions varied between 10 and 35%. The cells were cultured in round-bottom microtiter plates (Greiner, Nürtingen, F.R.G.) at a concentration of 2.5×10^5 cells/well in a volume of 200 µl. The medium was supplemented with 10% pooled carp-serum, previously inactivated at 56°C for 20 min. Triplicate cultures were incubated at 20°C in a humidified atmosphere containing 5% CO_2.

Measurement of DNA-synthesis. The cultures were pulsed at 68 h with 0.4 µCi ^{3}H-Thymidine (s.a. 5Ci/mMol, Radiochemical Centre, Amersham, England) in 20 µl medium. Sixteen hours later the cells were harvested on glass filters (Bioproducts, Walkersville, U.S.A.) with a Mash II Multiple Automated Sample Harvester (Dynatech, Nürtingen, F.R.G.), The filters were airdried and transferred to polyethylene vials (Lumac, Basel, Switserland). Finally, 1 ml scintillation fluid (Xylofluor, Baker, Phillipsburg, U.S.A.) was added to each vial and radio-activity was monitored in a Philips 4700 liquid scintillation spectrometer (Philips, Eindhoven, The Netherlands). All data are expressed in cpm ± sd.

RESULTS AND DISCUSSION

The leukocytes were stimulated at optimal mitogen concentrations as determined in preliminary experiments: PHA 50 µg/ml and LPS 200 µg/ml. Except for the thymocytes, the mitogenic effect of PHA and LPS on cells from spleen, mesonephros and pronephros resulted in a significant increase of the thymidine incorporation.

Various doses of oxyTC were added at the start of spleen cell cultures. As visualized in Fig. 1 the PHA response showed an oxyTC dependent decrease. This regards the suppression of ^{3}H-Thymidine incorporation into DNA. The 50% inhibition level was reached at 4-6 µg oxyTC/ml. Similar results were obtained using the mitogen LPS. The observed suppression of incorporation was not due to cytotoxic properties of the drug. This was measured by dye exclusion. It has been shown earlier (11, 12) that oxyTC severely impaired the in vivo immune response of carp. Under these circumstances serum levels of 1-15 µg oxyTC/ml were observed (11). At these concentrations mitochondrial protein synthesis will be inhibited (13). The conclusion seems to be justified that there is a good correlation between the in vivo experiments of Rijkers et al. (12) and our in vitro results.

Except for thymocytes, leukocytes from the spleen, pronephros and mesonephros showed a clear mitogenic response (Fig. 2). Although the cells originated from various lymphoid organs and the ability to respond to LPS was not similar, the DNA-synthesis was seriously suppressed in the presence of 10 µg oxyTC/ml. Obviously, there was no clear difference in cell-membrane permeability for oxyTC. The thymidine incorporation will be different from organ to organ because of the relative number of cells responding to a

certain mitogen. Whether this is true for the thymus remains unclear at the moment. The thymocytes respond poorly to LPS (Fig. 2) or PHA (not shown here) under these conditions: stimulation indices 2.0 and 1.2 respectively. It is observed that the suppressive effect of oxyTC is proportional to the height of the response. The lymphocyte will probably become unresponsive

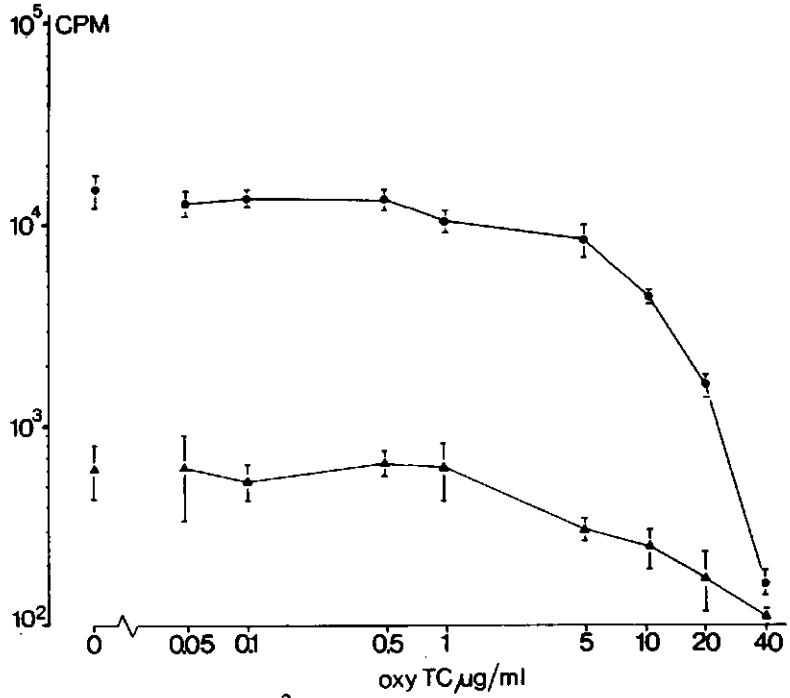


Fig. 1. Effect of oxyTC on ^{3}H-Thymidine incorporation into DNA. (● —— ●) Spleen cells stimulated with PHA, 50 µg/ml. (▲ — ▲) Control cultures.

after blocking of the mitochondrial protein synthesis by oxyTC.

The presence of oxyTC from the start of the culture caused a marked suppression of the DNA-synthesis. Addition of the drug at 72 h after start of pronephros cell cultures, seemed to be less effective (Fig. 3). This indicates that the early events in the response are most sensitive. The data obtained sofar suggest an interference of oxyTC with cell proliferation. In addition, the regulation of the immune response and memory formation are dependent on cell co-operation (14). It has been shown that non-lymphoid cells like monocytes and macrophages are also susceptible to antiobiotics (9, 15). Therefore, it is tempting to speculate that oxyTC has also an influence on cellular interactions. Because of the suppressive effects this drug may be a useful tool for studying the regulation of the immune repsonse.

But selective and cautious use of oxyTC is recommended in commercial fish farming.

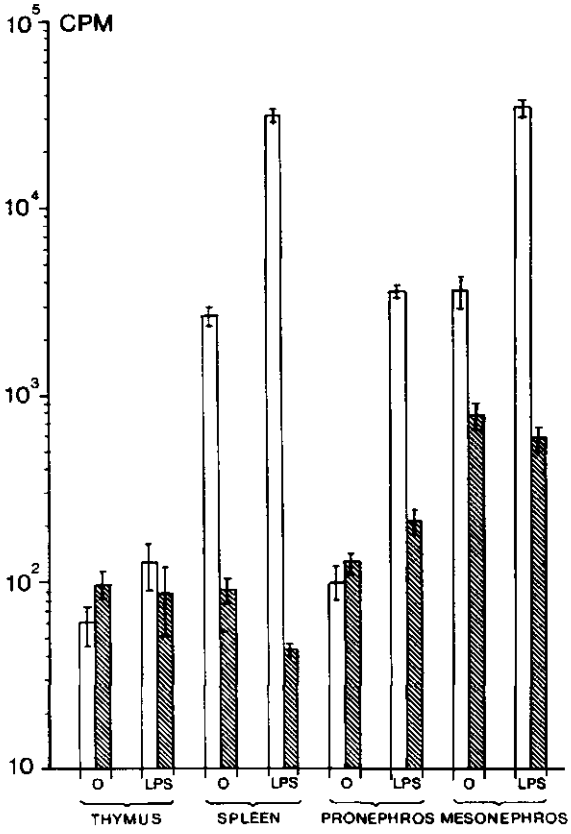


Fig. 2. Effect of oxyTC on the mitogenic response of leukocytes from thymus, spleen, pronephros and mesonephros. Open columns = control cultures, shaded columns = cultures with oxyTC (10 µg/ml). O = no mitogen added and LPS = 200 µg LPS/ml added.

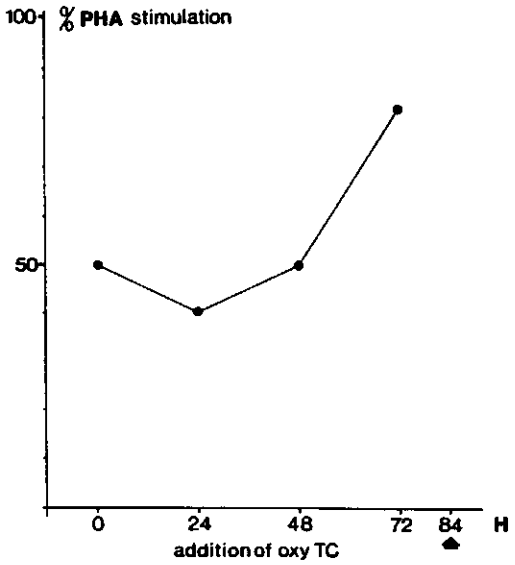


Fig. 3. Addition of oxyTC (10 µg/ml) at subsequent moments after starting the culture. Pronephros cells were stimulated with PHA, 50 µg/ml.

ACKNOWLEDGEMENTS

The authors thank Dr. W.B. van Muiswinkel and Dr. E. Egberts for helpful advice during the performance of this study and the preparation for the manuscript. Furthermore, we acknowledge Prof. C.C. Oosterlee for his interest and valuable suggestions. The authors wish to mention the technical support from the Dept. of Animal Physiology (Dr. J. van den Hoek). Mrs. A Tiemessen is kindly thanked for typing this manuscript.

REFERENCES

1. SCHAPERCLAUS, W., Erfolgreiche Bekämpfung der infectiösen Bauchwassersucht des Karpfens mit antibiotischen Mitteln in 11 Jahren, Z. Binnenfish. DDR, 14: 64-66, 1967.
2. BAUER, O.N., MUSSELIUS, V.A. and STRELKOV, Yu.-A., Diseases of Pond Fishes. Izdatel'stvo Kolos, Moskva, 220 pp., 1969.
3. GARROD, L.P., LAMBERT, H.P. and O'GRADY, F., Antibiotics and Chemotherapy. V. Livingston London, 149-166, 1973.

4. STEINBRUEGGE, J.M. and JUDSON, F.N., Type I Allergic Reaction to Orally Administered Tetracycline Hydrochloride. Sexually Transmitted Diseases. 193-194, 1980.
5. KROON, A.M. and ARENDZEN, A.J., The Inhibition of Mitochondrial Biogenesis by Antibiotics. Mitochondria and Biomembranes. Federation of European Biochemical Societies Eighth Meeting, Amsterdam, North-Holland American Elsevier, 28: 71-83, 1972.
6. KROON, A.M., DE VRIES, H. and NIJHOF, W., Protein Synthesis in Heart Mitochondria: Mechanism and Metabolic Aspects. Acta Cardiologica, 1: 1-13, 1976.
7. GIJZEL, W.P. and KROON, A.M., On The Protection of Bone Marrow Against Toxic Side Effects of Tetracyclines. J. Molecular Medicine, 3: 157-165, 1978.
8. DE JONGE, H., Toxicity of Tetracyclines in Rat-Small-Intestinal Epithelium. Biochemical Pharmacology, 22: 2659-2677, 1973.
9. KREUTZMANN, H.L., The Effects of Chloramphenicol and Oxytetracycline on Haematopoiesis in the European Eel. Aquaculture, 10: 323-334, 1977.
10. COOPER, E.L., Comparative Immunology. Prentice-Hall, Englewood Cliff, 338 pp., 1976.
11. RIJKERS, G.T., TEUNISSEN, A.G., VAN OOSTEROM, R. and VAN MUISWINKEL, W.B., The Immune System of Cyprinid Fish. The Immunosuppressive Effect of the Antibiotic Oxytetracycline in Carp. Aquaculture, 19: 177-189, 1980.
12. RIJKERS, G.T., VAN OOSTEROM, R. and VAN MUISWINKEL, W.B., The Immune System of Cyprinid Fish. Oxytetracycline and the Regulation of Humoral Immunity in Carp. Vet. Immunol. Immunopathol., 2: 281-290, 1981.
13. GIJZEL, W.P., STRATING, M. and KROON, A.M., The Biogenesis of Mitochondria. During Proliferation and Maturation of the Intestinal Epithelium of the Rat. Effects of Oxytetracycline. Cell Differentiation 1: 191-198, 1972.
14. UNANUE, E.R., The Regulation of the Immune Response by Macrophages. In: R. Van Furth (Editor) Mononuclear Phagocytes in Immunity, Infection and Pathology. Blackwell Scientific Publ., Oxford. pp. 721-738, 1975.
15. RHODES, M.W. and HSU, H.S., Effect of Kanamycin on the fate of Salmonella enteritidis, Within Cultured Macrophages of Guinea Pigs. J. Reticuloendothel. Soc., 15: 1-12, 1974.

THE INFLUENCE OF ANTIBIOTICS ON THE IMMUNE SYSTEM. II. MODULATION OF FISH LEUKOCYTE RESPONSES IN CULTURE

J.L. GRONDEL, A.G.M. GLOUDEMANS AND W.B. VAN MUISWINKEL

Veterinary Immunology and Immunopathology, 9 (1985) 251—260
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

THE INFLUENCE OF ANTIBIOTICS ON THE IMMUNE SYSTEM. II. MODULATION OF FISH LEUKOCYTE RESPONSES IN CULTURE

J.L. GRONDEL[1,2], A.G.M. GLOUDEMANS[1] and W.B. VAN MUISWINKEL[1]
Department of Experimental Animal Morphology & Cell Biology[(1)] and Department of Animal Husbandry[(2)], Agricultural University, P.O. Box 338, 6700 AH Wageningen, The Netherlands.

(Accepted 7 February 1985)

ABSTRACT

Grondel, J.L., Gloudemans, A.G.M. and van Muiswinkel, W.B., 1985. The influence of antibiotics on the immune system. II. Modulation of fish leukocyte responses in culture. Vet. Immunol. Immunopathol., 9: 251-260.

We report the immunomodulating effect of two tetracycline analogues (oxytetracycline and doxycycline) on mitogenic and allogeneic stimulation of carp (*Cyprinus carpio*) leukocytes *in vitro*. Both drugs interfered with ^{3}H-thymidine incorporation into the DNA of phytohaemagglutinin-stimulated pronephric leukocytes in a dose-dependent manner. Low concentrations of oxytetracycline delayed the mitogenic response, but did not reduce it.

In addition, several other antibiotics were tested for their possible interference with blastogenesis.

INTRODUCTION

Since the first isolation of tetracycline from *Streptomyces aureofaciens* in 1948 much work has been done on side effects of these drugs. Impaired phagocytic function of human leukocytes was observed after *in vitro* addition of chlortetracycline (Munoz and Geister, 1950). Interference with immunological defence mechanisms was caused by several analogues, including tetracycline (TC), doxycycline (DC), lymecycline (LC) and minocycline (MC).

Incubation of human neutrophils with TC or DC caused a decreased capacity to phagocytize yeast and bacteria. Leukocytes harvested from healthy donors after ingestion of TC also demonstrated a decreased phagocytic capacity for yeast (Forsgren *et al.*, 1974). In rats, it was shown that TC pretreatment had a suppressive effect on carbon clearance (Altura *et al.*, 1966). One of the properties of neutrophils is the directed migration induced by external stimuli. Both spontaneous and induced migration of human leukocytes *in vitro* was severely depressed by LC and DC (Belsheim *et al.*, 1979). Corresponding results were obtained in experiments on healthy volunteers given recommended dosages of the antibiotics and in patients treated with DC for post-operative infections. TC also inhibited the migration of human leukocytes *in vitro* (Forsgren and Schmeling, 1977; Forsgren *et al.*, 1978). In addition, mitogenic responses of human T and B lymphocytes were adversely affected by several tetracycline ana-

0165-2427/85/$03.30

logues (Banck and Forsgren, 1979; Thong and Ferrante, 1979).

Antibiotic immunomodulation is not restricted to the tetracyclines. The wide range of antibiotics which modulate immune responses *in vitro* as well as *in vivo* have been reviewed (Finch, 1980; Hauser and Remington, 1982). However, reports dealing with antimicrobial agents affecting the immune system of fish are scarce. Oxytetracycline (OxyTC) severely reduced the *in vivo* immune reponse of carp, evidenced by the prolongation of scale allograft survival and by suppression of the humoral response to sheep red blood cells (Rijkers *et.al.*, 1980, 1981). Immunosuppression has also been demonstrated in rainbow trout after feeding with pellets containing OxyTC (Anderson *et al.*, 1984; van Muiswinkel *et al.*, 1984). In a study designed to determine if antibiotics might directly influence immunocytes *in vitro*, we found that OxyTC inhibited mitogenic responses of carp leukocytes (Grondel and Boesten, 1982). Since OxyTC has widespread therapeutic applications in fish culture, it is important to understand the details of its immunomodulating effects. We have now done further work to obtain more insight into the mode of action of this drug, and have tested several other antibiotics for their possible interference with blastogenesis.

MATERIALS AND METHODS

Animals

Carp (*Cyprinus carpio* L.), 6-8 months of age, were bred in our laboratory and kept in aquaria with aerated running tapwater at a temperature of $22^{o} \pm 1^{o}C$. Animals were fed daily with pelleted dry food (K30, Trouw & Co., Putten, The Netherlands) by means of a "Scharflinger" automatic feeder.

Antibiotics

The following injectable antibiotics were used: oxytetracycline (Engemycine®, Mycofarm B.V., De Bilt, The Netherlands), doxycycline (Vibramycin®, Pfizer B.V., Rotterdam, The Netherlands), a combination of sulfatroxazole and trimethoprim (ratio 5:1, Leotrox®, Leo B.V., Emmen, The Netherlands), sulfadimethoxine (Intervet B.V., Boxmeer, The Netherlands), a combination of lincomycin and spectinomycin (ratio 1:2, Linco-Spectin®, Upjohn, Ede, The Netherlands), gentamicin (Gentocin-P®, A.C.F. Chemiefarma N.V., Maarssen, The Netherlands), ampicillin (Penbritin®, Beecham Farma B.V., Maarssen, The Netherlands), and chloramphenicol (Intervet B.V.). Powdered forms of sulfadimidine (Aesculaap B.V., Boxtel, The Netherlands), lincomycin-spectinomycin (ratio 1:2, Upjohn) and furaltadone (Furaltadone®, A.O.V. Cuyk, The Netherlands) were used. The solvent of the oxytetracycline solution was kindly provided by Gist-Brocades, Delft, The Netherlands. The commercially available antibiotics were freshly dissolved in

RPMI-1640 and added once at the start of the cultures.

Cell cultures

Standard RPMI-1640 culture medium (Flow, Irvine, Scotland) was buffered with 2.1 g/ml $NaHCO_3$ (pH 7.4), and supplemented with 2 mM L-glutamine (Merck, Darmstadt, F.R.G.), and 10% heat-inactivated pooled carp serum (PCS). Pronephric leukocytes were prepared and cultured as described before (Grondel and Boesten, 1982; Grondel and Harmsen, 1984). In brief, single cell suspensions were obtained by teasing the tissue through a nylon sieve, washed twice and finally resuspended in serum free culture medium. In all experiments cell viability was assessed using the trypan blue exclusion method. Cells were cultured for four days (unless otherwise specified) in round bottom microtiter plates (M24, Greiner, Nürtingen, F.R.G.) in a final volume of 0.2 ml per well in the presence of phytohaemagglutinin (50 µg/ml, PHA-P, Difco, Detroit, U.S.A.) or in medium only.

Two-way mixed leukocyte cultures were performed with peripheral blood leukocytes according to the method described by Gloudemans and Cohen (in prep.). Leukocytes were purified from heparinized blood by Percoll (Pharmacia, Uppsala, Sweden) cushion centrifugation. Cells collected from the interface were washed twice and finally resuspended in medium supplemented with 50 µM mercaptoethanol (Merck) and 0.2% PCS. Peripheral blood leukocytes (2.5×10^5) from each animal were cultured for 6 days in round-bottom microtiter plates under conditions identical to the pronephric leukocyte cultures mentioned above. In these experiments the individual variation was about 25%.

Measurement of DNA synthesis

DNA synthesis was assayed by ^{3}H-thymidine (^{3}H-TdR) incorporation after a 16-h pulse with 0.4 µCi ^{3}H-TdR (s.a. 5 Ci/mMol, Radiochemical Centre, Amersham, England) per well. Cells were harvested onto glass-fiber filters (Bioproducts, Walkersville, U.S.A.) using a multiple automated sample harvester (Mash II, Dynatech, Nürtingen, F.R.G.). The filters were dried and radioactivity was determined by scintillation spectrometry. Results are given for 2-4 representative experiments. Results of triplicate or quadruplicate cultures are expressed as the mean c.p.m. ± S.D., unless otherwise stated. The Student's t test was used for statistical evaluation.

RESULTS

Effect of tetracyclines on the mitogenic response of leukocytes

Both OxyTC and DC inhibited ^{3}H-thymidine incorporation into the DNA of PHA-stimulated pronephric leukocytes. The effects were dose-dependent (Figure 1).

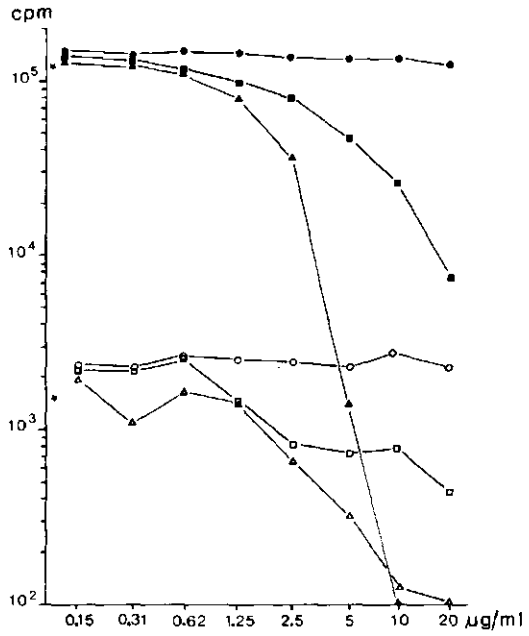


Fig. 1. The effect of OxyTC (■—■), DC (▲—▲) and the solvent of the OxyTC solution (●—●) on PHA-stimulated (closed symbols) and non-mitogen treated pronephric leukocytes (open symbols). Incorporation of ^{3}H-TdR in control cultures without antibiotics is indicated with asterisks (*). Cultures were harvested on day 4. Results are expressed as mean c.p.m. observed in triplicate cultures. The S.D. was about 10%.

At concentrations of 4-6 µg OxyTC and 1-2.5 µg DC per ml, the responses were reduced to about 50% of those of the mitogen-stimulated controls. DC was more suppressive than OxyTC. The difference between the two analogues may relate to the better lipid-solubility of doxycycline. Addition of Ca^{2+} in concentrations up to 1.7 mM to the culture medium, to compensate for the possible chelation of divalent cations by the drugs, did not relieve the impaired response (results not shown).

In order to exclude possible effects caused by the solvent, pronephric leukocytes were also exposed to various concentrations of the OxyTC solvent. Neither the background DNA synthesis of unstimulated control cells nor the same process in mitogen-activated leukocytes was significantly affected by the solvent (see Figure 1).

Kinetic change in the mitogenic response

PHA-stimulated pronephric leukocytes were cultured in the presence of 5, 10 or 15 µg OxyTC per ml. Cultures were harvested 2, 3, 4, 5, 6 or 7 days after initiation of the culture. ^{3}H-thymidine was added to the cultures 16 hours before harvesting. Figure 2 demonstrates a dose-dependent delay in the leukocyte response rather than a real suppressive effect by OxyTC. However, in the OxyTC treated cultures the peak was shifted to day 5 or later. Obviously, the impairment of cellular functions like DNA synthesis is not due to cytotoxicity as suggested in previous investigations (Grondel and Boesten, 1982).

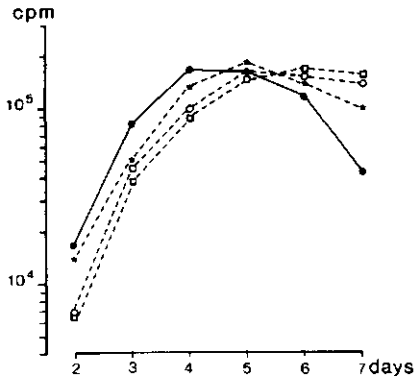


Fig. 2. Kinetics of thymidine uptake into PHA-stimulated pronephric leukocytes in the presence of 0 (● = control), 5 (★), 10 (○) or 15 (□) µg OxyTC per ml culture medium. Results are expressed as mean c.p.m. observed in quadruplicate cultures. The S.D. was about 10%.

Under our culture conditions the inhibitory effect on DNA synthesis was transient. The background level of ^{3}H-thymidine uptake by unstimulated control cells gradually decreases with time.

Effect of tetracyclines on the mixed leukocyte culture

In all experiments mentioned above, leukocytes were polyclonally activated by PHA. Stimulation of DNA synthesis without mitogens can be achieved by culturing peripheral blood or pronephric leukocytes from genetically dissimilar individuals (mixed leukocyte culture). Addition of the tetracycline analogues to such cultures at concentrations of 10 µg OxyTC or 5 µg DC per ml also reduced the ^{3}H-thymidine incorporation markedly: 61% and 98% for OxyTC and DC respectively (Figure 3).

The influence of different antibiotics on the mitogenic response

Different antibiotics were added in various concentrations to routine PHA stimulation tests (Figure 4). Five antibiotics [sulfatroxazole/trimethoprim (5:1), sulfadimethoxine, sulfadimidine, lincomycin/spectinomycin (1:2, injectable solution) and ampicillin] did not suppress the mitogenic response of the pronephric leukocytes. On the contrary, an increased incorporation of ^{3}H-thymidine was observed especially at low concentrations. Gentamicin and furaltadone

256

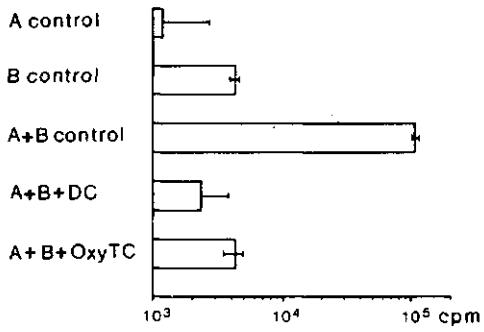


Fig. 3. The effect of OxyTC (10 µg/ml) or DC (5 µg/ml) on the DNA synthesis of allogeneic stimulated peripheral blood leukocytes (PBL). PBL of genetically dissimilar individuals (A and B) were cultured for 6 days. The results of quadruplicate cultures are expressed in c.p.m. ± S.D.

showed a dose-dependent inhibition of the mitogenic response. Thymidine uptake was stimulated by chloramphenicol at concentrations less than 5 µg/ml, whereas quantities equal to or exceeding this concentration became suppressive.

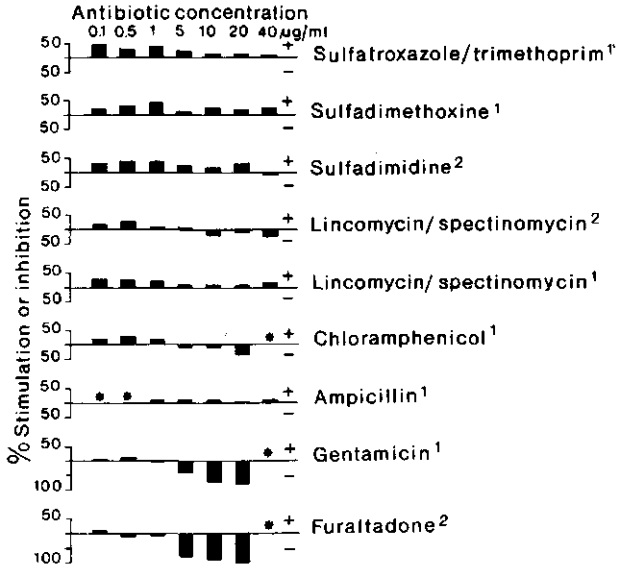


Fig. 4. Several antibiotics were tested for their possible interference with DNA synthesis. PHA-activated pronephric leukocytes were cultured for 4 days in the absence or presence of varying concentrations of different antibiotics. The results of triplicate cultures are expressed in percentage stimulation or inhibition compared with mitogen-treated control cultures. 1) injectable, 2) powder and *) not tested.

DISCUSSION

Our results show that OxyTC and DC affect mitogenic as well as allogeneic responses of carp leukocytes *in vitro*. This confirms our previous data showing that the incorporation of ^{3}H-thymidine into DNA of PHA- or LPS-stimulated leukocytes was depressed (Grondel and Boesten, 1982). Furthermore, the results show that OxyTC influences the kinetics of the response.

The difference between the two tetracycline analogues in their ability to suppress the *in vitro* response can be explained by their chemical structure and related properties (Neu, 1978).

For instance, the chelating properties of tetracyclines for divalent cations could interfere with the mitogenic response of leukocytes (Diamantstein and Odenwald, 1974). Our results are in agreement with those reported by Banck and Forsgren (1979) and Thong and Ferrante (1979) who showed that DC profoundly inhibited proliferative responses. At low concentrations DC suppressed both the mitogenic responses of human T and B lymphocytes and *in vitro* antibody production (Banck and Forsgren, 1979). The reversibility of the inhibitory effect was also studied by these authors: preincubation of the cells for 1 h in 10 µg DC per ml culture medium supplemented with 10% serum did not affect mitogenic responsiveness during subsequent culturing in the absence of tetracyclines (Thong and Ferrante, 1979). However, lymphocytes could not be restimulated when washed after two days of incubation with 50 µg DC per ml serum-free culture medium (Banck and Forsgren, 1979). The irreversible effect of this high drug concentration may be due to inhibition of cytoplasmatic protein synthesis (Banck and Forsgren, 1979; Gijzel and Kroon, 1978). At low doses (10 µg/ml), only mitochondrial protein synthesis appears to be inhibited (van den Bogert and Kroon, 1981). Thus, the discrepancy mentioned above can be explained by the different experimental conditions in these studies.

It should be emphasized that the active drug concentration will decrease with time after initial addition to the culture. Therefore, the immunosuppressive effect should be more striking if there were compensation for the loss of biological activity of the drug throughout the experiment.

In addition, our experiments showed that the kinetics of mitogenic responses were changed at low concentrations of OxyTC. In fact the suppression of the ^{3}H-TdR incorporation into DNA was transient. Similarly, immune responses may be delayed *in vivo*: treatment of carp with OxyTC affected the kinetics of the humoral response against sheep red blood cells (manuscript in preparation).

Chemotaxis and subsequent phagocytosis are important processes by which pathogenic microorganisms can be rendered inoffensive. It has been demonstrated that tetracyclines exert an adverse effect on these defence mechanisms. This

was observed in man, both *in vitro* (Forsgren *et al.*, 1974; Belsheim *et al.*, 1979; Forsgren and Schmeling, 1977; Forsgren *et al.*, 1978) and *in vivo* (Forsgren *et al.*, 1974; Belsheim *et al.*, 1979). Tetracyclines are bacteriostatic agents and interfere with bacterial protein synthesis at the 30S ribosomal level. The binding of tetracyclines to the ribosomes is reversible. Under appropriate conditions the microorganism can subsequently start growing again if the drug leaks out of the bacterium (Neu, 1978). Therefore, impairment of the immune system by bacteriostatic compounds may have serious implications for the outcome of therapy, especially when recovery needs a relatively long period.

Interactions between macrophages and lymphocytes are essential for the induction of a proper immune reponse. Macrophages have a variety of functions including uptake of foreign material (antigens), degradation and subsequent presentation of antigens to lymphocytes (Unanue, 1980). It is obvious that impairment of the initial processes in the immune response will decrease the effectiveness of the defence mechanisms. It has been demonstrated that macrophages accumulate a wide range of antibiotics, tetracycline included, when incubated in the presence of drug concentrations similar to clinically appropriate serum levels (Johnson *et al.*, 1980). As mentioned earlier, chemotactic and phagocytic processes can be affected by antibiotics. Investigations on the production of lymphokines revealed that the secretion of migration inhibition factor was suppressed by antibiotics, e.g. rifampicin (Serrou, 1974).

In conclusion, oxytetracycline and doxycycline interfere with the mitogenic and allogeneic response of carp leukocytes. Furaltadone, gentamicin and to a lesser extent chloramphenicol and lincomycin/spectinomycin also exerted suppressive effects when tested in the same system. It is interesting that the other tested antibiotics (sulfatroxazole/trimethoprim, sulfadimethoxine, sulfadimidine, injectable lincomycin/spectinomycin and ampicillin) enhanced the uptake of ^{3}H-TdR. Clearly, the leukocyte stimulation assay provides a sensitive tool for screening side effects of antibiotics. The antimicrobial agents which have been found to be responsible for immunomodulation *in vitro* need further investigation. However, it is beyond the scope of this study to subject all these compounds and solvents to detailed research.

These observations stress the notion that a careful choice has to be made before certain drugs are used. The prolonged application of tetracyclines mixed with food, as is usual in some fish culture systems for the prevention of diseases, should be avoided. On the other hand, these immunomodulating agents may be valuable tools for studying regulation of the immune response in fish.

ACKNOWLEDGEMENTS

We would like to thank Mrs. M.S.J. van der Wal - van Beckum for her skilful secretarial assistance and Mr. W.J.A. Valen for drawing the illustrations.

REFERENCES

Altura, B.M., Hershey, S.G., Ali, M. and Thaw, C., 1966. Influence of tetracycline on phagocytosis, infection and resistance to experimental shock: relationship to microcirculation. J. Reticuloendoth. Soc., 3: 447-457.

Anderson, D.P., Muiswinkel van, W.B. and Roberson, B.S., 1984. Effects of chemically induced immune modulation on infectious diseases of fish. In: M. Kende, J. Gainer and M. Chirigos (editors), Chemical regulation of immunity in Veterinary Medicine. A.R. Liss Inc., New York, pp. 187-211.

Banck, G. and Forsgren, A., 1979. Antibiotics and suppression of lymphocyte function *in vitro*. Antimicrob. Agents and Chemother., 16: 554-560.

Belsheim, J., Gnarpe, H. and Persson, S., 1979. Tetracyclines and host defense mechanisms: interference with leukocyte chemotaxis. Scand. J. Infect. Dis., 11: 141-145.

Bogert van den, J.J. and Kroon, A.M., 1981. Tissue distribution and effects on mitochondrial protein synthesis of tetracyclines after prolonged continuous intravenous administration to rats. Biochem. Pharmacol, 30: 1706-1709.

Diamantstein, T. and Odenwald, M.V., 1974. Control of the immune response *in vitro* by calcium ions. I. The antagonistic actions of calcium ions on cell proliferation and on cell differentiation. Immunology 27: 531-541.

Finch, R., 1980. Immunomodulating effects of antimicrobial agents. J. Antimicrob. Chemother., 6: 691-699.

Forsgren, A., Schmeling, D. and Quie, P.G., 1974. Effect of tetracycline on the phagocytic function of human leukocytes. J. Infect. Dis., 130: 412-415.

Forsgren, A. and Schmeling, D., 1977. Effect of antibiotics on chemotaxis of human leukocytes. Antimicrob. Agents Chemother., 11: 580-584.

Forsgren, A., Schmeling, D. and Banck, G., 1978. Effect of antibiotics on chemotaxis of human polymorphonuclear leukocytes *in vitro*. Infection 6 (suppl. 1): 8102-8106.

Gijzel, W.P. and Kroon, A.M., 1978. On the protection of bone marrow against toxic side effects of tetracyclines. J. Mol. Med., 3: 157-165.

Grondel, J.L. and Boesten, H.J.A.M., 1982. The influence of antibiotics on the immune system I. Inhibition of the mitogenic leukocyte response *in vitro* by oxytetracycline. Dev. Comp. Immunol., Suppl. 2, pp. 211-216.

Grondel, J.L. and Harmsen, E.G.M., 1984. Phylogeny of interleukins: growth factors produced by leukocytes of the cyprinid fish, *Cyprinus carpio* L. Immunology 52: 477-482.

Hauser, W.E. and Remington, J.S., 1982. Effects of antibiotics on the immune response. Am. J. Medicine, 72: 711-716.

Hesketh, T.R., Smith, G.A., Houslay, M.D., Warren, G.B. and Metcalfe, J.C., 1977. Is an early calcium flux necessary to stimulate lymphocytes? Nature, 267: 490-494.

Johnson, J.D., Hand, W.L., Francis, J.B., King-Thompson, N. and Corwin, R.W., 1980. Antibiotic uptake by alveolar macrophages. J. Lab. Clin. Med., 95: 429-439.

Munoz, J. and Geister, R., 1950. Inhibition of phagocytosis by aureomycin. Proc. Soc. Exp. Biol. Med., 75: 367-370.

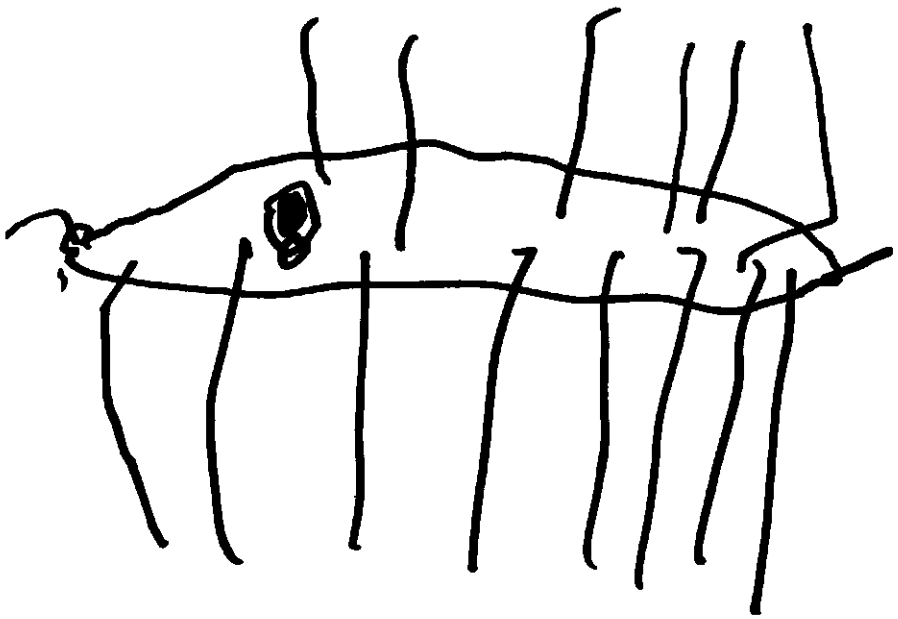
Muiswinkel van, W.B., Anderson, D.P., Lamers, C.H.J., Egberts, E., Loon van, J.J.A. and IJssel, J.P., 1984. Fish immunology and fish health. Fish Immunology, M.J. Manning & M.F. Tatner, eds., Proceedings of the Plymouth Meeting 1983, Academic Press, London (in press).

Neu, H.C., 1978. A symposium on the tetracyclines: A major appraisal. Bull. N.Y. Acad. Med., 54: 141-155.

Rijkers, G.T., Teunissen, A.G., Oosterom van, R. and Muiswinkel van, W.B., 1980. The immune system of cyprinid fish. The immunosuppressive effect of the antibiotic oxytetracycline in carp. Aquaculture, 19: 177-189.
Rijkers, G.T., Oosterom van, R. and Muiswinkel van, W.B., 1981. The immune system of cyprinid fish. Oxytetracycline and the regulation of humoral immunity in carp. Vet. Immunol. Immunophathol., 2: 281-290.
Serrou, B., 1974. Rifampicin and immunosuppression. Lancet, 11: 172.
Thong, Y.H. and Ferrante, A., 1979. Inhibition of mitogen-induced human lymphocyte proliferative responses by tetracycline analogues. Clin. Exp. Immunol., 35: 443-446.
Unanue, E.R., 1980. Cooperation between mononuclear phagocytes and lymphocytes in immunity. New England J. Med., 303: 977-985.

PHYLOGENY OF INTERLEUKINS: GROWTH FACTORS PRODUCED BY LEUCOCYTES OF THE CYPRINID FISH, CYPRINUS CARPIO L.

J.L. GRONDEL AND E.G.M. HARMSEN



Immunology 1984 **52** 477

Phylogeny of interleukins: growth factors produced by leucocytes of the cyprinid fish, *Cyprinus carpio* L.

J. L. GRONDEL & E. G. M. HARMSEN *Department of Experimental Animal Morphology & Cell Biology and Department of Animal Husbandry, Agricultural University, Wageningen, The Netherlands*

Accepted for publication 23 January 1984

Summary. Supernatants of phytohaemagglutinin (PHA)-activated pronephric leucocytes from carp (*Cyprinus carpio* L.) contain a lymphocyte growth factor which can induce a proliferative response of purified lymphoblasts but not freshly isolated leucocytes. The growth-promoting activity can be reduced by absorbing the supernatant with mitogen-activated blasts. In addition, increased incorporation of tritiated thymidine into PHA-activated blast cells is also induced by supernatants from two-way mixed leucocyte cultures. The data show that even at the evolutionary level of teleost fish, amplifying/regulatory leucocyte products exist. It is suggested that these factors play as important a role in the regulation of the immune response in fish as they do in mammals.

INTRODUCTION

Soluble factors formed by distinct populations of leucocytes play a pivotal role in the regulation of the immune response. In mammals, these regulatory products can be detected in supernatants of cultures of allogeneic leucocytes and mitogen-stimulated leucocytes (Gillis *et al.*, 1978; Andersson *et al.*, 1979; Miller *et al.*, 1980; Susskind & Faanes, 1981; Kern *et al.*, 1981). Depending on the assay system used, two factors, interleukin-1 (IL-1) and interleukin-2 (IL-2) can be distinguished. IL-1 is a macrophage-derived product (Smith *et al.*, 1980) that, together with mitogen or antigen, triggers the T-helper cell to produce IL-2 (Smith *et al.*, 1980; Farrar *et al.*, 1980). IL-2 exerts its activity only on activated cells (e.g. blasts) and maintains their proliferative capacity (Smith *et al.*, 1979). IL-2 influences antibody responses to T-dependent antigens positively (Leibson, Marrack & Kappler, 1981) and supports the maturation of cytotoxic precursor cells into effectors (Shaw *et al.*, 1978; Wagner *et al.*, 1980).

Although there is a relatively great phylogenetic distance between mammals and fish, this latter group of ectothermic vertebrates is characterized by well-developed humoral and cellular (Corbel, 1975) immune systems. Thymus, spleen, pronephros and mesonephros are the major lymphoid organs; bone marrow and lymph nodes are lacking. In carp, the pronephros and mesonephros are the major sites of antibody formation. The spleen plays a minor role (Rijkers, Frederix-Wolters & van Muiswinkel, 1980). Despite our knowledge of many facets of the immune system of fish, the existence and the role of modulating interleukin-like factors of fish has not been examined.

The purpose of the present study was to trace the phylogenetic ancestry of such amplifying/regulatory factors by determining whether they are produced by fish leucocytes.

Correspondence: Dr J. L. Grondel, Agricultural University, Dept. of Experimental Animal Morphology & Cell Biology, Zodiac, P.O. Box 338, 6700 AH Wageningen, The Netherlands.

MATERIALS AND METHODS

Animals

Outbred carp, 6–8 months of age, were raised in our laboratory. They were kept in aquaria with aerated running tapwater at a temperature of 22±1°. The animals were fed daily with pelleted dry food (K30, Trouw and Co, Putten, The Netherlands) by means of a 'Scharflinger' automatic feeder.

Cell cultures

Standard culture medium (RPMI-1640, Flow, Irvine, Scotland), diluted to carp tonicity (270 mosmol), was buffered with 2·1 g/litre $NaHCO_3$ (pH 7·4) and was supplemented with 2 mM L-glutamine (Merck, Darmstadt, F.R.G.), 100 μg/ml Streptomycin (Serva, Heidelberg, F.R.G.) and 100 IU/ml penicillin-G (Serva). This standard medium was further supplemented with 10% heat-inactivated pooled carp serum (PCS). Pronephric leucocytes were prepared as described earlier (Grondel & Boesten, 1982). In brief, MS-222 (Sandoz, Basel, Switzerland)-anaesthetized animals were bled before dissection to reduce contamination of the leucocyte suspensions with erythrocytes. Single cell suspensions were obtained by teasing the tissue (in serum-free culture medium) through a nylon sieve. The cells were washed twice in ice-cold culture medium without serum and cell viability was assessed using the trypan blue exclusion method. Cells were cultured in round-bottom microtitre plates (M24 ART, Greiner, Nürtingen, F.R.G.) in a volume of 0·2 ml per well. Triplicate cultures were incubated at 29° in a humidified atmosphere of 5% CO_2 plus 95% air. DNA-synthesis was assayed by [^{3}H]-thymidine ([^{3}H]-TdR) incorporation after a 16 hr pulse with 0·4 μCi [^{3}H]-TdR per culture (specific activity 5 Ci/mmol, Radiochemical Centre, Amersham, U.K.). Cells were harvested onto glass-fibre strips (Bioproducts, Walkersville, U.S.A.) using a Multiple Automated Sample Harvester (Mash II, Dynatech, Nürtingen, F.R.G.). The filters were dried and radioactivity was determined by scintillation spectrometry. All results are expressed as the arithmetic mean c.p.m. ± SD observed in triplicate cultures.

Production of active supernatants

Active supernatants (ASN) were obtained by incubating a 5 ml pronephric leucocyte suspension (5×10^6 cells per ml culture medium) in 25 cm^2 tissue culture flasks (no. 3050, Costar, Cambridge, U.S.A.) for 3 hr in the presence of 50 μg/ml phytohaemagglutinin (PHA-P, Difco, Detroit, U.S.A.). After this incubation at 29°, the mitogen-containing medium was discarded and the adherent monolayer of cells was rinsed three times with fresh medium at room temperature. Subsequently, the mitogen-activated cells were cultured for another 20 hr with 5 ml culture medium. The supernatants were collected, centrifuged at 1000 *g* for 20 min at 4°, sterilized by filtering through 0·2 μm membrane filters (Gelman, Michigan, U.S.A.) and stored at −20°. Control supernatants (CSN) of non-mitogen-treated leucocyte cultures served as controls. An IL-2-containing supernatant of a mouse cell line (EL-4) was kindly provided by Dr W. A. Buurman, Department of Surgery, Biomedical Centre, State University of Limburg, The Netherlands.

Preparation of blasts and assay for growth activity

In previous studies, blasts were characterized by their change in density and size during the mitogen activation relative to non-mitogen-treated control cells, using continuous Percoll gradients and staining techniques (A. van den Ouweland, personal communication). In addition, pronephric leucocytes ($2{\cdot}5 \times 10^7$ cells) were cultured for 4 days in 25 cm^2 culture flasks (Costar) in a total volume of 5 ml culture medium supplemented with 50 μg/ml PHA. After the incubation period, the PHA-activated cells were mechanically agitated by pipette, harvested and washed twice. In addition, the cell suspension was layered over a Percoll cushion (Pharmacia, Uppsala, Sweden) with a density of 1·055 g/ml and centrifuged for 20 min at 800 *g* at 4°. Blasts, collected from the interface, were washed twice, resuspended in culture medium and diluted in the same medium to the appropriate cell concentration. These cells were then subcultured in the presence of ASN, CSN, mitogen or medium only. Growth activity was determined by [^{3}H]-thymidine uptake (see above section on 'Cell cultures').

RESULTS

The assay for growth factor activity of a given supernatant was based on the ability of a supernatant to promote growth of blasts in the absence of mitogen. Due to their active state, freshly isolated PHA blasts incorporated significant levels of tritiated thymidine. Therefore, the number of blast cells per well was titrated so as to provide an assay population that by itself, only incorporated low levels of thymidine. Cultures that contained 1×10^5 or $0{\cdot}5 \times 10^5$ blasts

incorporated significant levels of tritiated thymidine on days 1 and 2 of culture and significantly reduced levels on days 3–6. Cultures that contained $2{\cdot}5 \times 10^4$ blasts, however, incorporated only low levels of thymidine on all days tested. Therefore, this cell number was used in the assay throughout this study.

Supernatant fluids were added at the start of the blast cell culture. Maximal activity of the supernatants, at 50% final concentration (0·1 ml cell suspension and 0·1 ml supernatant), progressively declined upon serial dilution (data not shown). For this reason growth activity found at 50% supernatant concentration is presented for all experiments.

Supernatants of cultures of pronephric leucocytes that had been incubated with PHA for only 3 hr maintained proliferative activity of PHA blasts for at least 5 days. Figure 1 presents results from one of two experiments that involved two different supernatants. In these experiments, both supernatants of non-mitogen-treated leucocyte cultures as well as fresh medium served as controls. Figure 1 shows that both types of control cultures incorporated only low levels of tritiated thymidine during the experiment. PHA by itself was mitogenic for blasts, but significantly less so than the active supernatant. Based on these kinetic data and to increase the sensitivity to different supernatant fluids, blasts were harvested in the logarithmic-phase (on day 3 and/or 4 of culture) in the subsequent experiments rather than on day 5.

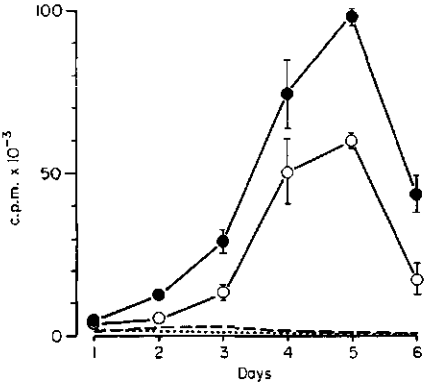


Figure 1. Kinetics of thymidine uptake into blast cells. Active supernatant (ASN) of PHA-activated pronephric leucocytes was tested on $2{\cdot}5 \times 10^4$ blasts per well at 50% final concentration (0·1 ml cell suspension and 0·1 ml ASN). PHA responsiveness was detected by culturing blast cells in the presence of a control supernatant (CSN) of non-mitogen-treated leucocyte culture supplemented with PHA (final concentration 50 μg/ml). Blasts cultured in CSN or medium only served as controls. ASN (●); CSN+PHA (○); CSN (– – –), fresh medium (· · · · ·).

Results suggest that leucocyte-formed growth factors, rather than any contaminating PHA, were responsible for the continued proliferation of PHA blasts. Titration experiments revealed that PHA agg-

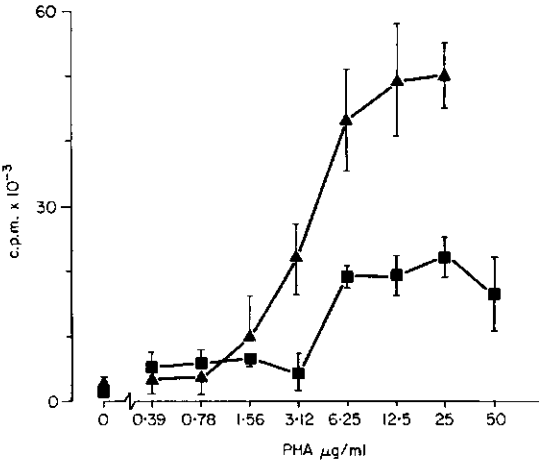


Figure 2. Proliferative response of blasts to various concentrations of PHA. Blast cells ($2{\cdot}5 \times 10^4$ per well) were cultured for 3 (■) and 4 (▲) days. Controls were cultured in medium without PHA.

lutinates sheep erythrocytes and is mitogenic for blast cells at concentrations higher than or equal to 0·39 μg/ml (mitogen titration in Fig. 2; agglutination data not shown). Since the four supernatants were neither haemagglutinating nor mitogenic for virgin pronephric leucocytes, any PHA contained in the supernatant was below the concentration detectable by these assays. As seen in Fig. 3, substances generated and released into the supernatant fluid during 24 and 48 hrs two-way mixed leucocyte cultures (MLC) also contained growth factors as assayed with PHA blasts. No mitogens were added to such cultures. It should be noted that MLC-generated supernatant fluid substances reported in Fig. 3 effected a 2·7-fold (24 hr MLC-supernatant) or 4·3-fold (48 hr MLC-supernatant) enhancement of tritiated thymidine incorporation compared with medium controls. Although significant, this increase was less than that seen for PHA-generated growth factors. The mixed leucocyte cultures were harvested on day 6 and stimulation indices around 40 and more were recorded. However, the highest levels of PHA stimulation are already achieved on the fourth day of culture. Therefore, it is conceivable that the difference between the growth activities can be explained by the polyclonal abilities of PHA. In contrast, an IL-2-containing supernatant of a mouse cell line (EL-4) was not able to activate carp blasts, indicating that these factors are probably specific for certain vertebrate groups.

Several observations suggest that blasts are the preferential targets of the growth factors. (i) The PHA-generated supernatants were mitogenic when added to PHA blasts. However, they were not stimulatory when added to cultures of freshly harvested (virgin) pronephric leucocytes (Fig. 4b). (ii) When an active supernatant supplemented with PHA (final concentration 50 μg/ml) was added to either virgin leucocytes or to PHA blasts, there was hardly any stimulation beyond that noted for the addition of PHA or supernatant separately (Fig. 4a). Adsorption

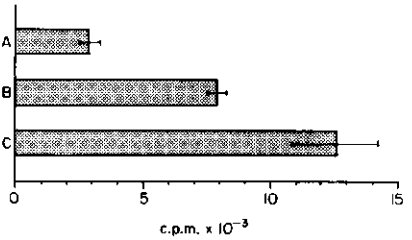


Figure 3. Proliferation of mitogen-activated blast cells in response to factors present in supernatant of a two-way mixed leucocyte culture. Pronephric leucocytes ($1{\cdot}25 \times 10^7$ cells) from each animal were cultured in 25 cm^2 flasks in a total volume of 5 ml culture medium. After 24 hr (B) and 48 hr (C) supernatants were harvested, centrifuged, filter-sterilized and tested at a final concentration of 50%. Control blasts were incubated with medium only (A). Cultures were harvested on day 3.

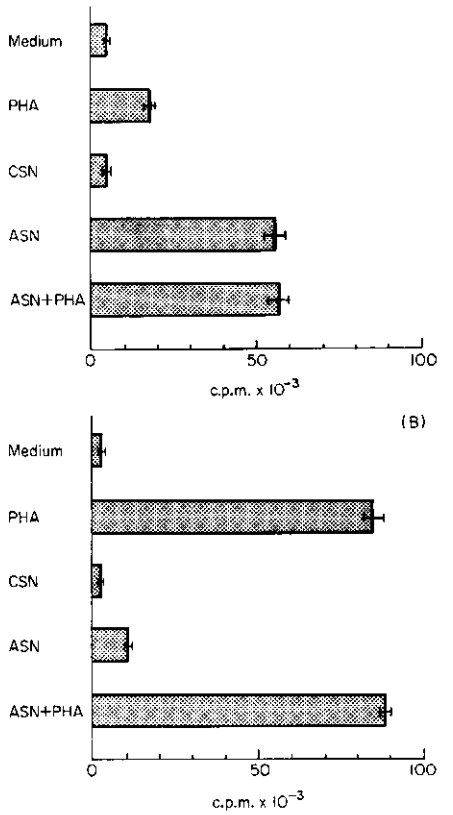


Figure 4. Effect of (active) supernatant (ASN) of PHA-stimulated pronephric leucocytes and control supernatants (CSN) or non-mitogen-treated leucocytes. Blast cells ($2{\cdot}5 \times 10^4$ per well, A) or virgin pronephric leucocytes ($2{\cdot}5 \times 10^5$ per well, B) were incubated for 3 (A) or 4 (B) days, respectively with medium only or medium supplemented with PHA (50 μg/ml), 50% CSN, 50% ASN or 50% ASN + PHA.

of a supernatant by PHA blasts reduced thymidine uptake by blasts by 36–48% in two experiments involving different supernatants. In contrast, incubation of one of these supernatants with virgin leucocytes did not significantly reduce the activity (Fig. 5).

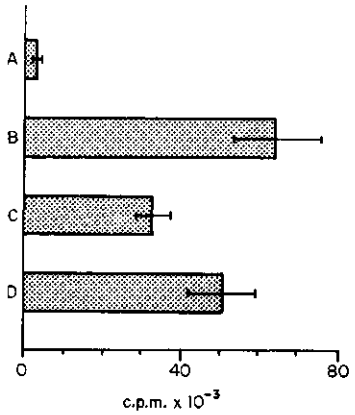


Figure 5. Effect of adsorption of growth promoting supernatants by PHA-stimulated blasts or virgin pronephric leucocytes. A cell-free supernatant (0·5 ml) was adsorbed by $2{\cdot}5 \times 10^7$ blasts (C) or by $2{\cdot}5 \times 10^7$ freshly prepared pronephric leucocytes (D) and was tested at a final concentration of 50% on $2{\cdot}5 \times 10^4$ blasts per well, cultured for 3 days. Adsorption was carried out by incubating the cells for 4 hr at 4°. The adsorbed supernatants were filter-sterilized after removing the cells by centrifugation. Blasts incubated with medium only (A) or with unadsorbed supernatant (B) served as controls. *P* value: for comparison between B and C, $<0{\cdot}01$; for B and D, not significant.

DISCUSSION

The study presented here was designed to investigate whether fish leucocytes form growth factors in response to mitogenic or allogeneic stimulation analogous to mammalian interleukins. For the production of the growth factor (or factors), a modification of the method described by Miller *et al.* (1980) for mice was used. Our procedure yields an active supernatant fluid free of mitogenic amounts of PHA without further processing. Freedom from mitogenic activity was determined by freshly isolated pronephric leucocytes being unable to respond to the active supernatant. This observation indicates both the absence of appropiate PHA concentrations as well as the inability of resting cells to respond to factors which sustain blast cell growth. The main problem in the procedures for the production of IL-2 in mammals is the presence of the lectin in the preparation and its possible interference with the growth-promoting activity. It was demonstrated that high levels of IL-2 were obtained by short-term treatment of murine and human lymphocytes with PHA or concanavalin A (Con A) (Spiess & Rosenberg, 1981; Wu *et al.*, 1982). Washing the IL-2-producing cells reduced the lectin concentration to levels which were insufficient to stimulate freshly isolated lymphocytes (Spiess & Rosenberg, 1981). Only activated lymphocytes were susceptible to IL-2.

In our experiments, supernatant fluids without mitogens were obtained by culturing allogeneic-stimulated carp leucocytes. The MLC-generated supernatant also contained a growth-promoting activity, although its activity was less compared to supernatants derived from PHA-stimulated carp leucocytes. It is conceivable that this difference relates to the polyclonal activation abilities of PHA.

For the detection of growth-promoting factors in supernatants lectin-activated blasts were used as indicator cells. Proliferation of those activated cells could be maintained for several days by addition of the growth factor. However, the indicator cells were still sensitive to PHA. Freshly isolated mouse T cell blasts also show a lectin responsiveness, but after repeated subculture those blasts become totally unresponsive to the mitogen used (Andersson *et al.*, 1979). At that time, their continuous proliferation will be dependent on the presence of IL-2.

The results of the adsorption experiments show that PHA-activated carp blasts can remove the growth factors from supernatants to a certain extent, suggesting the existence of receptors on these cells. This observation is consistent with the current view that only activated cells actually can adsorb IL-2 (Coutinho *et al.*, 1979; Kern *et al.*, 1981), whereas freshly isolated leucocytes remove only small amounts from the supernatants (Smith *et al.*, 1979).

Recently, factors with IL-2 activity have been reported for chickens (Schauenstein, Globerson & Wick, 1982). From a phylogenetic point of view, it was interesting to note the lack of any mutual cross-reactivity between murine and avian growth factors. Within mammals the absence of species specificity is reported (Gillis *et al.*, 1978; Coutinho *et al.*, 1979; Gillis, Smith & Watson, 1980). Mouse, rat and human IL-2 prep-

arations show identical biological activity when tested with a mouse assay system (Gillis *et al.*, 1980). On the other hand, the proliferation of human cells showed a preference for a homologous growth factor (Lafferty, Andrus & Prowse, 1980).

The results presented in this paper demonstrate that carp pronephric leucocytes produce soluble factors upon mitogenic or allogeneic stimulation. The abilities of these factors strongly resemble those of mammalian IL-2. However, the homology between these substances has yet to be determined biochemically. Whether the presence of macrophages and/or a soluble factor produced by these cells are required for the stimulation of carp leucocytes is under investigation.

ACKNOWLEDGMENTS

Prof. N. Cohen (Department of Microbiology, Division of Immunology, University of Rochester School of Medicin and Dentistry, Rochester, U.S.A.), Dr W. B. van Muiswinkel and Dr E. Egberts are gratefully thanked for their stimulating interest and advice during the performance of this study and preparation of the manuscript. We thank Mr W. J. A. Valen for drawing the illustrations.

REFERENCES

Andersson J., Grönvik K.O., Larsson E.L. & Coutinho A. (1979) Studies on T lymphocyte activation. I. Requirements for the mitogen-dependent production of T cell growth factors. *Eur. J. Immunol.* **9,** 581.

Corbel M.J. (1975) The immune response in fish: a review. *J. Fish Biol.* **7,** 539.

Coutinho A., Larsson E.L., Grönvik K.O. & Andersson A. (1979) Studies on T lymphocyte activation. II. The target cells for concanavalin A-induced growth factors. *Eur. J. Immunol.* **9,** 587.

Farrar J.J., Mizel S.B., Fuller-Farrar J., Farrar W.L. & Hilfiker M.L. (1980) Macrophage-independent activation of helper T cells. I. Production of interleukin 2. *J. Immunol.* **125,** 793.

Gillis S., Ferm M.M., Ou W. & Smith K.A. (1978) T cell growth factor: parameters of production and a quantitative microassay for activity. *J. Immunol.* **120,** 2027.

Gillis S., Smith K.A. & Watson J. (1980) Biochemical characterization of lymphocyte regulatory molecules. II. Purification of a class of rat and human lymphokines. *J. Immunol.* **124,** 1954.

Grondel J.L. & Boesten H.J.A.M. (1982) The influence of antibiotics on the immune system. I. Inhibition of the mitogenic leukocyte response *in vitro* by oxytetracycline. *Dev. Comp. Immunol.* Suppl. 2, 211.

Kern D.E., Gillis S., Okada M. & Henney C.S. (1981) The role of interleukin-2 (IL-2) in the differentiation of cytotoxic T cells: the effect of monoclonal anti-IL-2 antibody and absorption with IL-2 dependent T cell lines. *J. Immunol.* **127,** 1323.

Lafferty K.J., Andrus L. & Prowse S.J. (1980) Role of lymphokine and antigen in the control of specific T cell responses. *Immunol. Rev.* **51,** 279.

Leibson H.J., Marrack P. & Kappler J.W. (1981) B cell helper factors. I. Requirement for both interleukin 2 and another 40.000 mol wt factor. *J. exp. Med.* **154,** 1681.

Miller R.G., Lalande M.E., Derry H. & Paetkau V. (1980) Second signal requirements of cytotoxic T lymphocyte precursors. *Behring Inst. Mitt.* **67,** 41.

Rijkers G.T., Frederix-Wolters E.M.H. & van Muiswinkel W.B. (1980) The immune system of cyprinid fish. Kinetics and temperature dependence of antibody-producing cells in carp (*Cyprinus carpio*). *Immunology*, **41,** 91.

Schauenstein K., Globerson A. & Wick G. (1982) Avian lymphokines. I. Thymic cell growth factor in supernatants of mitogen stimulated chicken spleen cells. *Dev. Comp. Immunol.* **6,** 533.

Shaw J., Monticone V., Mills G. & Paetkau V. (1978) Effects of costimulator on immune responses *in vitro*. *J. Immunol.* **120,** 1974.

Smith K.A., Gillis S., Baker P.E. & McKenzie D. (1979) T cell growth factor-mediated T cell proliferation. *Ann. N.Y. Acad. Sci.* **332,** 423.

Smith K.A., Lachman L.B., Oppenheim J.J. & Favata M.F. (1980) The functional relationship of the interleukins. *J. exp. Med.* **151,** 1551.

Spiess P.J. & Rosenberg S.A. (1981) A simplified method for the production of murine T cell growth factor free of lectin. *J. immunol. Meth.* **42,** 213.

Susskind B.M. & Faanes R.B. (1981) Effects of γ-irradiation on lymphocyte subpopulations participating in the development of the cytotoxic T lymphocyte response. *J. Immunol.* **127,** 1485.

Wagner H., Röllinghoff M., Pfizenmaier K., Hardt C. & Johnscher G. (1980) T-T cell interactions during *in vitro* cytotoxic T lymphocyte (CTL) responses. II. Helper factor from activated Lyt 1^+ T cells in rate limiting (i) in T cell responses to nonimmunogenic alloantigen, (ii) in thymocyte responses to allogeneic stimulator cells, and (iii) recruits allo- or H-2-restricted CTL precursors from the Lyt 123^+ T subset. *J. Immunol.* **124,** 1058.

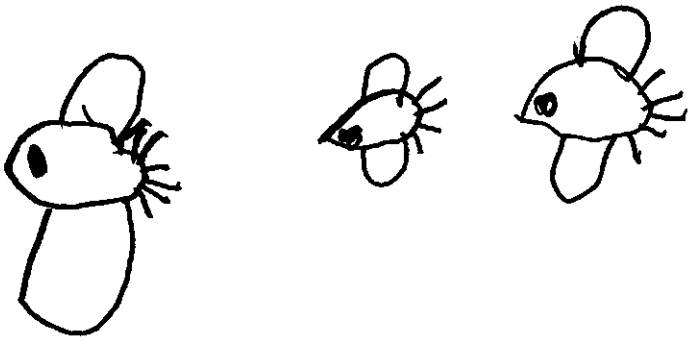
Wu Y., Erngerg J., Masucci M.G., Johnson D., Klein E. & Klein G. (1982) Human T cell growth factor (TCGF) produced by repeated stimulation of non-adherent human lymphocytes. *J. immunol. Meth.* **51,** 35.

Note added in proof: After this manuscript was accepted for publication, Caspi & Avtalion (1984) obtained results with respect to factors promoting the proliferation of carp T-like cells. In contrast to our results, they report that the proliferation of fish lymphoblasts can be sustained using IL-2 enriched preparations from mammalian lymphocytes.

Caspi R.R. & Avtalion R.R. (1984) Evidence for the existence of an IL-2 like lymphocyte growth promoting factor in a bony fish, *Cyprinus carpio*. *Dev. comp. Immunol.* (in press).

THE INFLUENCE OF ANTIBIOTICS ON THE IMMUNE SYSTEM. III. INVESTIGATIONS ON THE CELLULAR FUNCTIONS OF CHICKEN LEUKOCYTES IN VITRO

J.L. GRONDEL, G.C. ANGENENT AND E. EGBERTS



Veterinary Immunology and Immunopathology, 10 (1985) 307–316
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

THE INFLUENCE OF ANTIBIOTICS ON THE IMMUNE SYSTEM III. INVESTIGATIONS ON THE CELLULAR FUNCTIONS OF CHICKEN LEUKOCYTES IN VITRO

J.L. GRONDEL, G.C. ANGENENT and E. EGBERTS

Department of Experimental Animal Morphology & Cell Biology,

and Department of Animal Husbandry,

Agricultural University, P.O. Box 338, 6700 AH Wageningen, The Netherlands.

(Accepted 25 June 1985)

ABSTRACT

Grondel, J.L., Angenent, G.C. and Egberts, E., 1985. The influence of antibiotics on the immune system. III. Investigations on the cellular functions of chicken leukocytes in vitro. Vet. Immunol. Immunopathol., 10: 307-316.

Tetracyclines are bacteriostatic antibiotics widely used in veterinary medicine. It is reported here that the tetracycline analogue oxytetracycline (OxyTC) interferes with the mitogenic response of chicken leukocytes. A dose-dependent suppression of lectin stimulation of peripheral blood and spleen leukocytes was observed. The kinetics of the DNA-synthetic activity were seriously affected. The antimicrobial agent does not seem to have any effect on interleukin 2 (IL-2) production, whereas the uptake of ^{3}H-thymidine by IL-2-dependent T cell blasts was severely reduced. It is concluded that the T cell blast is the target for OxyTC.

INTRODUCTION

An effective immune response is subject to amplification and regulation. Specialized cells are responsible for the production of factors which play a crucial role in this complex regulatory mechanism. A T cell-dependent immune response is amplified by the action of interleukin 1 (IL-1) and interleukin 2 (IL-2) on T cell subsets. Both factors appear to be proliferative and/or differentiative signals for T and B lymphocytes during the course of the response (Smith et al., 1980; Oppenheim and Gery, 1982). The process of amplification is dependent upon both the level of interleukin synthesis and induction of receptors. The antigenic specificity of the immune response appears to be preserved at the level of receptor induction. The properties of IL-2 and its receptor have recently been reviewed by Robb (1984).

Interleukins have been isolated and characterized in many mammalian species. Recently, the existence and role of interleukin-like factors have been demonstrated in birds (Schauenstein et al., 1982; Hayari et al., 1982; Schnetzler et al., 1983) and fish (Grondel and Harmsen, 1984; Caspi and Avtalion, 1984), emphasizing the phylogenetic importance of amplifying/regulary factors for a regular immune reaction.

Drugs can be valuable tools for the study of the regulation of the immune response. Some antibiotics have been shown to interfere with the immunological defense mechanisms. The effects of a wide range of antimicrobial agents on the immune response in vitro as well as in vivo have been reviewed by Finch (1980) and Hauser and Remington (1982), and concern different mammalian species.

0165-2427/85/$03.30

Chemotaxis and subsequent phagocytosis, important neutrophil functions, are suppressed by several tetracycline analogues (Belsheim et al., 1979; Forsgren et al., 1974; Forsgren and Schmeling, 1977; Forsgren et al., 1978). Furthermore, it has been demonstrated that these bacteriostatic substances affect the mitogenic response of leukocytes (Banck and Forsgren, 1979; Thong and Ferrante, 1979; Grondel and Boesten, 1982; Grondel et al., 1985).

In vivo investigations with fish have revealed that both cellular and humoral immunity are suppressed during oxytetracycline (OxyTC) treatment (Rijkers et al., 1980; Rijkers et al., 1981).

This investigation focusses on the immunomodulating action of OxyTC on chicken leukocytes in vitro. The study was performed in order to obtain information about the possible interference of OxyTC with the early events during the mitogenic response of spleen leukocytes.

MATERIALS AND METHODS

Animals

White Leghorn pullets aged 5 to 9 weeks were used. The animals were kept in battery cages and received water and food ad libitum without antibiotics.

Culture medium and mitogens

The culture medium RPMI-1640 was buffered with $NaHCO_3$ (2.1 g/l, pH 7.4) and supplemented with 100 IU/ml penicillin (Serva, Heidelberg, F.R.G.), 100 µg/ml streptomycin (Serva) and 2 mM glutamine (Merck, Darmstadt, F.R.G.). This medium was further supplemented with 2 µg/ml bovine serum albumin (BSA; Povite Products, Oss, The Netherlands) and 1% heat inactivated fetal calf serum (FCS; Gibco, Glasgow, Scotland).

Phytohaemagglutin (PHA-P) and lipopolysaccharide (LPS) grade b (E.coli 055: B5) were obtained from Difco (Detroit, U.S.A.) and concanavalin A (Con A) was obtained from Sigma (St. Louis, U.S.A.). Dilutions of the mitogens were made in culture medium. Unless specified otherwise, the following mitogen concentrations were used in the microcultures throughout the study: Con A 1.25 µg/ml and PHA 100 µg/ml.

The antibiotic oxytetracycline (OxyTC) was purchased as Engemycin^R from Mycofarm, De Bilt, The Netherlands. The solvent for OxyTC was kindly provided by Gist Brocades, Delft, The Netherlands. OxyTC and the solvent were dissolved in culture medium and added only once at the start of the cultures.

Cell cultures

Single cell suspensions were obtained by teasing spleen tissue through a nylon sieve. The cells were washed twice in ice-cold culture medium.

Heparinized blood was taken from the wing vein and diluted 1:1 with culture

medium. The cell suspension was layered over a 63% Percoll cushion (Pharmacia, Uppsala, Sweden) and centrifuged for 20 min at 500 x g and 4°C. Peripheral blood leukocytes (PBL) were collected from the interface, washed twice, and resuspended in culture medium. The cell viability was assessed using the trypan blue exclusion method.

Spleen cells or PBL (2.5×10^5) were cultured at 40°C in roundbottom micro-titre plates (M24 ART, Greiner, Nürtingen, F.R.G.) in a volume of 0.2 ml per well. Triplicate cultures were incubated for two days at 40°C in a humidified atmosphere of 5% CO_2 plus 95% air. DNA synthesis was assayed by (^{3}H)-thymidine (^{3}H-TdR) incorporation after a 16 h pulse with 0.4 µCi ^{3}H-TdR per culture (specific activity 5 Ci/mmol; Radiochemical Centre, Amersham, U.K.). Cells were harvested onto glass-fibre strips (Bioproducts, Walkersville, U.S.A.) using a Multiple Automated Sample Harvester (Mash II; Dynatech, Nürtingen, F.R.G.). The filters were dried, and radioactivity was determined by scintillation spectrometry. All results are expressed as arithmetic mean c.p.m. ± S.D.

Production of IL-2-containing supernatants

IL-2-containing supernatants were obtained by incubating a 25 ml spleen cell suspension (5×10^6 per ml culture medium) in 75-cm^2 tissue culture flasks (Costar, Cambridge, U.S.A.) for 3 h in the presence of 10 µg/ml Con A. After the incubation at 40°C, the mitogen-containing medium was discarded and the adherent monolayer of cells was rinsed three times with warm (40°C) medium with FCS or BSA. Subsequently, the mitogen-activated spleen cells were cultured for another 20-24 h with 15 ml culture medium. The supernatants were collected, centrifuged at 1000 x g for 20 min at 4°C, sterilized by filtering through 0.2-µm membrane filters (Gelman, Michigan, U.S.A.), and stored at -20°C. Supernatants of non-mitogen- treated cultures served as controls.

Supernatants containing OxyTC were intensively dialyzed in the cold against RPMI-1640 for at least 24 h.

Assay for IL-2 activity

IL-2 activity was assayed using Con A-activated blasts as the indicator cell population. A 25-ml spleen cell suspension (5×10^6 ml culture medium) was incubated for two days in the presence of 10 µg/ml Con A. The non-adherent cells were washed twice with medium containing 25 mM α-methyl-D-mannoside (Sigma), layered over a 50% Percoll cushion and centrifuged for 20 min at 800 x g and 4°C. The T cell blasts were collected from the interface, washed twice and resuspended in medium with 20% FCS, without BSA.

IL-2 activity was determined by culturing 10^5 cells per well for 2 days in the presence of 50% supernatant; final FCS conc. 10%. Growth activity was determined by (^{3}H)-thymidine uptake (see above section on "Cell cultures").

RESULTS

To investigate whether OxyTC affects mitogenesis of T cells, OxyTC was added in various concentrations to cultures of freshly isolated leukocytes. This resulted in a suppression by OxyTC of the mitogenic response of leukocytes obtained from either spleen or peripheral blood in a dose-dependent manner. The DNA synthesis of both spleen cells and PBL stimulated by Con A and PHA, respectively, was reduced to 50% at an antibiotic concentration ranging from 12.5 to 25 µg/ml. The solvent was also tested at various dilutions comparable to the amounts present in the stimulation test. The solvent did not affect the mitogenic response. At the 50% inhibition level neither OxyTC nor the solvent were cytotoxic as determined by the trypan blue exclusion method at successive intervals after the initiation of the culture (results not shown).

To investigate whether OxyTC influences the kinetics of the mitogenic response, PHA-stimulated PBL were cultured in the presence of 25 µg OxyTC/ml. The control and antibiotic-treated cultures were pulsed for 16 h with ^{3}H-TdR before harvesting on subsequent days. The kinetics of ^{3}H-TdR incorporation by lectin-activated PBL showed that the DNA synthesis was severely suppressed by OxyTC (see figure 1).

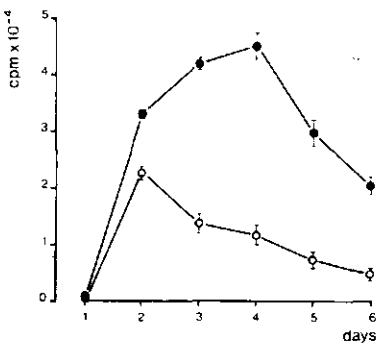


Fig. 1. Kinetics of thymidine incorporation into PHA-stimulated PBL in the presence of OxyTC (25 µg/ml, open symbols) or medium (= control, closed symbols). The results of triplicate cultures are expressed as mean c.p.m. ± S.D.

After two days of culture the label uptake was reduced to about 70% of the control cultures, and an even sharper decline was observed on the following days. Cultures treated with the solvent appeared to be unaffected.

In the former experiments, OxyTC was added only once at the beginning of the incubation period. Therefore, experiments were set up to examine the effects of OxyTC on PBL which had already passed the initiation process. OxyTC was administered to the cultures at serial intervals after lectin induction. As shown in figure 2, the inhibitory effect was most pronounced during the early stages of the mitogen-induced T cell proliferation.

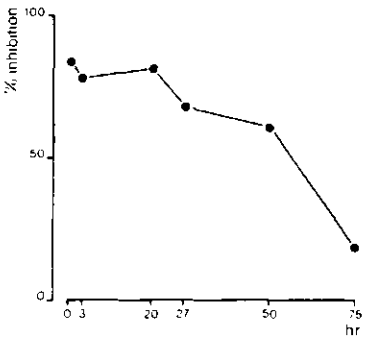


Fig. 2. OxyTC (25 µg/ml) was administered to the cultures at serial intervals after PHA stimulation. The results of triplicate PBL cultures are expressed as percentages of inhibition compared with mitogen-treated control cultures.

Since it has been shown that interleukins are an essential component of a proper mitogenic response, we tested whether OxyTC interferes with the IL-2 dependence of mitogenesis.

Supernatants derived from Con A-stimulated spleen cell cultures were tested for their growth-promoting effect on T cell blasts. The factor produced and tested under these conditions is analogous to the mammalian IL-2 (Schauenstein et al., 1982). In the presence of stimulated spleen cell-derived supernatants the blasts showed an increased incorporation of ^{3}H-TdR (see figure 3A). Addition of Con A to such cultures did not influence the radioactive label uptake significantly. Supernatants which were stimulatory for T cell blasts were not mitogenic for freshly isolated spleen cells (figure 3B). The latter, however, could be activated with the addition of Con A to an IL-2-containing supernatant (results not shown). Furthermore, supernatants from non-mitogen-treated spleen cells were not able to induce mitogenesis of T cell blasts.

In addition, the IL-2 activity could be adsorbed to T cell blasts. Freshly isolated spleen cells as a source of resting cells did not remove significant amounts of activity (results not shown).

To determine the kinetics of IL-2 production in the presence of OxyTC, supernatants of Con A-induced spleen cell cultures were harvested at different time intervals. To exclude the inhibitory effect of OxyTC, the collected supernatants were intensively dialyzed against RPMI-1640. Thereafter, they were filter sterilized and tested for their IL-2 activity on T cell blasts. Figure 4A shows that maximal activity in control supernatants, which were not dialyzed, was obtained around 21 h. This corresponds to the time chosen in the standard procedure to collect IL-2 containing supernatants from Con A-induced spleen cell cultures.

However, when control supernatants from cultures of the same spleen cell preparation were dialyzed, an enhanced growth-promoting activity on T cell blasts was observed (see figure 4B). Although the peak activity was usually found in

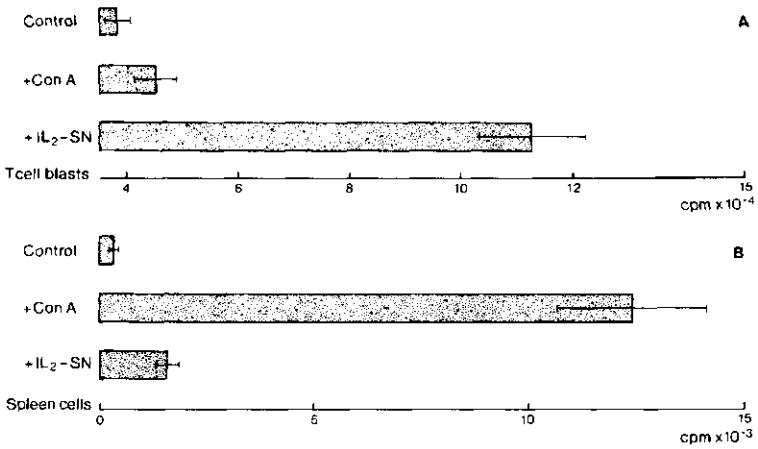


Fig. 3. The growth-promoting effect of supernatants derived from Con A-stimulated spleen cell cultures (IL2-SN) on T cell blasts (A) and spleen cells (B). Results are expressed as mean c.p.m. ± S.D. observed in triplicate cultures.

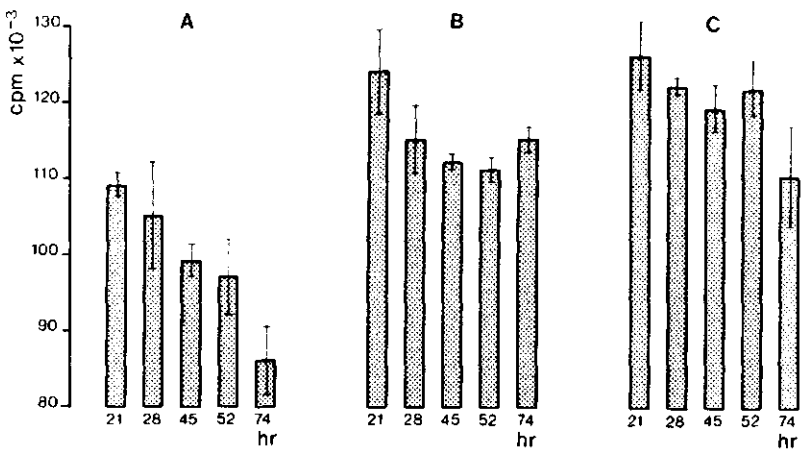


Fig. 4. Supernatants were harvested at different time intervals from Con A-activated spleen cells cultured in the absence (A,B) or presence (C) of 25 µg/ml OxyTC. Their growth-promoting effect on T cell blasts was tested without dialysis (A), or after dialysis (B,C).

21-h supernatants, the decline of the growth-promoting effect was much less pronounced than in control preparations. As shown in figure 4C, the production of IL-2 was not significantly affected in the presence of OxyTC.

Control cultures of T cell blasts cultured for a period of 48 h always show a background level of DNA synthesis. This is due to their active state immediately after their isolation. The label uptake in these control cultures was influenced by OxyTC to a degree comparable with mitogen-induced spleen cell cultures. In the presence of an IL-2-containing supernatant (for control stimulation, see figure 5B) the DNA synthesis was severely suppressed by OxyTC. The 50% inhibition level was already reached at a concentration of ± 10 µg/ml (see figure 5A).

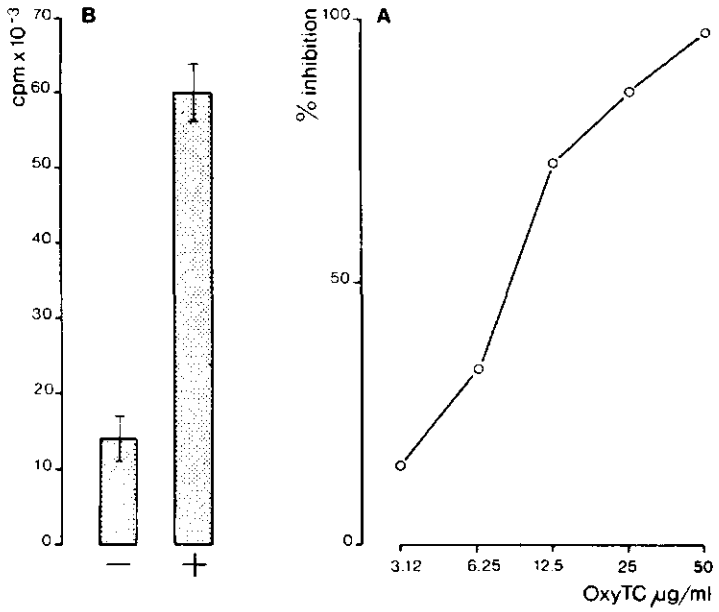


Fig. 5. A. The effect of OxyTC on the incorporation of ^{3}H-TdR into the DNA of T cell blasts in the presence of an IL-2-containing supernatant. B. Incorporation of ^{3}H-TdR in control cultures with medium (-) or with the IL-2-containing supernatant (+). Results are expressed as mean c.p.m. ± S.D. of triplicate cultures.

DISCUSSION

Investigations on the immune system as a target for side effects of drugs concern mainly mammalian species. It has been shown for several antibiotics that antimicrobial treatment in vitro as well as in vivo, results in immunomodulation (Finch, 1980; Hauser and Remington, 1982). The results reported in this paper show that avian leukocytes are affected by the bacteriostatic substance OxyTC.

A dose-dependent inhibition of the mitogen-induced DNA synthesis of both PBL and spleen leukocytes was observed. Our results are in agreement with previous investigations on mitogenic responses of fish leukocytes (Grondel and Boesten, 1982; Grondel et al., 1985), and with results reported by Banck and Forsgren (1979) and Thong and Ferrante (1979) who showed a profound inhibition of the proliferative response of human leukocytes in the presence of the tetracycline analogue doxycycline.

It has been reported that the stability of OxyTC depends upon the pH of the medium (van den Bogert, 1983). The anti-microbial activity of OxyTC dissolved in 0.15M NaCl (20°C, pH 7.2) was reduced to 50% within 24 h. Only at a pH value of 3 or less will the antibiotic be stable for several days. Of course, these acid conditions are incompatible with culturing cells. Therefore, the assumption seems to be justified that the active drug concentration will decrease with time in our culture system (40°C, pH 7.4).

In addition, OxyTC interferes with the protein synthesis of sensitive bacteria and, at relatively low concentrations, with certain eukaryotic cells. It appears that both events are reversible (Neu, 1978; de Vries and Kroon, 1970). Despite the instability of the drug and the reversible action on protein synthesis, the mitogenic response of chicken PBL seems to be blocked rather than delayed. In fish, it also has been observed that treatment of leukocyte cultures with OxyTC affects the kinetics of the mitogenic reaction, but here, the peak of maximum activity is just delayed (Grondel et al., 1985).

It should be emphasized that an immune response is a complex interplay of specialized cells and factors, and is subject to strict control. An immune reaction is a dynamic process. Therefore, during the course of the response the conditions are continuously changing. Events like antigen uptake and processing by macrophages, and the release of amplifying/regulatory substances, interact with the expansion of specific T cells (Unanue, 1980). In other words, using the DNA synthesis of proliferating T cells as a parameter of immune reactivity corresponds to looking at an instant picture. However, immunomodulating drugs can directly or indirectly affect the outcome of that picture. From our results it can be concluded that the early stages of the mitogenic response of chicken T lymphocytes are most sensitive to OxyTC. Therefore, we tested whether the production of interleukin-2, a T cell growth-promoting factor, and/or the DNA synthesis of IL-2-dependent T cell blasts were suppressed by the antibacterial agent.

The results described show that OxyTC does not seem to have any effect on the formation of IL-2. On the other hand, in the presence of both OxyTC and an IL-2-containing supernatant the label uptake by growth-factor-dependent blasts was severely reduced.

Various explanations are possible, e.g. the synthesis and/or expression of functionally active IL-2 receptors could be inhibited. The most likely explanation at the moment is the lack of energy, needed for these rapidly dividing T cell blasts, caused by the inhibition of mitochondrial protein synthesis. Evidence for impaired cell proliferation by tetracycline has been put forward in experiments with mammalian cell cultures (Leezenberg et al., 1979). Furthermore, it has been shown that OxyTC depressed the antibody response as well as the delayed-type hypersensitivity response to sheep red blood cells, a T cell-dependent antigen (van den Bogert and Kroon, 1982). From these experiments, it has been suggested that the formation of T cells was reduced.

So far, the T cell or T cell blast seems to be the target for the interference of OxyTC with the immune system. On the other hand, there are several investigations dealing with the impairment of chemotaxis and subsequent processes, e.g. phagocytosis, by tetracycline analogues (Munoz and Geister, 1950; Forsgren et al., 1974, 1978; Forsgren and Schmeling, 1977; Belsheim et al., 1979). Those observations stress that the role of neutrophils and macrophages in the immune response, in relation to side effects of drugs, needs to be clarified.

Since tetracyclines are widely used as antibiotics in veterinary medicine, we recommend that their prolonged application should be avoided.

ACKNOWLEDGEMENTS

The authors wish to thank Mr. M.G.B. Nieuwland for his technical assistance.

REFERENCES

Banck, G. and Forsgren, A., 1979. Antibiotics and suppression of lymphocyte function in vitro. Antimicrob. Agents and Chemother. 16: 554-568.

Belsheim, J., Gnarpe, H. and Persson, S., 1979. Tetracyclines and host defense mechanisms: interference with leukocyte chemotaxis. Scand. J. Infect. Dis. 11: 141-145.

Bogert, C. van den, and Kroon, A.M., 1982. Effects of oxytetracycline on in vivo proliferation and differentiation of erythroid and lymphoid cells in the rat. Clin. Exp. Immunol. 50: 327-335.

Bogert, J.J. van den, 1983. Mitochondrial protein synthesis as target in cancer chemotherapy. Thesis. State University, Groningen, The Netherlands.

Caspi, R.R. and Avtalion, R.R., 1984. Evidence for the existence of an IL-2 like lymphocyte growth-promoting factor in a bony fish, Cyprinus carpio. Dev. Comp. Immunol. 8: 51-60.

Finch, R., 1980. Immunomodulating effects of antimicrobial agents. J. Antimicrob. Chemother. 6: 691-699.

Forsgren, A., Schmeling, D. and Quie, P.G., 1974. Effect of tetracycline on the phagocytic function of human leukocytes. J. Infect. Dis. 130: 412-415.

Forsgren, A. and Schmeling, D., 1977. Effect of antibiotics on chemotaxis of human leukocytes. Antimicrob. Agents Chemother. 11: 580-584.

Forsgren, A., Schmeling, D. and Banck, G., 1978. Effect of antibiotics on chemotaxis of human polymorphonuclear leukocytes in vitro. Infection 6 (suppl. 1): S102-S106.

Grondel, J.L. and Boesten, H.J.A.M., 1982. The influence of antibiotics on the immune system I. Inhibition of the mitogenic leukocyte response in vitro by oxytetracycline. Dev. Comp. Immunol. (suppl. 2): 211-216.

Grondel, J.L. and Harmsen, E.G.M., 1984. Phylogeny of interleukins: Growth factors produced by leukocytes of the cyprinid fish, Cyprinus carpio L. Immunology 52: 477-482.

Grondel, J.L., Gloudemans, A.G.M. and Muiswinkel, W.B. van, 1985. The influence of antibiotics on the immune system. II. Modulation of fish leukocyte responses in culture. Vet. Immunol. Immunopathol. 9: 251-260.

Hauser, W.E. and Remington, J., 1982. Effects of antibiotics on the immune response. Am. J. Medicine 72: 711-716.

Hayari, Y., Schauenstein, K. and Globerson, A., 1982. Avian lymphokines, II: interleukin-1 activity in supernatants of adherent splenocytes of chickens. Dev. Comp. Immunol. 6: 785-789.

Leezenberg, J.A., Wesseling, H. and Kroon, A.M., 1979. Possible cytostatic action of tetracyclines in the treatment of tumors of the nasopharynx and larynx. Eur. J. Clin. Pharmacol. 16: 237-241.

Munoz, J. and Geister, R., 1950. Inhibition of phagocytosis by aureomycin. Proc. Soc. Exp. Biol. Med. 75: 367-370.

Neu, H.C., 1978. A symposium on the tetracyclines: A major appraisal. Bull. N.Y. Acad. Med. 54: 141-155.

Oppenheim, J.J. and Gery, I., 1982. Interleukin-1 is more than an interleukin. Immunology Today. 3: 113-119.

Rijkers, G.T., Teunissen, A.G., Oosterom, R. van, and Muiswinkel, W.B. van, 1980. The immune system of cyprinid fish. The immunosuppressive effect of the antibiotic oxytetracycline in carp. Aquaculture 19: 177-189.

Rijkers, G.T., Oosterom, R. van, and Muiswinkel, W.B. van, 1981. The immune system of cyprinid fish. Oxytetracycline and the regulation of the humoral immunity in carp. Vet. Immunol. Immunopathol. 2: 281-290.

Robb, R.J. 1984. Interleukin-2: The molecule and its function. Immunology Today. 5: 203-209.

Schauenstein, K., Globerson, A. and Wick, G., 1982. Avian lymphokines I: Thymic cell growth factor in supernatants of mitogen-stimulated chicken spleen cells. Dev. Comp. Immunol. 6: 533-540.

Schnetzler, M., Oommen, R., Nowak, J.S. and Franklin, R.M., 1983. Characterization of chicken T cell growth factor. Eur. J. Immunol. 13: 560-566.

Smith, K.A., Lachman, L.B., Oppenheim, J.J. and Favata, M.F., 1980. The functional relationship of the interleukins. J. Exp. Med. 151: 1551-1556.

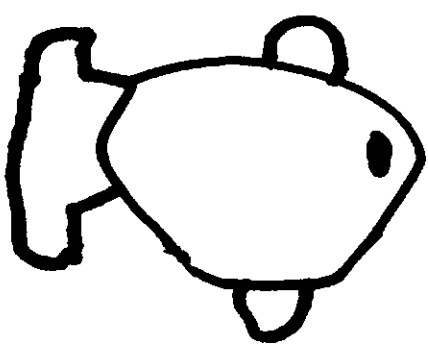
Thong, Y.H. and Ferrante, A., 1979. Inhibition of mitogen-induced human lymphocyte proliferative responses by tetracycline analogues. Clin. Exp. Immunol. 35: 443-446.

Unanue, E.R., 1980. Cooperation between mononuclear phagocytes and lymphocytes in immunity. New England J. Med. 303: 977-985.

Vries, H. de, and Kroon, A.M., 1970. On the effect of chloramphenicol and oxytetracycline on the biogenesis of mammalian mitochondria. Biochimica et Biophysica Acta 204: 531-541.

THE INFLUENCE OF ANTIBIOTICS ON THE IMMUNE SYSTEM. IV. IMMUNO-PHARMACOKINETIC INVESTIGATIONS ON THE PRIMARY ANTI-SRBC RESPONSE IN CARP (CYPRINUS CARPIO L.) AFTER OXYTETRACYCLINE INJECTION

J.L. GRONDEL, J.F.M. NOUWS AND W.B. VAN MUISWINKEL



SUMMARY

Immunomodulating effects of oxytetracycline (OxyTC) in fish, birds and mammals have been reported in a number of publications. Interference with the immune system may have serious implications for the outcome of therapy. The present study was performed to obtain further details on the immunomodulating effects of OxyTC in carp Cyprinus carpio L. The immunological study was extended by investigating the pharmacokinetic behaviour of OxyTC. OxyTC at a dosage of 60 mg/kg was administered 5 times intraperitoneally with a dosage interval of 3 days; the first injection was given 1 day before immunization. Investigations on the kinetics of the primary anti-sheep red blood cell (SRBC) response, during a short-term OxyTC treatment, revealed that the kinetics of the plaque forming cell (PFC) response was not significantly affected by OxyTC nor its solvent. In contrast, the number of plaque forming cells was significantly decreased. The anti-SRBC antibody production was delayed by 2-4 days in both experimental groups (OxyTC and solvent). However, within 12-14 days post immunization the same antibody levels were detected as in the control group, indicating the temporal effect of OxyTC on the immune response.

The pharmacokinetic data showed that high OxyTC plasma levels (> 50 µg/ml) were detected about 10 h after a single i.p. injection. A mean plasma elimination half-life ($T\frac{1}{2}$) of 34.5 h was calculated. This is longer compared to that reported for mammals.

The differences in pharmacokinetic behaviour of drugs between mammals and fish, and even between fish species may explain the susceptibility of the immune system in fish for modulation.

INTRODUCTION

Rearing fish at high densities demands management practices and husbandry techniques which can cope with numerous problems, of which disease is the most serious. The usual methods employed for the control of diseases include vaccination for prophylaxis and antimicrobial therapy for treatment (Austin 1984; Austin 1985).

In diseased animals the balance between natural defence mechanisms and pathogenic pressure has been disturbed, favouring the development of the micro-organisms. When dealing with bacterial infections, antibiotics applied

orally, via baths or parenterally (i.m., i.p.), are valuable tools. The bactericidal or bacteriostatic action of the drug enables the immune system to mount an appropriate response against the invading organism. The infective agent will be eliminated as a result of the combined action of the immune system and the antibacterial compound. However, immunomodulating effects of several antibiotics in fish, birds and mammals have been described and recently reviewed (Grondel & Van Muiswinkel 1986). For instance, long-term treatment of carp, Cyprinus carpio L., with oxytetracycline (OxyTC), either by feeding or injection, resulted in a seriously depressed cellular and humoral immunity (Rijkers, Van Oosterom & Van Muiswinkel 1981).

The objective of this study was to obtain more information on immune response kinetics in carp during short-term treatment with OxyTC. Furthermore, the immunological study was extended by investigating the pharmacokinetic behaviour of this drug.

MATERIALS AND METHODS

Animals

Common carp, 6-8 months of age, were bred in our laboratory and kept in aquaria with aerated running tap water at a temperature of 20°C ± 1°C. Animals were fed daily with pelleted dry food (K30, Trouw & Co., Putten, The Netherlands), at 2.5% of their body weight, by means of a "Scharflinger" automatic feeder. Body weight ranged from 100 to 200 gram.

Antibiotic

Oxytetracycline (OxyTC) was purchased as Engemycine^{R} from Mycofarm, De Bilt, The Netherlands. The solvent for OxyTC (polyvinylpyrrolidone) was kindly provided by Gist Brocades, Delft, The Netherlands.

Experimental protocol

Animals were divided into three groups. Control animals received (PBS) or the solvent of OxyTC in a volume equivalent to the antibiotic dosage (60 mg/kg). PBS, OxyTC and solvent were injected intraperitoneally (i.p.). The first dosage was administered 1 day before immunization and the subsequent

injections were given on day 2, 5, 8 and 11 post immunization (short-term antibiotic treatment), unless otherwise stated.

Antigen and immunization

Sheep red blood cells (SRBC) were obtained from the Department of Animal Husbandry, Agricultural University, Wageningen. Cells were washed 3 times with phosphate buffered saline (PBS, pH 7.2) before use. 10^9 SRBC were injected intramuscularly (i.m.) in the dorsal region.

Hemolytic plaque and hemagglutination assay

Plaque forming cells (PFC) from the anterior kidney (pronephros) and spleen were determined using the hemolytic plaque assay adapted for carp as described previously (Rijkers, Teunissen, Van Oostrom & Van Muiswinkel 1980). Bream, Abramis brama L., serum was used as a complement source. PFC slides were incubated at 25°C for 4 h and the results were expressed as plaque forming cells per 10^6 white cells (PFC/10^6 WC). Serum antibody titres to SRBC were determined using the standard method of hemagglutination in microtitre plates.

The Student's t test was used to compare the means of Ab titres and PFC data. If $p < 0.05$ the difference was considered significant.

Pharmacokinetic experiment

Carp were kept individually in tanks with aerated tap water (20°C). A single dose of OxyTC was administered i.p. (60 mg/kg). Heparinized blood samples (0.2 ml) from the caudal vein were taken at regular time intervals. After sampling the water (6, 12, 24, 30, 49 and 61 h) the tanks were refreshed. Water and plasma samples were frozen at -20°C pending analysis.

Determination of OxyTC concentrations

The large plate agar-diffusion method was used. OxyTC concentrations in plasma and water were determined according to the method described previously (Nouws & Vree 1983a; Nouws, Van Ginneken & Ziv 1983b). Briefly, the samples were assayed with Bacillus subtilis BGA (10^4 spores/ml agar) or Bacillus cereus var. mycoides (ATCC 6941) plates (0.1 ml of a 24-h broth

culture/200 ml agar). Standard II Nähr-agar (Merck 7883), supplemented with 0.2% KH_2PO_4, the pH of the agar being 6.0, was used. The B. subtilis plates were incubated overnight at 30°C, and those with B. mycoides were incubated overnight at 25°C.

The OxyTC concentrations were calculated by standard curves obtained by antibiotic standards prepared in pooled carp plasma.

Protein binding of OxyTC was determined in plasma samples using the micropartition system^R (MPS-1, Amicon; no. 4010). Samples were centrifuged for 30 min at 2000 rpm. OxyTC was determined as described above.

Pharmacokinetic analysis was performed by standard procedures (Baggot 1977). The peak OxyTC concentration (C_{max}) and peak time (T_{max}) for each carp were read from the concentration-time curve. The area under the plasma curve (AUC t = 0 up to t = 104 h) was calculated using the trapezoid rule. The half-life ($T\frac{1}{2}$) of OxyTC disposition was determined.

RESULTS

Kinetics of the primary response

We investigated the kinetics of the PFC response in the anterior kidney (pronephros) and of the hemagglutinating antibody production in order to determine whether the in vivo primary anti-SRBC response was inhibited by a short-term OxyTC treatment.

The peak of the primary PFC response of the control animals was detected on day 10 post immunization (Fig. 1A). At the same day the PFC numbers were reduced approximately 63% and 47% by injecting OxyTC and solvent, respectively. The PFC response of the solvent and OxyTC-treated carp was strongly inhibited (Fig. 1A).

An alteration of the kinetics was demonstrated by the results of the hemagglutination assay (Fig. 1B). Antibodies were present in all groups at day 10. However, they were less in the OxyTC and solvent groups compared to the control group. Control levels of anti-SRBC antibodies in serum of OxyTC-treated animals were observed with a delay of 2-4 days. In solvent-injected fish the same phenomenon was observed but to a lesser extent (Fig. 1B). For both experimental groups control values were detected from day 12 (solvent) or 14 (OxyTC) onwards.

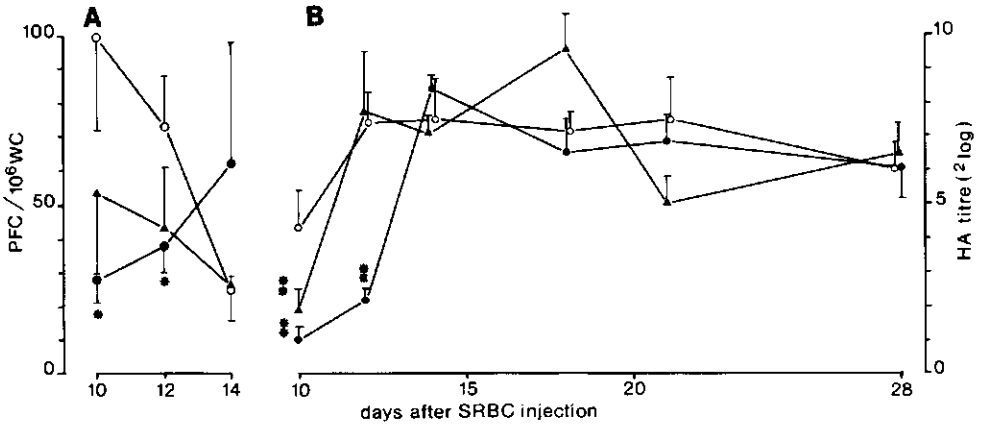


FIGURE 1. Influence of short-term OxyTC treatment on the primary anti-SRBC response in carp. Animals were injected 5 times with OxyTC (60 mg/kg/3 days, ●————●), solvent (▲————▲) or PBS (○————○) starting 1 day before immunization with SRBC. The PFC number in the pronephros was determined on day 10, 12 and 14 (A). Each point represents the arithmetic mean ± 1 SE (n=7). Serum antibody levels were determined on day 10 and subsequent days (B). These antibody titres are expressed as ^{2}log dilutions. Each point represents the arithmetic mean ± 1 SE (n=10). * = $p < 0.025$, ** = $p < 0.01$.

OxyTC sensitive immunological processes

A short-term treatment, consisting of multiple OxyTC injections, may affect all immunological processes. No distinction can be made between the different developmental stages of a response. The results sofar provide information about the development of the response during an antibiotic treatment. OxyTC was injected before immunization until the measurements (PFC-assay) were carried out. In order to obtain data about antibiotic sensitive stages, OxyTC treatment was started at predetermined moments during the primary anti-SRBC response (Fig. 2).

An expected inhibition (pronephros: ±70%; spleen: ±80%) was observed when carp received the first OxyTC injection 1 day before immunization (Fig. 2, column A). However, no significant suppression of the PFC response was detected in the pronephros and spleen from carp which received the first OxyTC dosage and SRBC simultaneously (Fig. 2, column B). In contrast, the group of animals (column F) which received only 1 OxyTC injection on day 11

showed a significant reduction of the PFC response in both spleen and pronephros. For the pronephros, intermediate results were obtained in group C, D and E, even though, these carp also received their last injection on day 11. However, the PFC numbers in the spleen for the same groups (C,D,E) were significantly decreased. (Fig. 2).

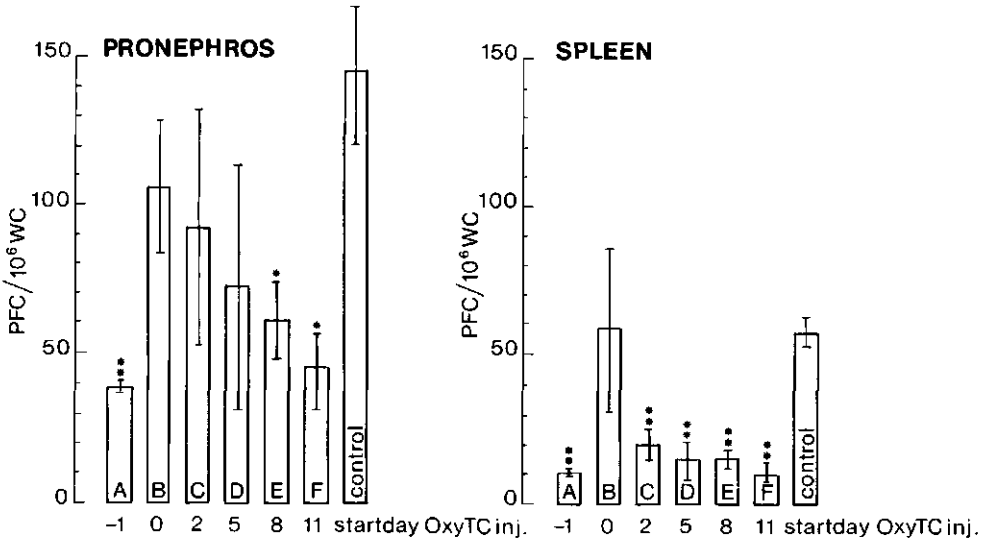


FIGURE 2. Effect of short-term OxyTC treatment (60 mg/kg/3 days) starting at fixed moments before or during the primary anti-SRBC response. On day 12 the PFC numbers in pronephros and spleen were determined. Day 0 represents the moment of immunization with SRBC. Six groups of animals (A - F, n=4) received the first injection of the treatment on day -1, 0, 2, 5, 8, or 11. Therefore, the animals have had 5 (A), 5 (B), 4 (C), 3 (D), 2 (E) or 1 (F) injection, respectively. Control animals (n=6) were treated 5 times with PBS. Each bar represents the arithmetic mean ± 1 SE. * = $p < 0.025$, ** = $p < 0.01$.

The hemagglutination data (Table 1) are in agreement with the inhibition profile of the PFC response in pronephros and spleen (Fig. 2). The lowest hemagglutination titres were detected in group A. However, no significant influence of the OxyTC treatment was detected on the height of the anti-SRBC titre in group B. These animals received OxyTC injections on day 0, 2, 5, 8 as well as day 11 (Table 1).

TABLE 1

The effects of short-term OxyTC treatment on the production of anti-SRBC antibodies in serum on day 12.

Experimental groups (n=4) *)						
A	B	C	D	E	F	Control
1.0±0.4**	4.5±0.2	3.5±0.9	2.0±0.6	2.7±1.1	2.5±0.6	3.5±0.8#

*) See fig. 2 for further explanation. **) $p<0.05$ #) n=6

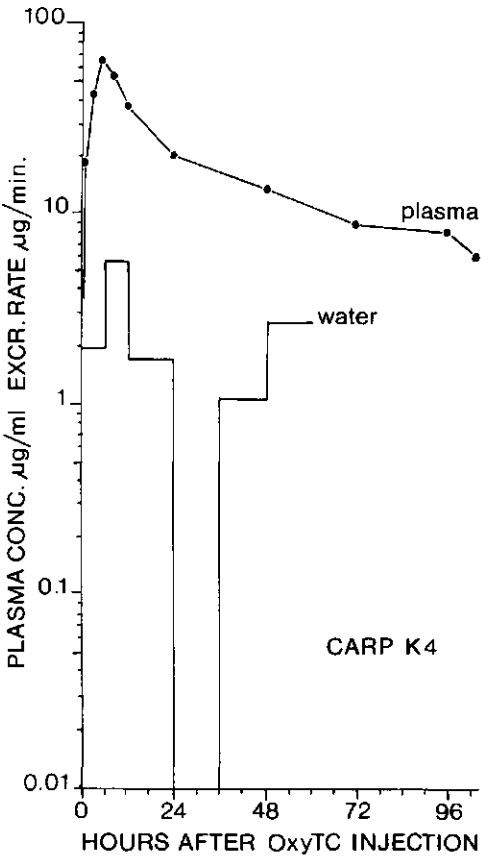


FIGURE 3. Plasma concentration curve and excretion rate of OxyTC. A single OxyTC dosage (60 mg/kg) was administered intraperitoneally. Carp were kept individually in tanks with stagnant aerated water at 20°C. Water and blood samples were taken at regular time intervals. After sampling the tanks were refreshed.

OxyTC plasma disposition

Figure 3 shows the plasma disposition and excretion rate of OxyTC for one representative carp. Ten hours after a single i.p. OxyTC injection (60 mg/kg) a maximum plasma concentration was detected (C_{max} = ±70 µg/ml). The plasma elimination half-life time ($T_{\frac{1}{2}}$) was 47 hours. A mean plasma elimination half-life value of 34.5 ± 2.5 hours could be calculated from data obtained from several experiments in which carp (n = 22) were i.p. injected with OxyTC. The total drug recovery, expressed as percentage of the dosage applied, was 33% within 61 hours post injection. Plasma protein binding determinations revealed a binding percentage of 25.1 ± 1.77 (n=8).

DISCUSSION

Immunomodulating effects of OxyTC in fish, birds and mammals have been described in a number of publications (Rijkers et al. 1980; Rijkers et al. 1981; Van den Bogert & Kroon 1982; Grondel, Gloudemans & Van Muiswinkel 1985a; Grondel, Angenent & Egberts 1985b; Van Muiswinkel, Anderson, Lamers, Egberts, Van Loon & IJssel 1985). In carp, the primary anti-SRBC response was inhibited after long-term treatment with OxyTC, either by feeding or injection of the antibiotic. Whereas, a continuous antibiotic treatment did not affect the secondary response as measured by counting the PFC numbers at the peak day of the response (Rijkers et al. 1981).

The present extension of these in vivo studies examined the kinetics of the PFC response during an antibiotic treatment and the anti-SRBC antibody production on subsequent days. It was shown that the kinetics of the PFC response of the pronephros was not significantly changed by the OxyTC treatment, in contrast to the PFC numbers. The antibody production was 2 - 4 days delayed. Surprisingly, the solvent of OxyTC (polyvinylpyrrolidone) had an inhibitory effect. Although this suppressive action was statistically not significant, the reduced anti-SRBC antibody levels on day 10 confirmed the influence of the solvent on the immune response. The antibody titres of both experimental groups reached control values with 2 - 3 days delay, indicating the temporal effect of both solvent and OxyTC on the response.

The reversible action of OxyTC is in agreement with studies which suggest a non-permanent bond of tetracyclines to ribosomes (Neu 1978; Vries de & Kroon 1970). If the antibiotic diffuses out of the bacterium the organism

can subsequently start growing. The recovery of the immune response within 3 days after the ultimate injection illustrates well that the immune system has responded to the SRBC in almost all respects. Most likely, activated B cells will proceed with proliferating and differentiating into plasma cells at a normal rate when OxyTC concentrations are decreasing. T cell activation, proliferation and subsequent production of lymphokines precedes B cell maturation (Ewijk van, Rozing, Brons & Klepper 1977; Ziegler & Unanue 1981; Chesnut, Colon & Grey 1982; Grey & Chesnut 1985; Miedema & Melief 1985). The in vivo studies in carp do not suggest a serious interference with helper cell activity.

An immune response is initiated by an antigen and comprises all kinds of cell biological events which are precisely adjusted to one another. Apparently, OxyTC treatment disturbs one or more of these events, resulting in a delay of the antibody response. A differential sensitivity was observed in experiments in which OxyTC treatment started at fixed moments during the development of the immune response. In carp, the primary anti-SRBC response was most suppressed when OxyTC treatment was started one day before immunization. Comparable results were obtained when animals received a single injection on the 11^{th} day after immunization, just 1 day before the PFC-assay was performed (Fig. 2). In the latter experiment, the conditions allow the immune system to develop plasma cells actively secreting antibodies at least to some extent. However, the numbers of PFC were strongly reduced on the 12^{th} day. This could be explained by arrest of differentiation of activated B cells and inhibition of antibody synthesis when animals receive OxyTC for the first time.

The pharmacokinetic data show that high OxyTC plasma levels (> 50 µg/ml) were detected about 10 hours after a single i.p. injection. At these concentrations OxyTC may interfere with cytoplasmic protein synthesis as reported for mammalian cells, provided that the drug enters the cell (Gijzel & Kroon 1978). Furthermore, at instantaneous high concentrations, binding with divalent cations may occur and may subsequently disturb Ca^{2+} dependent cell processes including immunomodulation (Diamantstein & Odenwald 1974). In rats, continuously infused with OxyTC, the kinetics of the primary anti-SRBC antibody response was not influenced. However, the amount of antibodies was reduced. A blockade of T cell proliferation was proposed, based on experiments in which the presence of OxyTC was varied during the immune response. Constant low OxyTC levels (± 10 µg/ml) could be achieved by using infusion techniques (20 mg/kg/day). Multiple OxyTC injections (60

mg/kg) will cause strong fluctuations in serum levels as can be concluded from the pharmacokinetic data.

In mammals, the mechanism of *in vitro* handling of antigen by macrophages includes uptake, processing and antigen presentation to appropriate helper cells and requires about 60 min (Ziegler & Unanue 1981; Chesnut *et al.* 1982). It was shown that particulate antigen (*Listeria monocytogenes*) uptake and processing were distinct events with regard to temperature and energy dependence (Ziegler & Unanue 1981). Unlike antigen processing, antigen uptake could occur in the presence of inhibitors of oxidative and glycolytic metabolism. From these results it may be concluded that *in vivo* uptake of particulate antigens (e.g. erythrocytes) by macrophages will occur in carp treated with OxyTC. However, the subsequent rate of antigen processing will depend on the antibiotic concentration. If the first OxyTC injection of the treatment was administered simultaneously with SRBC, no inhibition could be observed (Fig. 2). OxyTC plasma levels which may interfere significantly with antigen processing, were detected only several hours after immunization, which suggests that antigen processing and presentation can occur at least to some extent. From the moment of immunization, relatively high antibiotic plasma concentrations can be expected when OxyTC is administered 24 hours before immunization. Subsequently, the initiation of the immune response is delayed, but not abrogated.

Pharmacokinetic data provide more insight into the action of drugs upon immunomodulation. For OxyTC, several pharmacokinetic parameters are presented upon i.p. injection. A strikingly long plasma elimination half-life time was observed compared to that reported for mammals (Nouws *et al.* 1983a+b). In carp, variable maximum concentrations were obtained due to the route of administration. In certain occasions, the gut was accidently damaged or perforated which resulted in a 100% recovery from the aquarial water within 12 hours post injection.

Antibiotic distribution across the body is heterogeneous and depends on for instance the composition of the organs/tissues and the biochemical characteristics of the drug. Preliminary results showed that OxyTC was accumulated and retained in the pronephros, one of the main lymphoid organs in carp. Such a specific affinity for lymphoid tissues could explain the interference with immune responses. Pharmacokinetic data on tissue distribution, plasma disposition and drug metabolism can differ markedly between mammals, birds and fish (Nouws, Vree, Breukink, Van Miert & Grondel 1986; Grondel, Nouws & Haenen 1986). Moreover, extreme differences can be

expected between fish species with respect to temperature, age, species and disease status.

The combined action of immune system and antimicrobial drug will increase the defensive potential. Therefore, it is emphasized that an immuno-pharmacokinetic approach is needed to prevent immunotoxicological events and to optimize the usage of antibiotics in fish culture. Consequently, the fundamental aspects of this type of research will support an effective management.

ACKNOWLEDGEMENTS

The authors gratefully acknowledge the technical assistance of E.G.M. Harmsen and the support of O.L.M. Haenen. Furthermore, we thank M. Nieuwland (Dept. of Animal Husbandry, Wageningen) for providing the antigens.

REFERENCES

Austin, B. (1984) The control of bacterial fish diseases by antimicrobial compounds. In: Antimicrobials in agriculture. Woodbine, M. (ed.), London: Butterworths, 255-268.

Austin, B. (1985) Antibiotic pollution from fish farms: effects on aquatic microflora. Microbiological Sciences, **2**, 113-117.

Baggot, J.D. (1977) Principles of drug disposition in domestic animals. W.B. Saunders Co., Philadelphia/London/Toronto.

Chesnut, R.W., Colon, S.M. & Grey, H.M. (1982) Requirements for the processing of antigens by antigen-presenting B cells. I. Functional comparison of B cell tumors and macrophages. Journal of Immunology **129**, 2382-2388.

Diamantstein, T. & Odenwald, M.V. (1974) Control of the immune response in vitro by calcium ions. I. The antagonistic actions of calcium ions on cell proliferation and on cell differentiation. Immunology **27**, 531-541.

Ewijk van, W., Rozing, J., Brons, N.H.C. & Klepper, D. (1977) Cellular events during the primary immune response in the spleen. Cell and Tissue Research **183**, 471-489.

Gijzel, W.P. & Kroon, A.M. (1978) On the protection of bone marrow against toxic side effects of tetracyclines. Journal of Molecular Medicine **3**,

157-165.

Grey, H.M. & Chesnut, R.W. (1985) Antigen processing and presentation to T cells. Immunology Today **6**, 101-106.

Grondel, J.L., Gloudemans, A.G.M. & Van Muiswinkel, W.B. (1985a) The influence of antibiotics on the immune system. II. Modulation of fish leukocytes responses in culture. Veterinary Immunology and Immunopathology **9**, 251-260.

Grondel, J.L., Angenent, G.C. & Egberts, E. (1985b) The influence of antibiotics on the immune system. III. Investigations on the cellular functions of chicken leukocytes in vitro. Veterinary Immunology and Immunopathology **10**, 251-260.

Grondel, J.L. & Van Muiswinkel, W.B. (1986) Immunological defence mechanisms as a target for antibiotics. In: Van Miert (Ed.); Comparative Veterinary Pharmacology, Toxicology and Therapy. 3rd EAVPT Congress, Ghent, Belgium. MTP Press Ltd, Lancaster, in press.

Grondel, J.L., Nouws, J.F.M. & Haenen, O.L.M. (1986) Fish and antibiotics. Pharmacokinetics of sulphadimidine in carp (Cyprinus carpio). Veterinary Immunology and Immunopathology **12**, 281-286.

Miedema, F. & Melief, C.J.M. (1985) T-cell regulation of human B cell activation. Immunology Today **6**, 258-259.

Neu, H.C. (1978) A symposium on the tetracyclines: a Major appraisal. Bulletin of the New York Academy of Medicine **54**, 141-155.

Nouws, J.F.M. & Vree, T.B. (1983a) Effect of injection site on the bioavailability of an oxytetracycline formulation in ruminant calves. The Veterinary Quarterly **5**, 165-170.

Nouws, J.F.M., Van Ginneken, C.A.M. & Ziv, G. (1983) Age-dependent pharmacokinetics of oxytetracycline in ruminants. Journal of Veterinary Pharmacology and Therapy **6**, 59-66.

Nouws, J.F.M., Vree, T.B., Breukink, H.J., Van Miert, A.S.P.J.A.M., & Grondel, J.L. (1986) Pharmacokinetics, hydroxylation and acetylation of sulphadimidine in different species of animals, birds, fish, reptiles and mollusks. In: Van Miert (Ed.); Comparative Veterinary Pharmacology, Toxicology and Therapy, 3rd EAVPT congress, Ghent, Belgium. MTP Press Ltd, Lancaster, in press.

Rijkers, G.T., Teunissen, A.G., Van Oosterom, R., & Van Muiswinkel , W.B. (1980) The immune system of Cyprinid fish. The immuno-suppressive effect of the antibiotic oxytetracycline in carp. Aquaculture **19**, 177-189.

Rijkers, G.T., Van Oosterom, R. & Van Muiswinkel, W.B. (1981) The immune

system of Cyprinid fish. Oxytetracycline and the regulation of the humoral immunity in carp. Veterinary Immunology and Immunopathology **2**, 281-290.

Van den Bogert, C. & Kroon, A.M. (1982) Effects of oxytetracycline on in vivo proliferation and differentiation of erythroid and lymphoid cells in the rat. Clinical Experimental Immunology **50**, 327-335.

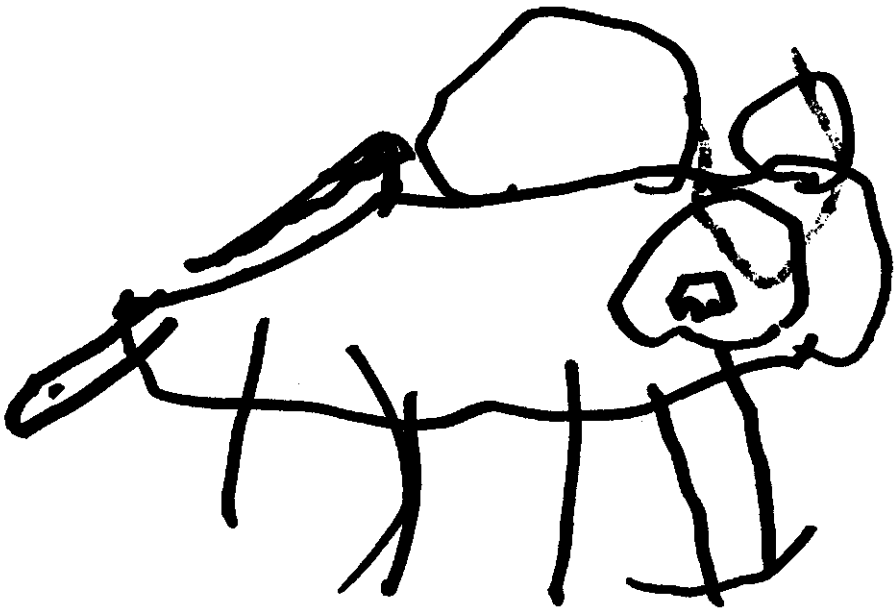
Van Muiswinkel, W.B., Anderson, D.P., Lamers, C.H.J., Egberts, E., Van Loon, J.J.A. & IJssel, J.P. (1985) Fish Immunology and Fish Health. Fish Immunology, M.J. Manning & M.F. Tatner, eds., Proceedings of the Plymouth Meeting 1983, Academic Press, London.

Vries de, H. & Kroon, A.M. (1970) On the effect of chloramphenicol and oxytetracycline on the biogenesis of mammalian mitochondria. Biochimica et Biophysica Acta **204**, 531-541.

Ziegler, K. & Unanue, E.R. (1981) Identification of a macrophage antigen-processing event required for I-region-restricted antigen presentation to T lymphocytes. Journal of Immunology **127**, 1869-1879.

PHARMACOKINETICS AND TISSUE DISTRIBUTION OF OXYTETRACYCLINE IN CARP (CYPRINUS CARPIO L.) FOLLOWING DIFFERENT ROUTES OF ADMINISTRATION

J.L. GRONDEL, J.F.M. NOUWS, M. DE JONG, A.R. SCHUTTE AND F. DRIESSENS



SUMMARY

The objective of this pharmacokinetic study was to investigate absorption, distribution, elimination and bioavailability of oxytetracycline (OTC) in carp Cyprinus carpio L. after different routes of administration. OTC was administered intravenously (i.v.), intramuscularly (i.m.) and orally at 60 mg/kg body weight. OTC levels were determined in plasma and several tissues. Analysis of the plasma drug concentration-time curves following i.v. OTC injection revealed three distinct phases. A three-compartment open model was used to derive pharmacokinetic parameters. Compared to mammals, a very extended final elimination half-life was observed (139.8 ± 38.1 h). Following i.m. OTC administration, C_{max} was 56.8 ± 10.9 µg OTC/ml at 14 h post injection. The Vd area was 2.1 ± 0.66 l/kg. Extreme differences were observed with respect to bioavailability following i.m. and oral administration; approximately 80 and 0.6 %, respectively.

Following i.m injection tissue OTC determinations revealed that the drug was accumulating in pronephros, bone tissue and scales. After 21 days the OTC concentrations were 2.9 ± 0.8, 5.2 ± 0.3 and 4.7 ± 3.1 µg/ml, respectively. In tissue samples from the dorsal region (muscle), including the injection, site OTC could not be demonstrated at that time.

The pharmacokinetic data are discussed in relation to the susceptibility of the immune system of fish for modulation.

INTRODUCTION

Oxytetracycline (OTC) is a commonly used antibiotic in commercial aquaculture of freshwater and marine fish species. OTC is a bacteriostatic compound with a broad antibacterial activity against both Gram-positive and Gram-negative micro-organisms, both aerobic and anaerobic species (Neu 1978).

The drug may be used for prophylaxis or therapy of bacterial infections. Several diseases are described, i.e. carp erythrodermatitis, columnaris disease, edwardsiellosis, enteric redmouth disease, furunculosis, on which occasion tetracyclines are recommended drugs for treatment (Austin 1984). OTC oral dosage regimes range from 50-100 mg/kg of fish per day for 3-14 days depending upon the infection.

In the U.S.A. only three antibacterial agents, i.e. sulfamerazine, sulfadimethoxine/ormetoprim and oxytetracycline, are approved for use in

aquaculture. Considering the varied fish species, diseases and rearing conditions, the choice is very limited. New products may provide better therapeutic treatments which are more focussed on the infective organism and the specific farm conditions.

Antibiotics may inhibit growth or even kill the bacterium depending on the mode of action and concentration. However, the immunological defence mechanism has to eliminate the infective agent. Antibiotic interference with the immune system will decrease the defensive capacity of the animal. It was recently reviewed that OTC and other compounds may interfere with immunological processes in fish, birds and mammals (Grondel & Van Muiswinkel 1986b). In this context pharmacokinetic studies are important, because data are needed concerning the behaviour of drugs in ectothermic animals as fish. The objective of this pharmacokinetic study was to investigate plasma disposition, distribution, bioavailability and elimination of OTC in carp after different routes of administration.

MATERIALS AND METHODS

Animals

Carp Cyprinus carpio L. were bred in our laboratory and kept in aquaria with aerated tap water at a temperature of 20°C. Animals were fed daily with pelleted dry food (K30, Trouw & Co., Putten, The Netherlands), at 2.5% of their body weight, by means of a "Scharflinger" automatic feeder.

Antibiotic treatments

Animals were divided into four groups. Two groups of 5 carp each received oxytetracycline (OTC) by an intravenous (i.v., caudal vein) or intramuscular (i.m., dorsal region) injection. An injectable veterinary formulation of OTC (Engemycine^{R}, Mycofarm, De Bilt, The Netherlands) was administered at a dose of 60 mg/kg. In the third group (n=6) carp were kept individually in tanks with stagnant aerated tap water (20°C). OTC was administered orally to these animals. The antibiotic was mixed with dry food and pelleted. The initial calculated dose was 60 mg OTC/kg.

Heparinized blood samples (0.2 ml) from the caudal vein were taken at regular time intervals for all groups and were centrifuged for 10 min at 800 x g. Water samples were collected immediately after feeding (t=0) with

antibiotic containing pelleted food (3rd group). Moreover, after sampling the tanks were refreshed. Water and plasma samples were frozen at -20°C pending analysis.

In the 4th group carp were sacrificed at regular time intervals after a single i.m. injection with OTC (60 mg/kg) to determine antibiotic concentrations in different tissues. OTC levels were determined in liver, spleen, mesonephros, pronephros, muscle (at the injection site), muscle (dorsal region) and skin. The tissues were carefully minced, weighted and diluted 1:3 (w:v) in phosphate buffered saline (PBS, pH 6). To scales or pieces of bone 1M HCl was added (1:1; w:v) and incubated with pepsine for 1 h at 37°C. Afterwards, the acid pepsine solution was adjusted to pH 6. Tissue samples were frozen at -90°C. Before analysis the tissue samples were thawed, centrifuged (1300 x g, 5 min), the supernatants were collected and appropriate dilutions were made with PBS.

Determination of OTC concentrations

The large plate agar-diffusion method was used. OTC concentrations in plasma, water and supernatants were determined according to the method described by Nouws & Vree 1983b and Nouws, Van Ginneken & Ziv 1983a. Briefly, the samples were assayed with Bacillus subtilis BGA (10^4 spores/ml) or Bacillus cereus var. mycoides (ATCC 6941) plates (0.1 ml of a 24-h broth culture/200 ml agar). Standard II Nähr-agar (Merck 7883), supplemented with 0.2% KH_2PO_4 pH 6.0, was used. The B subtilis plates were incubated overnight at 30°C, and those with B mycoides were incubated overnight at 25°C. The OTC concentrations were calculated by standard curves obtained from antibiotic standards prepared in pooled carp plasma, homologous tissue homogenates or PBS.

Pharmacokinetic analysis

Plasma OTC concentrations for each animal in the i.v. and i.m. experimental groups were analyzed according to the standard procedures described by Baggot (1977). Additional calculations from the i.v. and i.m. OTC concentration-time data were performed according to the procedures described by Nouws, Vree, Termond, Lohuis, Van Lith, Binkhorst and Breukink 1985.

The intravenous plasma OTC concentrations of each carp were analyzed with

the NONLIN Program (Metzler, Elfring & McEwen 1974) for best fit to the three-compartment pharmacokinetic model by weighted least squares regression analysis. The plasma OTC concentration-time curve (according to the three compartment model) can be adequately described by the tri-exponential equation: $C_p = A^\circ.e^{-\alpha.t} + B^\circ.e^{-\beta.t} + C^\circ.e^{-\gamma.t}$
By means of the parameters A°, B°, C°, α, β and γ the pharmacokinetic parameters K_{12}, K_{21}, K_{13}, K_{31} and K_{el} can be calculated.

Additional calculations of Vd area (apparent distribution volume), body clearance (Cl_b) and distribution volume of the central compartment (V1) were performed in a model-independent way according to Nouws et al. 1983a.

The peak OTC concentration (C_{max}) and peak time (T_{max}) for each carp were read from the concentration-time curve. The area under the curve (AUC) was calculated using the trapezoid rule.

RESULTS

The mean plasma OTC concentrations following i.v. and i.m. administration are presented in Table 1. Individual plasma OTC concentration-time profiles are shown in Fig. 1 (i.v., carp F5 and i.m.,carp G1). Pharmacokinetic values for oxytetracycline administered intravenously are presented in Table 2.

The distribution phase revealed a half-life ($T\frac{1}{2}\ \alpha$) of 3.5 ± 1.7 h; the extrapolated zero-time OTC concentration (A°) was 265.7 ± 48.2 µg/ml following i.v. administration. The final elimination half-life ($T\frac{1}{2}\gamma$) was 139.8 ± 38.1 h. The apparent volume of distribution, Vd area (i.v.), and the distribution volume of the central compartment (V1) were 2.10 ± 0.66 and 0.19 ± 0.03 litre/kg, respectively.

The initial steep decline of the plasma OTC concentration (α = 0.2553 ± 0.0059 h^{-1}), following i.v. administration, resulted mainly from distribution to two tissue compartments (T2 and T3). The rate of diffusion to the peripheral compartment (K_{12}, V1 -> T2) was about 29-fold higher than to the deeper compartment (K_{13}, V1 -> T3). Moreover, the OTC diffusion from the central compartment to the peripheral compartment was 2.66-fold (K_{12}/K_{21}) faster than the back diffusion (Table 2). The final elimination phase (γ = 0.0053 ± 0.0014 h^{-1}) constitutes primarily the release of drug from the deep compartment (T3 -> V1). The rate of back diffusion (K_{31}, T3 -> V1) from this compartment was about 9-fold slower than that of the peripheral compartment (K_{21}, T2 -> V1; Table 2).

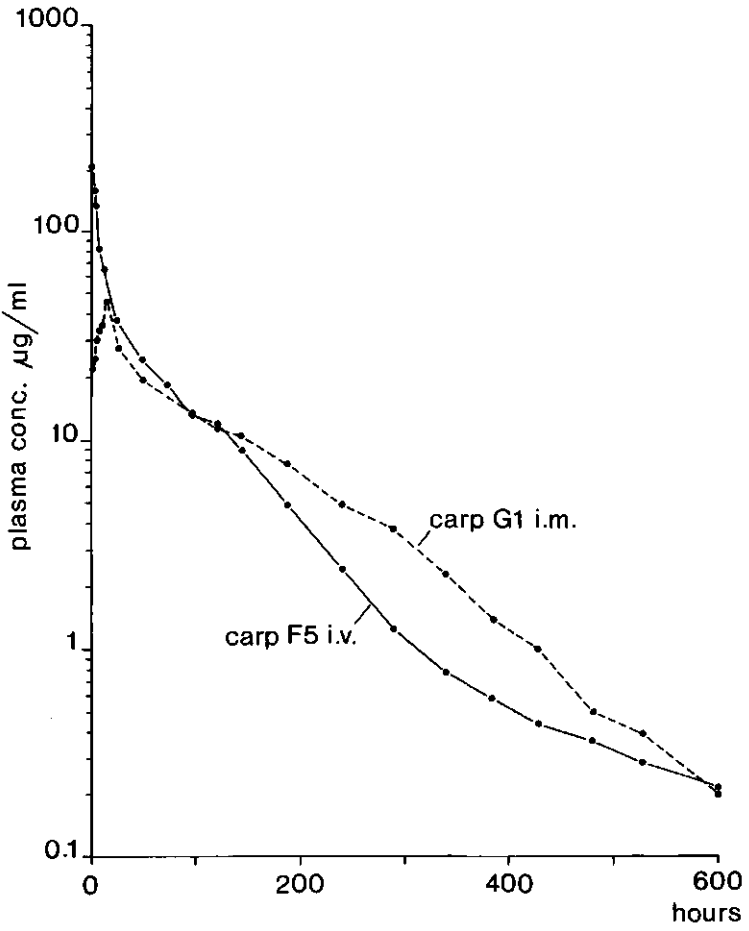


FIGURE 1. Plasma concentrations of oxytetracycline in a carp following a single intravenous (carp F5) or intramuscular (carp G1) injection of 60 mg/kg. Carp were kept at 20°C.

Fourteen hours after intramuscular injection of OTC (T_{max}) mean peak values (C_{max}) were observed of 56.8 ± 10.9 µg/ml. The absorption phase revealed a half-life of ± 12 h. A biphasic elimination phase was observed. The final elimination half-life was 78.6 ± 5.3 h. The bioavailability (t = 0–600 h) achieved with the i.m. route was about 80%.

In contrast to the i.v. and i.m. route of administration, the observed plasma OTC concentrations were extremely low in carp which received the antibiotic by oral application (Table 3, Fig. 2). In four out of six animals only traces of OTC (< 0.07 µg/ml) could be detected in plasma. The dose

TABLE 1

Antibiotic concentration in plasma of carp following intravenous or intramuscular administration of oxytetracycline

Temp. 20°C		
Dose (mg/kg) 60		
	I.V.	I.M.
No. of animals	5	5
Weight ± sd (g)	372 ± 158	336 ± 92
	Plasma oxytetracycline concentration	
Time (h)	(µg/ml ± sd)	
1.0	247.5 ± 48.2	22.8 ± 16.3
2.0	202.2 ± 30.4	26.6 ± 17.1
4.0	145.6 ± 28.1	29.3 ± 12.6
7.0	100.9 ± 20.0	32.7 ± 10.8
10.0	75.8 ± 16.0	37.2 ± 13 1
14.0	49.9 ± 7.7	56.8 ± 10.9
24.0	41.2 ± 4.7	31.8 ± 5.7
48.0	29.5 ± 4.3	23.5 ± 4.8
72.0	21.5 ± 1.9	20.0 ± 4.6
96.0	15.3 ± 3.6	15.1 ± 4.4
120.0	12.8 ± 2.6	13.4 ± 3.9
144.0	10.0 ± 2.3	11.2 ± 3.2
191.0	5.7 ± 1.1	8.5 ± 2.2
240.0	3.3 ± 0.8	4.9 ± 0.9
288.0	1.8 ± 0.4	3.4 ± 0.8
336.0	1.2 ± 0.3	2.2 ± 0.6
384.0	0.8 ± 0.2	1.5 ± 0.4
430.0	0.6 ± 0.2	1.1 ± 0.4
480.0	0.44 ± 0.10	0.53 ± 0.2
528.0	0.33 ± 0.05	0.34 ± 0.1
600.0	0.22 ± 0.02	0.18 ± 0.0

TABLE 2

Pharmacokinetic values for oxytetracycline administered intravenously to **carp** (mean ± sd)

Temp.	20°C	
Dose (mg/kg)	60	
No. of animals		5
Weight	(g)	372 ± 158
A°	(µg/ml)	265.7 ± 48.2
B°	(µg/ml)	51.2 ± 2.9
C°	(µg/ml)	6.2 ± 4.7
α	(h^{-1})	0.2553 ± 0.0059
β	(h^{-1})	0.0142 ± 0.0035
γ	(h^{-1})	0.0053 ± 0.0014
$T_{\frac{1}{2}\alpha}$	(h)	3.5 ± 1.7
$T_{\frac{1}{2}\beta}$	(h)	50.8 ± 9.9
$T_{\frac{1}{2}\gamma}$	(h)	139.8 ± 38.1
K_{el}	(h^{-1})	0.0552 ± 0.0093
K_{12}	(h^{-1})	0.1518 ± 0.0472
K_{21}	(h^{-1})	0.0564 ± 0.0096
K_{12}/K_{21}		2.66 ± 0.41
K_{13}	(h^{-1})	0.0052 ± 0.0044
K_{31}	(h^{-1})	0.0061 ± 0.0021
K_{13}/K_{31}		0.78 ± 0.36
$AUC_{0-\infty}$	(µg.h/ml)	5862 ± 439
Cl_b	(ml/min/kg)	0.17 ± 0.01
Vd area	(l/kg)	2.10 ± 0.66
Vl	(l/kg)	0.19 ± 0.03
C_p°	(µg/ml)	323.1 ± 53.9

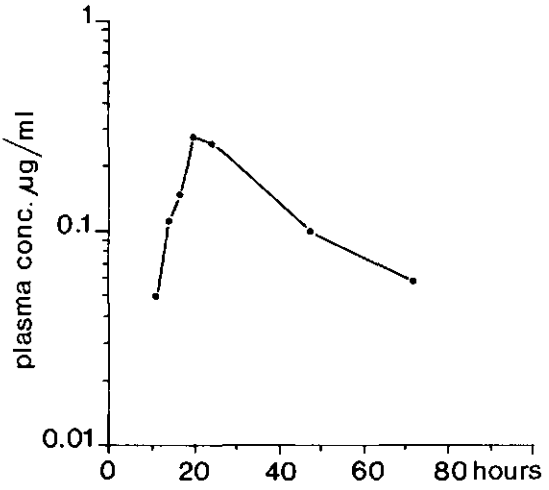


FIGURE 2. Plasma concentrations of oxytetracycline in carp (F6) following a single oral administration of 34 mg OTC/kg. Carp were kept at 20°C.

TABLE 3

Antibiotic concentration in plasma of carp following oral administration of oxytetracycline (mean ± sd)

Temp. 20°C	
No. of Animals	6
Weight (g)	103.5 ± 5.5
Dose (mg/kg)	24.5 ± 9.9
Plasma oxytetracycline concentration (µg/ml)	
Time (h)	
1	*
11	0.05 (n=1)
14	0.088 ± 0.045
17	0.078 ± 0.038
20	0.11 ± 0.09
24	0.097 ± 0.083
48	0.085 ± 0.021 (n=2)
72	0.06 (n=1)
96	*

* below detection limit

administered was 60 mg OTC/kg. However, the actual dose taken up appeared to be about half this dose (Table 3). This could be calculated from the data obtained from water samples which were taken immediately after uptake of the antibiotic containing pellets. In two out of six carp the first detectable plasma OTC concentration was observed 11 h post injection (p.i.).The C_{max} varied between 0.07 and 0.28 µg/ml, and was achieved between 14 and 20 h. The bioavailability achieved with the oral route corrected for the dose was only 0.6 %.

TABLE 4

Antibiotic concentration[1] in different tissues of **carp** following intramuscular administration of oxytetracycline (mean ± sd)

Temp.	20°C				
Dose (mg/kg)	60				
Tissue	Time (h)				
	48**	96*	230*	312*	505*
Liver	6.77	4.64	1.61	1.84	2.33
Spleen	1.55	0.89	2.63	2.79	2.73
Mesonephros	1.19	1.87	2.67	4.17	4.40
Pronephros	1.64	2.31	6.73	12.70	18.80
Muscle	0.55	0.55	0.90	1.25	#
Muscle[2]	1.70	1.56	-	-	#
Skin	0.96	0.73	2.52	3.16	3.71
Bone	1.4	1.00	2.93	5.43	36.30
Scale	2.23	-	-	24.50	39.70
plasma[3]	14.77 ± 3.5	4.65 ± 2.2	2.20 ± 1.3	0.45 ± 0.1	0.17 ± 0.09

1 Mean ratio = $\frac{\text{Tissue OTC conc.}}{\text{Plasma OTC conc.}}$

2 Site of injection

3 Mean concentration (µg/ml) ± sd

* n=2 ** n=3

\# below detection limit

\- not determined

In Table 4 the tissue/plasma concentration ratios are presented following i.m. administration of a single OTC dose (60 mg/kg). The data of plasma and five tissues are depicted graphically in Figure 3. Forty-eight hours p.i. the maximum OTC concentration was found in the liver. After 96 h an equilibrium was achieved (ratio= 2). Tissue/plasma concentration ratios of 3 and 4 were shown in spleen and mesonephros, respectively. Obviously, the ratio at the injection site was initially high compared to muscle from the dorsal non-injected region. However, 505 h p.i. OTC could not be detected in both tissues. Accumulation of OTC was observed in pronephros, scales and bone. No equilibrium was achieved during the experimental period. At 505 h p.i. OTC concentrations in pronephros, scales and bone were detected of 2.9 ± 0.8, 4.7 ± 3.1 and 5.2 ± 0.28 µg/ml, respectively.

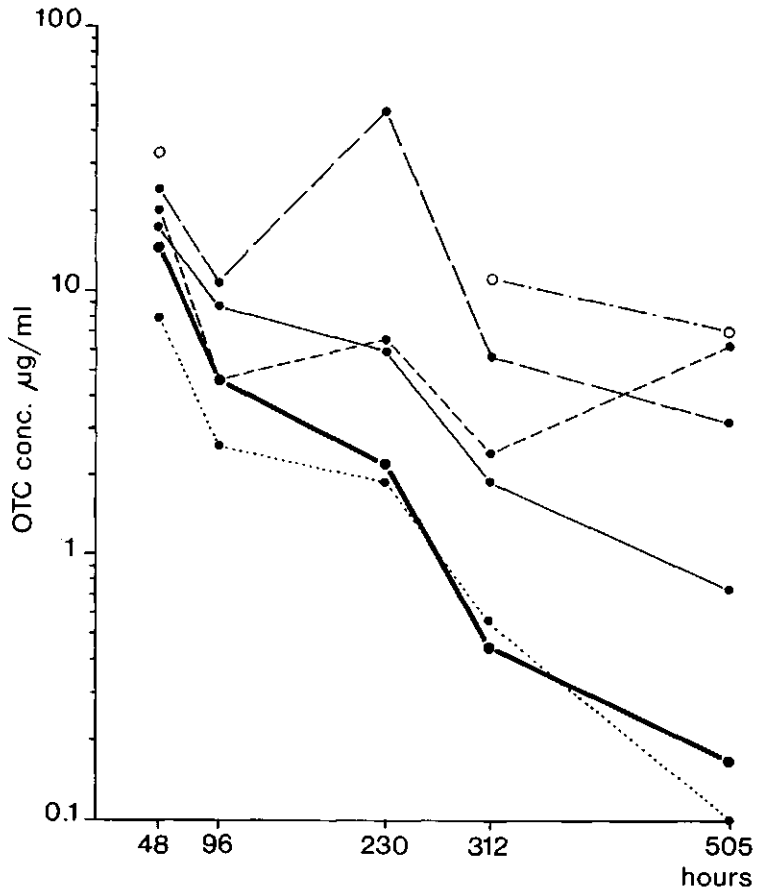


FIGURE 3. Mean plasma and tissue concentrations of oxytetracycline following a single intramuscular injection of 60 mg/kg. Plasma (●━━━━●) Pronephros (●—— ——●); Mesonephros (●————●); Scale (○—·—○) Bone (●– – – –●); Muscle (●·····●)

DISCUSSION

The analysis of the plasma drug concentration-time profiles after OTC was administered intravenously showed three distinct phases. The initial decline of plasma OTC concentration reflects mainly the distribution from the central (V1) to the peripheral compartment (T2) with the rate constants K_{12} (0.1518 ± 0.0472 h^{-1}). Diffusion of the drug to a second (deeper) compartment (T3) is expressed by the rate constant K_{13}. Apparent distribution equilibrium was observed after 336 h and constituted elimination of OTC with a relatively long half-life ($T_{\frac{1}{2}\gamma}$ = 139.8 ± 38.1 h) compared to mammals (6-9 h; Baggot 1977; Nouws et al. 1985).

The pharmacokinetics of OTC in carp differed widely from mammals (Baggot 1977; Nouws et al. 1985; Mevius, Vellenga, Breukink, Nouws, Vree & Driessens 1986). This difference in pharmacokinetic behaviour of OTC may be explained by the slower penetration rate into the peripheral compartments (T2 and T3) and vice versa, other tissue composition and blood perfusion. Moreover, renal clearance constitutes only glomerular filtration in freshwater species in contrast to mammals (both glomerular and tubular secretion). The glomerular filtration rate in freshwater species is low (about 2-7 ml/hr/kg; Hickman & Trump 1969). Because of the low tubular permeability to filtered plasma water the urine is dilute. Its volume balance the quantity of water entering the body (Hickman & Trump 1969). The passive filtration process in carp in conjunction with the slow release of the accumulated OTC in pronephros, bone tissue and scales contribute to the extended elimination half-life.

Differences in pharmacokinetic behaviour following different routes of administration (i.v., i.m., oral) were observed with respect to bioavailability and peak concentration (C_{max}). The intraperitoneal route of administration of OTC is discussed elsewhere (Grondel, Nouws & Van Muiswinkel 1986c). The lowest bioavailability was achieved in carp receiving antibiotic containing food. The maximum plasma OTC concentration (C_{max}) was 0.28 µg/ml at 20 h after oral administration of a dose of 34 mg OTC/kg. The oral bioavailability (0.6%) was 7 times lower than e.g. in pigs (Mevius et al. 1986). Also low OTC levels (0.26 µg/ml) were detected in serum of adult sockeye salmon (Oncorhynchus nerka) after a single dose (10 mg OTC/fish) administered in a gelatine capsule (Strasdine & McBride 1979).

Serious doubt has to be expressed about the therapeutic efficacy of the low OTC levels achieved in our oral trial. The minimum inhibitory OTC

concentration (MIC) for common fish pathogens varies widely: Aeromonas salmonicida 0.25 µg/ml; A liquefaciens 0.5 µg/ml; Pseudomonas fluorescens 5.0 µg/ml; Flexibacter columnaris 8 µg/ml (Herman 1969).

There is a tendency of tetracyclines to bind and to form complexes with substances in the alimentary tract which implies that absorption might be slow and incomplete (Notari 1975; Neu 1978). To avoid food interactions one may change the composition of the food ingredients (a special diet for medication) or increase the amount of OTC per kg food. Indeed, with doses of 4000 - 16000 mg OTC/kg food OTC levels were achieved which varied considerably in different tissues of rainbow trout Salmo gairdneri (0.5 µg/ml in muscle and 13.5 µg/ml in gills; Keck, Martell & Gerard 1984). However, it is emphasized that the pharmacokinetic behaviour of OTC may vary impressively with respect to both fish species and temperature (manuscript in prep.). Furthermore, drug-induced damage of the intestinal epithelium might occur at high concentrations (De Jonge, 1973) and a low absorption rate. Moreover, one has to consider the fact that ±15% of the teleost species are stomachless, including carp. Protein digestion and absorption are mainly performed in the anterior 60% of the digestive tract as shown for grasscarp Ctenopharyngodon idella (Stroband 1980). In contrast to fish species with a stomach and mammals, strong pH variations were absent (pH= ±7).

Although the Vd value was relatively large, it does not indicate that OTC is distributed homogeneous in different tissues and organs. The results show that OTC was accumulated in pronephros, bone tissue and scales. A readily reversible binding means that certain extravascular compartments may serve as a pool of active drug. However, considerable OTC concentrations were detected in these tissues even after 505 h; 2.9 ± 0.8, 5.2 ± 0.3 and 4.7 ± 3.1 µg/ml, respectively. This indicates a strong binding or complexation and consequently a minor availability of the drug. The ability of bone tissue of fish to accumulate OTC was mentioned by Herman (1969). Furthermore, whole body autoradiography studies in rainbow trout showed that tritium labelled tetracycline was still present in the skeleton 21 days after administration (Ingebrigtsen, Nafstad & Maritim 1985).

In tissue samples from the dorsal region, including the injection site, OTC could not be demonstrated after 505 h. Obviously, the OTC levels reached values beneath the detection limit of the microbiological assay (0.05 µg/ml). No irritating effect was observed at the injection site using this OTC formulation.

High OTC concentrations were detected in the pronephros. The drug

accumulated in this important lymphoid and hemopoietic organ. Immunomodulating effects of OTC in fish are reported (Grondel, Gloudemans & Van Muiswinkel 1985; Grondel et al. 1986c) and the influence of antibiotics on the immune system in general has been reviewed recently (Grondel and Van Muiswinkel, 1986b).

From a practical point of view oxytetracycline is administered most easily by mixing the drug with food. However, it is obvious that several pharmacological and immunological disadvantages should be taken into consideration when OTC is chosen for therapeutic use in fish farming.

Immuno-pharmacological research in fish should be directed toward antibiotics which would be rapidly and completely absorbed and distributed throughout the proper tissues without accumulation or interfering with immunological defence mechanisms. For the establishment of proper withdrawal times with respect to consumption the half-life of the drug must be short.

It is emphasized that ectothermic animals are adapted to relatively low temperatures. Extreme temperature differences do occur depending upon geographical location and seasonal variations. Fluidity measurements of plasma membranes of lymphocytes isolated from both cold- and warm-acclimated pinfish Lagodon rhomboides showed no differences (Abruzzini, Ingram & Clem 1982). Consequently, the membrane composition must be different to ensure optimal physiological processes. The biochemical adaptation to the environmental temperature may have implications for the pharmacokinetic behaviour of a drug and for the susceptibility of the immunological defence mechanisms to toxic damage.

Pharmacokinetic data on tissue distribution, plasma disposition and drug metabolism are scarce in fish and can differ markedly from endothermic animals as mammals and birds (Nouws, Vree, Breukink, Van Miert & Grondel 1986; Grondel, Nouws & Haenen 1986a). Moreover, extreme differences in pharmacokinetic behaviour of drugs between fish species can be expected with respect to species, age and disease status. Therefore, a critical immuno-pharmacological approach of this subject is a prerequisite for a rational use of antibiotics in the treatment of fish.

ACKNOWLEDGEMENTS

We are grateful to Mr. E. Termond (Department of Clinical Pharmacy, St. Radboud Hospital, Nijmegen, The Netherlands) for the performance of the NONLIN computer program.

REFERENCES

Abruzzini, A.F., Ingram, L.O. & Clem, L.W. (1982) Temperature-mediated processes in teleost immunity: homeoviscous adaptation in teleost lymphocytes. Proceedings of the Society for Experimental Biology and Medicine **169**, 12-18.

Austin, B. (1984) The control of bacterial fish diseases by antimicrobial compounds. In Woodbine, M. (ed.), Antimicrobials in agriculture. Butterworths, London, pp. 255-268.

Baggot, J.D. (1977) Principles of drug disposition in domestic animals. W.B. Saunders & Co., Philadelphia/London/Toronto. 238 pp.

De Jonge, H. (1973) Toxicity of tetracyclines in rat-small-intestinal epithelium. Biochemical Pharmacology **22**, 2659-2677.

Grondel, J.L., Gloudemans, A.G.M. & Van Muiswinkel, W.B. (1985) The influence of antibiotics on the immune system. II. Modulation of fish leukocyte responses in culture. Veterinary Immunology Immunopathology **9**, 251-260.

Grondel, J.L., Nouws, J.F.M. & O.L.M. Haenen (1986a) Fish and antibiotics. Pharmacokinetics of sulphadimidine in carp (Cyprinus carpio). Veterinary Immunology Immunopathology **12**, 281-286.

Grondel, J.L. & Van Muiswinkel, W.B. (1986b) Immunological defence mechanisms as a target for antibiotics. A review. In: Van Miert (Ed.); Comparative Veterinary Pharmacology, Toxicology and Therapy, 3rd EAVPT Congress, Ghent, Belgium. MTP Press Ltd., Lancaster, in press.

Grondel, J.L., Nouws, J.F.M. & Van Muiswinkel (1986c) The influence of antibiotics on the immune system. IV. Immuno-pharmacokinetic investigation on the primary anti-SRBC response in carp (Cyprinus carpio) after oxytetracycline injection. Journal of Fish Diseases (in press).

Herman, R.L. (1969) Oxytetracycline in fish culture, a review. Bureau of Sport Fisheries and Wildlife, U.S. Department of Interior, Washington D.C., Technical Papers **31**, 9 pp.

Hickman, C.P. & Trump, B.F. (1969) Fish Physiology. Volume I. Hoar & Randall (Eds.). Academic Press, New York. pp 91-239.

Ingebrigtsen, K., Nafstad, I. & Maritim, A. (1985) The distribution of ^{3}H-tetracycline after a single oral dose in the rainbow trout (Salmo gairdneri) as observed by whole body autoradiography. Acta Veterinaria Scandinavia **26**, 428-430.

Keck, G., Martell, C. & Gerard, J.P. (1984) Pharmacologie des poissons. Etude pharmacocinetique de l'oxytetracycline (OTC) chez la truite arc-en-ciel. Revue Medicine Veterinair **135**, 217-228.

Metzler, C.M., Elfring, C.L. & McEwen, A. (1974) I.: A package of computer Programs for pharmacokinetic modelling. Biometrics **30** (3), 562-570.

Mevius, D.J., Vellenga, L., Breukink, H.J., Nouws, J.F.M., Vree, T.B. & Driessens, F. (1986) Pharmacokinetics and renal clearance of oxytetracycline in piglets following intravenous and oral administration. The Veterinary Quarterly (in press).

Neu, H.C. (1978) A symposium on the tetracyclines: a Major appraisal. Bulletin of the New York Academy of Medicine **54**, 141-155.

Notari, R.E. (1975) Biopharmaceutics and Pharmacokinetics. M. Dekker Inc., New York. 285 pp.

Nouws, J.F.M., Van Ginneken, C.A.M. & Ziv, G. (1983a) Age-dependent pharmacokinetics of tetracycline in ruminants. Journal of Veterinary Pharmacology and Therapy **6**, 59-66.

Nouws, J.F.M. & Vree, T.B. (1983b). Effect of injection site on the bioavailability of an oxytetracycline formulation in ruminant calves. The Veterinary Quarterly **5**, 165-170.

Nouws, J.F.M., Vree, T.B., Termond, E., Lohuis, J., Van Lith, P., Binkhorst, G.J. & Breukink, H.J. (1985) Pharmacokinetics and renal clearance of oxytetracycline after intravenous and intramuscular administration to dairy cows. The Veterinary Quarterly **7**, 296-305.

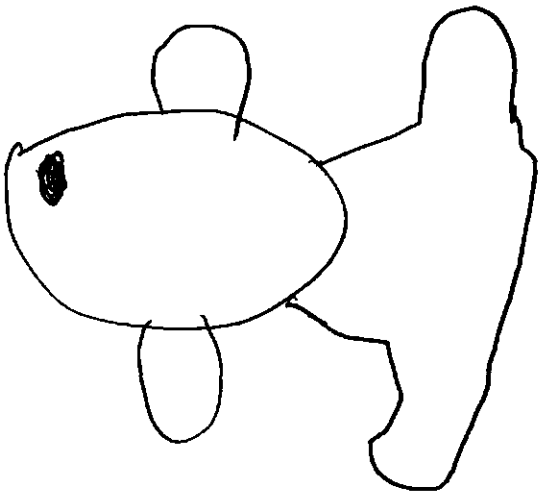
Nouws, J.F.M., Vree, T.B., Breukink, H.J., Van Miert, A.S.J.P.A.M. & Grondel, J.L. (1986) Pharmacokinetics, hydroxylation and acetylation of sulphadimidine in man, ruminants, horses, pigs and fish. In : Van Miert (Ed.); Comparative Veterinary Pharmacology, Toxicology and Therapy, 3rd EAVPT Congress, Ghent, Belgium. MPT Press Ltd., Lancaster, in press.

Strasdine, G.A. & McBride, J.R. (1979) Serum antibiotic levels in adult sockeye salmon as a function of route of administration. Journal of Fish Biology **15**, 135-140.

Stroband, H.W.J. (1980) Structure and function of the digestive tract of the grasscarp. Histological and Morphological Thesis, Agricultural University, Wageningen, The Netherlands.

COMPARATIVE PHARMACOKINETIC INVESTIGATIONS IN RAINBOW TROUT (SALMO GAIRDNERI) AND AFRICAN CATFISH (CLARIAS GARIEPINUS) FOLLOWING OXYTETRACYCLINE ADMINISTRATION

J.L. GRONDEL, J.F.M. NOUWS, A.R. SCHUTTE AND F. DRIESSENS



SUMMARY

A comparative pharmacokinetic study was conducted in rainbow trout (Salmo gairdneri) and African catfish (Clarias gariepinus) following intravenous (i.v.) and intramuscular (i.m.) administration of oxytetracycline (OTC) at 60 mg/kg body weight. Trout and catfish were kept in tanks with aerated tap water at a constant temperature of 12°C and 25°C, respectively. Concentrations of OTC were determined in plasma samples which were collected at regular time intervals.

The two- and three-compartment open model adequately described the plasma drug levels in African catfish and rainbow trout following i.v. OTC administration, respectively. A eight fold higher extrapolated zero time concentration (C_p^0 = 753.3 ± 290.7 µg/ml) was observed in trout followed by an initial steep decline in plasma levels compared to catfish (C_p^0 = 86.4 ± 10.9 µg/ml). A significant difference was observed between the two species with respect to the relatively large apparent distribution volumes (Vd area) after i.v. OTC administration (trout: 2.1 ± 0.3; catfish: 1.33 ± 0.22 l/kg). The mean final elimination half-lives of both fish species were extended compared to mammals (trout: 89.5; catfish: 80.3 h). A mean maximum plasma concentration (C_{max} = 56.9 µg/ml) was observed in trout at 4 h after i.m. administration of OTC. Whereas, in catfish a lower C_{max} (± 43.4 µg/ml) was determined at about 7 h. No significant difference was observed with respect to bioavailability following i.m. administration of OTC (trout: 85%; catfish: 86%).

The pharmacokinetic data of OTC and its immuno-modulating abilities are discussed. Investigation of new products in different fish species is proposed in order to achieve an optimal use in agreement with the marked physiological differences in this animal group.

INTRODUCTION

Oxytetracycline (OTC) is one of the most widely used antibiotics for prophylactic and therapeutic purposes in fish farming. It is administered to a wide range of fish species, both freshwater and marine species. However, pharmacokinetic data which describe drug behaviour in fish are limited and may differ markedly between species.

Drug absorption is an important clinical parameter. The physicochemical properties of the drug and the physiological conditions determine the

distribution throughout the tissues. The extent to which a drug is absorbed is correlated with the systemic availability and consequently determines the therapeutical efficacy. The physiological conditions may vary quite considerably depending upon, for instance, temperature when considering ectothermic animals (Smit, Hattingh & Ferreira 1981). An extended elimination half-life was observed for oxytetracycline in carp (Cyprinus carpio) at 20°C (Grondel, Nouws & Van Muiswinkel 1986a; Grondel, Nouws, De Jong, Schutte & Driessens 1986b) and for gentamicin in channel catfish (Ictalurus punctatus) at 22°C (Rolf, Setser & Walker 1986) when compared with the situation in mammals. The characteristic deposition of tetracyclines in bones and scales of fish was used for marking animals (Weber & Ridgway 1962). However, it has been demonstrated in carp that accumulation of oxytetracycline also occurred in an immunological important organ; pronephros (Grondel et al., 1986a+b). Interference with immunological defence mechanisms will decrease the defensive potential against invading pathogens.

Because of the widespread therapeutic use of oxytetracycline and its immuno-modulating abilities a comparative study was conducted to obtain pharmacokinetic data from 2 economic important species, one a typical coldwater and the other a warmwater fish (trout and catfish).

MATERIALS AND METHODS

Animals

Outbred rainbow trout (Salmo gairdneri) were obtained from the Organization for Improvement of Inland Fisheries (O.V.B., Lelystad, The Netherlands) and kept individually in tanks with aerated tap water at a temperature of 12°C. African catfish (Clarias gariepinus) were bred at our university (Fish accommodation "De Haar") and kept in tanks with aerated water at a temperature of 25°C. Rainbow trout and African catfish were acclimatized for 8 and 2 weeks, respectively. Animals were fed daily with pelleted dry food (Trouvit, Trouw & Co., Putten, The Netherlands).

Antibiotic treatments

Both fish species were divided into two groups. One group of both species (n=4) received oxytetracycline (OTC) by an intravenous (i.v., caudal vein)

injection. To the second group (n=3) OTC was administered intramuscularly (i.m., dorsal region). An injectable veterinary formulation of OTC (Engemycine^R, Mycofarm, De Bilt, The Netherlands) was administered at a dose of 60 mg/kg. Heparinized blood samples (0.2 ml) were taken from the caudal vein at regular time intervals for all four groups and were centrifuged for 10 min at 800 x g. Plasma samples were frozen at -20°C pending analysis

Determination of OTC concentrations

The large plate agar-diffusion method was used. OTC concentrations in plasma were determined according to the method described by Nouws, Van Ginneken & Ziv 1983a; Nouws & Vree 1983b). Briefly, the samples were assayed with Bacillus subtilis BGA (10^4 spores/ml) or Bacillus cereus var. mycoides (ATCC 6941) plates (0.1 ml of a 24-h broth culture/200 ml agar). Standard II Nähr-agar (Merck 7883) was used, supplemented with 0.2% KH_2PO_4, pH 6.0. The B. subtilis plates were incubated overnight at 30°C, and those with B. mycoides were incubated overnight at 25°C. The OTC concentrations were calculated by standard curves obtained by antibiotic standards prepared in pooled trout or catfish plasma.

Pharmacokinetic analysis

Plasma OTC concentrations for each animal in the i.v. and i.m. experimental groups were analyzed according to the standard procedures described by Baggot (1977). Additional calculations from the i.v. and i.m. OTC concentration-time data were performed according to the procedures described by Nouws, Vree, Termond, Lohuis, Van Lith, Binkhorst & Breukink 1985.

The intravenous plasma OTC concentrations of each catfish and trout were analyzed with the NONLIN Program (Metzler, Elfring & McEwen 1974) for the best fit to the two- or three-compartment pharmacokinetic model. The plasma OTC concentration-time curve (according to the two- or three-compartment model) can be adequately described by the equations:

$C_p = A^\circ.e^{-\alpha.t} + B^\circ.e^{-\beta.t}$ or $C_p = A^\circ.e^{-\alpha.t} + B^\circ.e^{-\beta.t} + C^\circ.e^{-\gamma.t}$, respectively.

By means of the parameters A°, B°, C°, α, β, and γ the pharmacokinetic parameters K_{12}, K_{21}, K_{13}, K_{31} and K_{el} can be calculated.

Additional calculations of Vd area (apparent volume of distribution), body clearance (Cl_b) and distribution volume of the central compartment (V1)

were performed in a model-independent way according to Nouws et al., 1983a.

The peak OTC concentration (C_{max}) and peak time (T_{max}) for each animal were read from the concentration-time curve. The area under the curve (AUC) was calculated using the trapezoid rule.

TABLE 1

Antibiotic concentrations in plasma of **African catfish** following intravenous and intramuscular administration of oxytetracycline

Temp. 25°C		
Dose (mg/kg) 60		
	I.V.	I.M.
No. of animals	4	3
Weight ± sd (g)	293 ± 45	290 ± 43
	Plasma oxytetracycline concentration	
Time (h)	(µg/ml ± sd)	
1.0	80.6 ± 15.7	32.8 ± 19.1
2.0	69.8 ± 11.6	36.4 ± 20.2
4.0	60.8 ± 11.9	35.1 ± 13.9
7.0	54.4 ± 14.1	36.1 ± 15.0
10.0	52.0 ± 14.6	34.7 ± 15.3
14.0	51.2 ± 8.9	33.8 ± 8.4
24.0	35.0 ± 11.5	29.7 ± 6.4
48.0	27.3 ± 4.2	24.1 ± 8.2
72.0	24.3 ± 4.6	22.1 ± 5.8
96.0	19.5 ± 3.9	19.3 ± 5.1
120.0	15.1 ± 3.4	14.3 ± 6.6
144.0	13.1 ± 2.2	11.3 ± 4.9
192.0	9.0 ± 2.3	10.2 ± 1.2
240.0	5.8 ± 1.9	6.6 ± 1.3
288.0	3.7 ± 1.1	3.9 ± 1.3
384.0	1.3 ± 0.3	1.7 ± 0.5
434.0	0.84 ± 0.2	0.98 ± 0.3
480.0	0.58 ± 0.1	0.60 ± 0.3
528.0	0.43 ± 0.1	0.44 ± 0.2
600.0	0.30 ± 0.1	0.36 ± 0.2

RESULTS

Mean plasma OTC concentrations following i.v. and i.m. administration for African catfish and rainbow trout are presented in Table 1 and 2, respectively. Individual semi-logarithmic plots of plasma OTC concentration versus time after i.v. and i.m. injection are demonstrated in Figure 1 and 2. Pharmacokinetic values for oxytetracycline administered i.v. of both groups of animals are presented in Table 3 and 4.

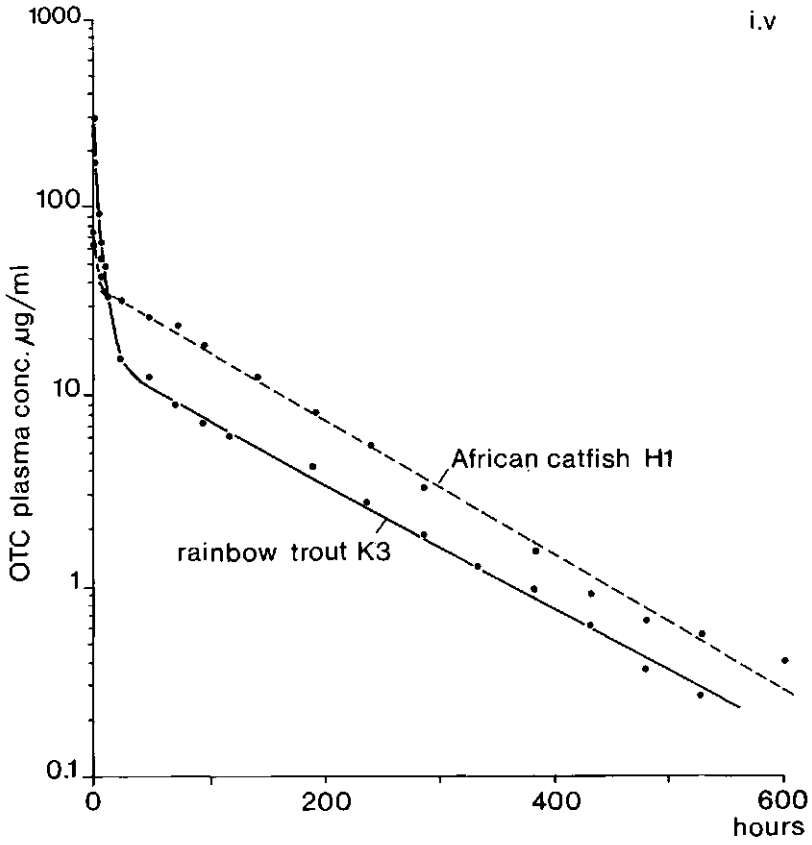


Figure 1. Plasma concentrations of oxytetracycline (OTC) in a rainbow trout (K3) and an African catfish (H1) following a single intravenous injection of 60 mg OTC/kg. The animals were kept at 12°C and 25°C, respectively.

Comparisons of plasma OTC concentrations following i.v. administration were performed for both fish species with the computer-generated two- and three-exponential concentration-time plots. For catfish, it was shown that the curves were best described by the two-compartment model. When OTC was

injected i.v. into catfish a biphasic curve was observed (Fig. 1) and an extrapolated mean zero time concentration (C_p^0) could be calculated of 86.4 µg/ml (Table 3). In contrast, the results of trout

TABLE 2

Antibiotic concentrations in plasma of **rainbow trout** following intravenous and intramuscular administration of oxytetracycline

Temp.	12°C	
Dose (mg/kg)	60	
	I.V.	I.M.
No. of animals	4	3
Weight ± sd (g)	323 ± 9	390 ± 55
	Plasma oxytetracycline concentration	
Time (h)	(µg/ml ± sd)	
1.0	293.2 ± 55.6	26.9 ± 7.5
2.0	171.1 ± 30.4	43.0 ± 15.0
4.0	100.0 ± 35.2	56.9 ± 13.5
7.0	70.2 ± 11.7	50.9 ± 14.1
10.0	50.0 ± 11.5	46.3 ± 10.0
14.0	37.8 ± 3.3	36.2 ± 10.6
24.0	21.4 ± 5.5	19.5 ± 4.7
48.0	13.7 ± 2.6	12.2 ± 2.0
72.0	10.0 ± 1.8	10.5 ± 2.4
96.0	8.2 ± 1.6	9.1 ± 2.7
120.0	6.2 ± 0.9	7.7 ± 3.7
192.0	4.2 ± 0.7	5.6 ± 3.7
240.0	2.8 ± 0.4	4.2 ± 3.4
288.0	1.7 ± 0.2	2.4 ± 1.4
336.0	1.2 ± 0.2	1.9 ± 1.0
384.0	0.9 ± 0.2	1.4 ± 0.8
434.0	0.62 ± 0.2	1.0 ± 0.7
480.0	0.33 ± 0.1	0.68 ± 0.5
528.0	0.29 ± 0.1	0.51 ± 0.4
600.0	0.15 ± 0.0	0.26 ± 0.2

revealed a triphasic curve which was optimal described by a three-compartment model. The extrapolated mean zero time concentration (C_p^o) was about 8-fold higher and reflected a significant difference ($p<0.01$) between the distribution volumes of the central compartment (trout: ± 0.09 l/kg; catfish: 0.7 l/kg, Table 3 and 4). Moreover, an initial steep decline in plasma OTC levels was observed in trout. Differences were also observed with respect to the apparent volumes of distribution following i.v.

TABLE 3

Pharmacokinetic values for oxytetracycline administered intravenously to **African catfish** (mean ± sd)
Two-compartment open model

Temp.	25°C	
Dose (mg/kg)	60	
No. of animals		4
Weight	(g)	293 ± 45
A^o	(µg/ml)	42.5 ± 8.5
B^o	(µg/ml)	43.9 ± 9.1
α	(h^{-1})	0.1841 ± 0.1212
β	(h^{-1})	0.0087 ± 0.0004
$T\frac{1}{2}\alpha$	(h)	5.2 ± 3.2
$T\frac{1}{2}\beta$	(h)	80.3 ± 3.9
K_{el}	(h^{-1})	0.0164 ± 0.0028
K_{12}	(h^{-1})	0.0844 ± 0.0710
K_{21}	(h^{-1})	0.0919 ± 0.0483
K_{12}/K_{21}		0.69 ± 0.49
$AUC^{o}_{-\infty}$	(µg.h/ml)	5369 ± 1102
Cl_b	(ml/min/kg)	0.19 ± 0.03
Vd area	(l/kg)	1.33 ± 0.22
V1	(l/kg)	0.70 ± 0.09
C_p^o	(µg/ml)	86.4 ± 10.9

administration of OTC (trout: ± 2.10 l/kg; catfish: 1.33 l/kg). Body clearance was determined as Cl_b = dose (i.v.)/$AUC^{o}_{-\infty}$ and revealed that trout cleared a larger volume of blood (Table 3 and 4). The mean final elimination half-lives were similar, but differed markedly compared with mammals.

The initial steep decline of plasma OTC concentration following i.v. administration in both species, is mainly a result of distribution from the central compartment (V1) to tissue compartments (T2 in catfish and T2 + T3 in trout). The rate of diffusion (K_{12}) from the central compartment (V1) to the peripheral tissue compartment (T2) in trout was about 7 times faster

TABLE 4

Pharmacokinetic values for oxytetracycline administered intravenously to **rainbow trout** (mean ± sd)
Three-compartment open model

Temp.	12°C	
Dose (mg/kg)	60	
No. of animals		4
Weight	(g)	323 ± 9
A°	(µg/ml)	600.8 ± 247.4
B°	(µg/ml)	134.9 ± 85.9
C°	(µg/ml)	17.5 ± 3.6
α	(h^{-1})	1.3475 ± 0.6339
β	(h^{-1})	0.1230 ± 0.0428
γ	(h^{-1})	0.0078 ± 0.0008
$T\frac{1}{2}\alpha$	(h)	0.6 ± 0.2
$T\frac{1}{2}\beta$	(h)	6.3 ± 2.6
$T\frac{1}{2}\gamma$	(h)	89.5 ± 8.7
K_{el}	(h^{-1})	0.1749 ± 0.0132
K_{12}	(h^{-1})	0.6484 ± 0.3665
K_{21}	(h^{-1})	0.3826 ± 0.2390
K_{12}/K_{21}		1.78 ± 0.41
K_{13}	(h^{-1})	0.2304 ± 0.0920
K_{31}	(h^{-1})	0.0182 ± 0.0029
K_{13}/K_{31}		12.36 ± 3.6
$AUC^{\circ}_{-\infty}$	(µg.h/ml)	3759 ± 306
Cl_b	(ml/min/kg)	0.27 ± 0.02
Vd area	(l/kg)	2.10 ± 0.30
V1	(l/kg)	0.09 ± 0.04
C°_p	(µg/ml)	753.3 ± 290.7

than in catfish. Moreover, the OTC diffusion from the central compartment to the peripheral compartment was 1.78-fold (K_{12}/K_{21}) faster than the back diffusion (Table 4). Whereas, in catfish the mean back diffusion rate (K_{21}, T2 -> V1) was not significantly different from the forward diffusion rate (K_{12}, V1 -> T2, Table 3). The back diffusion rate (K_{21}, T2 -> V1) was 4 times slower than in trout. In trout, the rate of diffusion to the peripheral compartment (K_{12}, V1 -> T2) was about 2.8-fold higher than to the deeper compartment (K_{13}, V1 -> T3).

The final elimination phase (trout: ± 0.0078 h^{-1}; catfish: 0.0087 h^{-1}) constitutes primarily the release of drug from the deep compartment (T3) in trout and the tissue compartment (T2) in catfish, respectively. The rate of back diffusion (K_{31}, T3 -> V1) in trout was ± 12 times slower than the forward diffusion (K_{13}) and was 21-fold slower than that of the peripheral compartment to the central compartment (K_{21}, T2 -> V1; Table 4).

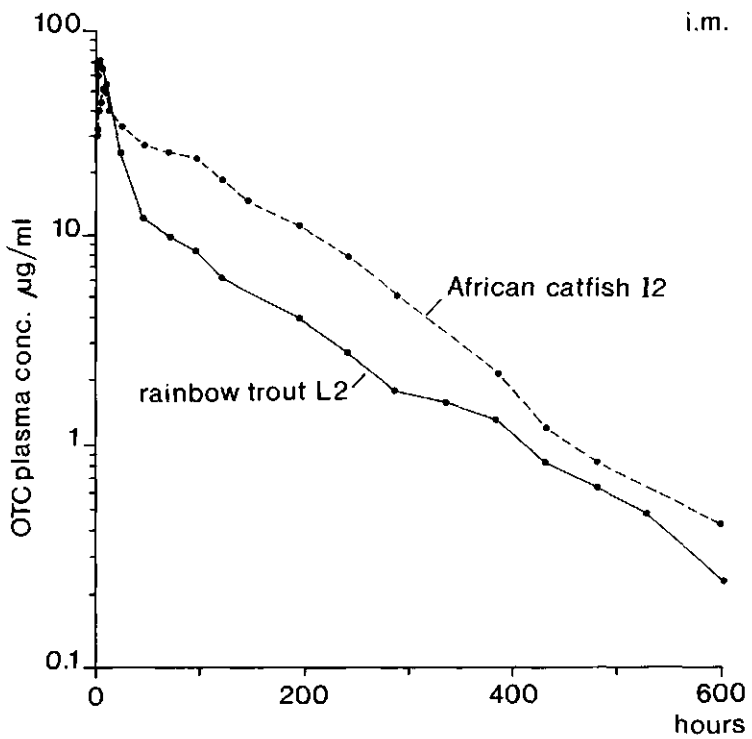


Figure 2. Plasma concentrations of oxytetracycline (OTC) in a rainbow trout (L2) and an African catfish (I2) following a single intramuscular injection of 60mg OTC/kg. The animals were kept at 12°C and 25°C, respectively.

Following i.m. OTC injection comparable differences were observed. In trout C_{max} (56.9 ± 13.5 µg/ml) was reached ± 4 h post administration. Whereas, in catfish C_{max} was lower (43.4 ± 16.3 µg/ml) and detected at a later moment: ± 7 h. The absorption phase revealed half-lives of ± 4 h and ± 1 h post administration in catfish and trout, respectively. A biphasic elimination phase was observed in both fish species. The final elimination half-lives were: trout: 94.7 ± 16.0 h; catfish: 74.4 ± 10.1 h. The bioavailability (t = 0-600 h) achieved with the i.m. route was about 85% for both species.

After i.m. administration of OTC (dorsal region) a swelling was observed at the injection site in catfish and lasted for 2-5 days.

DISCUSSION

In this comparative study of OTC in rainbow trout (Salmo gairdneri) and african catfish (Clarias gariepinus) considerable differences in pharmacokinetic behaviour were observed. From the data showing time-related changes in serum OTC concentrations, following i.v. injection, several pharmacokinetic parameters could be calculated, which described the behaviour of OTC in both species.

A two- and three-compartment open model adequately described the plasma drug levels in African catfish and rainbow trout following i.v. administration of OTC, respectively. The extrapolated zero time (C_p^0) for the OTC concentration in trout was relatively high compared to catfish. This reflects a small distribution volume of the central compartment (V1). In carp (Cyprinus carpio) at 20°C, an intermediate distribution volume was observed following i.v. administration of OTC at 60 mg/kg (C_p^0 = 290 ± 37; V1 = 0.21 ± 0.02 l/kg; Grondel et al., 1986b). Here too, a three-compartment open model could be applied which optimally described the plasma OTC concentrations.

Furthermore, species differences were observed with respect to blood clearance and apparent volumes of distribution (Table 3 and 4). The apparent volume of distribution (Vd area) of OTC in trout was relatively large (± 2.1 l/kg). This parameter provides an idea about the extent of distribution. However, it does not indicate in which way OTC is distributed throughout the different tissues and organs. In this respect, it is interesting to mention that OTC accumulated in bone tissue, scales and pronephros of carp (Grondel

et al., 1986b). The nephros is an important immunological organ in bony fish (Grondel et al., 1986a).

Striking differences were observed with respect to the rate constants (K_{12}, trout: ± 0.6484 h^{-1} ; catfish: ± 0.0844 h^{-1}) which explained in part the steep decline of OTC levels in trout compared with catfish. The different rate constant values (K_{12}, K_{21} and K_{13}, K_{31}) emphasize a species dependent behaviour of a particular drug. This could be explained by tissue composition, blood perfusion and mode of life.

In fish, marked differences in vascularization have been described with respect to the type of muscles: white, pink and red (Boddeke, Slijper & Van Der Stelt 1959; Mosse 1978; Akster 1981). Furthermore, the ratio white/red muscle varies and is related to the mode of life of each species. For instance, the muscles of pike (Esox lucius) and perch (Perca fluviatilis) have nearly exclusively broad white muscle fibres. Whereas, in carp and trout a fairly high amount of narrow red muscle fibres have been demonstrated (Boddeke et al., 1959). Unfortunately, data on African catfish are not available.

The final elimination half-life for OTC in both fish species were similar, but were extended compared to mammals (Baggot, 1977; Nouws et al., 1983a+b; Nouws, Vree, Breukink, Van Miert & Grondel 1986). A relatively long half-life (± 15 h) was also observed for gentamicin in channel catfish (Ictalurus punctatus) at 22°C following i.m. administration (Rolf et al., 1986). However, comparative studies of sulphadimidine and its metabolites between a wide range of species (e.g. man, horses, pigs, ruminants, laying-hens and carp) revealed a half-life for carp at 20°C which was far from extended. The elimination half-lives were 7.7, 9.5, 9-11, 3.5, 3.5, and 17.5 h, respectively (Nouws et al., 1986). Furthermore, comparison of the half-life values of chloramphenicol in carp (20°C) and rainbow trout (12°C) following i.m. injection of the drug yielded similar data (9 -10 h; Grondel and Nouws, unpublished data). The extended half-life of OTC could be explained by the elimination of the drug by a passive diffusion process at the glomerular site and the gills. Whereas, in mammals active processes are involved.

Many factors determine the pharmacokinetic behaviour of a drug within the body. The ambient temperature and corresponding metabolic rate seems to be an obvious explanation for the extended drug half-live in ectothermic animals. However, from the limited data available today it may be concluded that half-life differences are not merely due to the ambient temperature.

This becomes clear when fishes with a different environmental adaptation are compared at the same temperature.

The elimination process constitutes of excretion and biotransformation. Drug metabolism increases the clearance from the body. In many fish species the major biotransformation reactions have been observed (e.g. hydroxylation, dealkylation, hydrolysis and conjugation reactions; Guarino & Lech 1986). However, the amount and composition of enzymes available may differ markedly between species. Oxytetracycline metabolism is minimal in mammals and is excreted in the urine. It has been demonstrated for sulphadimidine in carp that this compound could be converted to hydroxylated and acetylated metabolites. The main metabolite was N_4-sulphadimidine, but composed only 2% of the dose excreted (Nouws et al., 1986).

Comparison of the serum level-time curves, obtained after giving OTC i.m. at the same dosage rate (60 mg/kg) to catfish and trout, showed that the drug was most absorbed by catfish, based on the relative areas under the curve (AUC t = 0-600 h). Significant differences in the bioavailability were not observed, in contrast to peak concentration (C_{max}) and peak time (T_{max}) which reflects the rate of absorbtion from the injection site. In carp at 20°C intermediate peak concentrations (C_{max} = 40.8 ± 12.2 µg/ml) were detected at an extended time; T_{max} = 14.0 h (Grondel et al., 1986b).

In conclusion, OTC is used over a wide range of fish species. However, clear differences were observed with respect to the pharmacokinetic behaviour of this drug in rainbow trout and African catfish. Consequently, the therapeutic regimes should be adapted for each fish species. However, the immuno-modulating abilities of tetracyclines and perhaps also other drugs (Grondel & Van Muiswinkel 1986c), emphasize the need to investigate any new product.

ACKNOWLEDGEMENT

We are grateful to Mr. E. Termond (Department of Clinical Pharmacy, St. Radboud Hospital, Nijmegen, The Netherlands) for the performance of the NONLIN computer program. The biotechnical support of Mr. S.H. Leenstra and Mr. P. van Kleef is gratefully acknowledged. Furthermore, we thank Mr. F. Samuels (O.V.B., Lelystad, The Netherlands) for providing rainbow trout.

REFERENCES

Akster, H.A. (1981). Ultrastructure of muscle fibres in head and axial muscles of perch (Perca fluviatilis L.) A quantitive study. Cell and Tissue Research **119**, 111-131.

Baggot, J.D. (1977). Principles of drug disposition in domestic animals. W.B. Saunders & Co., Philadelphia/London/Toronto.

Boddeke, R., Slijper, E.J. & Van Der Stelt, A. (1959) Histological characteristics of the body-musculature of fishes in connection with their mode of life. Proceedings of the Koninklijke Nederlandse Akademie voor Wetenschappen Serie C **62**, 576-588.

Grondel, J.L., Nouws, J.F.M. & Van Muiswinkel, W.B. (1986a). The influence of antibiotics on the immune system. IV. Immuno-pharmacokinetic investigation on the primary anti-SRBC response in carp (Cyprinus carpio) after oxytetracycline injection. Journal of Fish Diseases, in press.

Grondel, J.L., Nouws, J.F.M., De Jong, M., Schutte, A.R. & Driessens F. (1986b). Pharmacokinetics and tissue distribution in carp (Cyprinus carpio) following different routes of administration of oxytetracycline. Submitted for publication.

Grondel, J.L. & Van Muiswinkel, W.B. (1986c). Immunological defence mechanisms as a target for antibiotics. A review. In: Van Miert (Ed.); Comparative Veterinary Pharmacology, Toxicology and Therapy, 3rd EAVPT Congress, Ghent, Belgium. MPT Press Ltd., Lancaster, in press.

Guarino, A.M. & Lech, J.J. (1986). Metabolism, Disposition and Toxicity of drugs and other xenobiotics in aquatic species. Veterinary and Human Toxicology, **28**, Suppl. 1, 38-44.

Metzler, C.M., Elfring, C.L. & McEwen, A. (1974) I.: A package of computer Programs for pharmacokinetic modelling. Biometrics 562-570. Upjohn Co, Kalamazoo, USA.

Mosse, P.R.L. (1978) The distribution of capillairies in the somatic musculature of two vertebrate types with particular reference to teleost fish. Cell and Tissue Research **187**, 281-303.

Nouws, J.F.M., Van Ginneken, C.A.M. & Ziv, G. (1983a). Age-dependent pharmacokinetics of tetracycline in ruminants. Journal of Veterinary Pharmacology and Therapy **6**, 59-66.

Nouws, J.F.M. & Vree, T.B. (1983b). Effect of injection site on the bioavailability of an oxytetracycline formulation in ruminant calves. The Veterinary Quarterly **5**, 165-170.

Nouws, J.F.M., Vree, T.B., Termond, E., Lohuis, J., Van Lith, P., Binkhorst, G.J. & Breukink, H.J. (1985) Pharmacokinetics and renal clearance of oxytetracycline after intravenous and intramuscular administration to dairy cows. The Veterinary Quarterly **7**, 296-305.

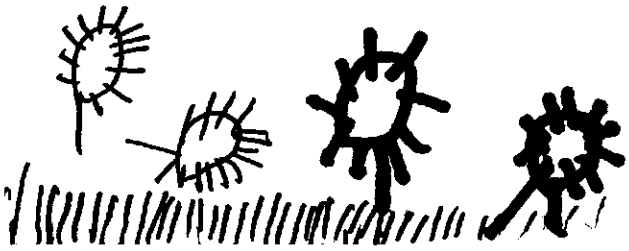
Nouws, J.F.M., Vree, T.B., Breukink, H.J., Van Miert, A.S.J. P.A.M. & Grondel, J.L. (1986). Pharmacokinetics, hydroxylation and acetylation of sulphadimidine in man, ruminants, horses, pigs and fish. In : Van Miert (Ed.); Comparative Veterinary Pharmacology, Toxicology and Therapy, 3rd EAVPT Congress, Ghent, Belgium. MPT Press Ltd., Lancaster, in press.

Rolf, L.L., Setser, M.D. & Walker, J.L. (1986). Pharmacokinetics and tissue residues in channel catfish (Ictalurus punctatus) given intracardiac and intramuscular injections of gentamicin sulfate. Veterinary and Human Toxicology **28**, Suppl. 1, 25-31.

Smit, G.L., Hattingh, J. & Ferreira, J.T. (1981). The physiological responses of blood during thermal adaptation in three freshwater fish species. Journal of Fish Biology **19**, 147-160.

Weber, D.D. & Ridgway, G.J. (1962). The deposition of tetracycline drugs in bones and scales of fish and its possible use for marking. The Progressive Fish Culturist **24**, 150-155.

SUMMARY



SUMMARY

Studies on antibiotics, oxytetracycline (OxyTC) in particular, are presented in this thesis with respect to the influence of these drugs on the immune system of carp and chickens. Special attention was paid to the pharmacokinetic behaviour of OxyTC.

Immunology

Carp (Cyprinus carpio) leukocytes from spleen, pronephros and mesonephros showed a clear in vitro mitogenic response. The DNA synthesis of PHA-stimulated pronephric cells was reduced to about 50% of the mitogen-activated controls at therapeutic concentrations of 4 - 6 µg OxyTC/ml. Doxycycline (DC) was even more suppressive: 50% inhibition at 1 - 2.5 µg/ml. The difference between the two tetracycline analogues may relate to the better lipid-solubility of DC. In addition, OxyTC (10 µg/ml) and DC (5 µg/ml) markedly reduced the incorporation of ^{3}H-thymidine into alloantigen-stimulated pronephric leukocytes by 61% and 98%, respectively. Furthermore, it was demonstrated that OxyTC changed the kinetics of the mitogenic response at relative low concentrations (5 - 15 µg/ml). A dose-dependent delay of 1 - 2 days was observed. In fact, the antibiotic suppression was transient. Obviously, the impairment of cellular functions like DNA synthesis is not due to cell killing.

The immuno-modulating effects of OxyTC was also demonstrated for chicken peripheral blood (PBL) and spleen leukocytes. However, the kinetics of the ^{3}H-thymidine incorporation by lectin-activated PBL showed that the DNA synthesis was severely suppressed in stead of delayed. The production of the amplifying/regulatory factor interleukin 2 (IL-2) was not affected in the presence of the antimicrobial agent. Whereas, the uptake of ^{3}H-thymidine by IL-2-dependent T cell blasts was strongly reduced. It was concluded that the T cell blast is one of the targets for OxyTC.

Investigations in carp on the kinetics of the primary anti-SRBC antibody response, during a short-term OxyTC treatment in vivo, revealed that the kinetics of the plaque forming cell response was not significantly affected by OxyTC. In contrast, the number of plaque forming cells was significantly decreased. Furthermore, the anti-SRBC antibody production was delayed by 2 - 4 days. However, within 12 - 14 days post immunization the same antibody levels were detected as in the control group, indicating the temporary effect of OxyTC on the immune response.

Pharmacokinetics

The objective of the pharmacokinetic studies was to investigate plasma disposition, distribution, bioavailability and elimination of OxyTC in carp, rainbow trout (Salmo gairdneri) and African catfish (Clarias gariepinus).

Analysis of the plasma drug concentration time curves following intravenous (i.v.) OxyTC administration (60 mg/kg) revealed distinct phases. The two- and three-compartment open model adequately described the plasma drug levels in catfish and trout, respectively. A three-compartment model was also used for carp to derive pharmacokinetic parameters. Compared with mammals, an extended average final elimination half-live was observed in all three fish species following i.v. OxyTC administration: carp: 139.8 h; trout: 89.5 h; catfish: 80.3 h. Between the three species, marked differences were observed with respect to the rate diffusion constants, distribution volume of the central compartment, apparent distribution volume and body clearance.

A bioavailability was observed of 80 - 86% following intramuscular (i.m.) injection of OxyTC (60 mg/kg). Whereas, a bioavailability of only 0.6% was achieved in carp with the oral route of administration.

Immunology and Pharmacokinetics

Oxytetracycline interfered with the immune response in carp. However, data were needed concerning the pharmacokinetic behaviour of this drug in carp. Tissue distribution studies following a single i.m. OxyTC injection (60 mg/kg) revealed that the drug was accumulating in pronephros, bone tissue and scales. After 21 days the average OxyTC concentrations were 2.9, 5.2 and 4.7 µg/ml, respectively. The pronephros is an important lymphoid and haemopoietic organ in fish. Furthermore, the pharmacokinetic data showed that high plasma levels (> 50 µg/ml) were detected over a relatively long time period. These data provide more insight into the action of OxyTC upon immuno-modulation.

Immuno-pharmacokinetic research

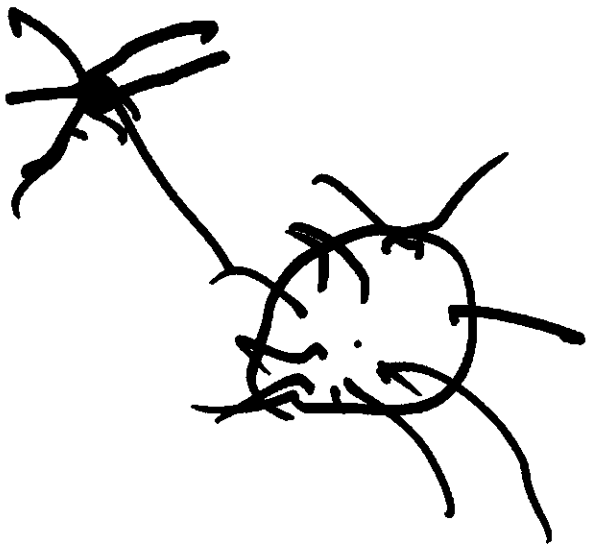
When defence mechanisms fail to prevent the establishment of infective micro-organisms in the host, the consequence will be disease. Under these conditions antibiotics are valuable tools for the therapy of bacterial infections. Interference of an antibiotic with the immunological responses will decrease the defensive potential. Therefore, it is important to exclude any immuno-suppression by antimicrobial drugs in animals and man. This is

obvious, because the defence mechanisms have to ensure the final elimination of pathogens.

In this context pharmacokinetic studies, including tissue distribution, are of great importance. These studies provide data concerning the behaviour of drugs within the body. Extreme differences can be expected with respect to animal species, age and disease status.

The combined action of immune system and antimicrobial drugs will increase the defensive capacity. Therefore, it is emphasized that an immuno-pharmacological approach is a prerequisite to prevent immuno-toxicological events and to optimize the use of antibiotics. Consequently, the fundamental aspects of this type of research will support an effective human and veterinary medical management.

SAMENVATTING



IMMUNOLOGISCHE AFWEERSYSTEMEN EN ANTIBIOTICA

Inleiding

Aan het vermogen van dieren om zich te verweren tegen ziekten worden in de intensieve dierhouderij hoge eisen gesteld. De intensieve visteelt is hierin geen uitzondering. Integendeel, in deze tak van houderij komen een aantal bijzondere aspecten aan bod die de visgezondheid direct of indirect beïnvloeden.

Met name de kwaliteit van het water is hierbij van groot belang (zie figuur 1). Veranderingen veroorzaakt door vervuiling van het oppervlaktewater kunnen desastreuze gevolgen hebben voor de gezondheid. De viskweker is niet of nauwelijks in staat controle op dergelijke invloeden uit te oefenen. Deze controle is wel uitvoerbaar in recirculatie-systemen waarbij het om een beperkt aantal te meten schadelijke stoffen gaat, o.a. ammoniak, nitriet en metaalionen (bijv. koper). Het zal duidelijk zijn dat water tevens een uitstekend transportmedium is voor ziektekiemen.

fig. 1

Een ander aspect is de temperatuur. De temperatuur en temperatuurs-veranderingen beïnvloeden in sterke mate het vermogen van koudbloedige dieren (o.a. vissen) op infecties te reageren. Dit kan o.a. tot uiting komen in de infectie-gevoeligheid tijdens de verschillende seizoenen. In scherp contrast hiermee staan de vogels met hun constante lichaams-temperatuur, onafhankelijk van het milieu. Bovendien is deze temperatuur relatief hoog: 41°C.

De weerstand van mens en dier tegen infectie-ziekten kan men opgebouwd denken uit een aantal verdedigingslinies. Deze barrières, die micro-organismen moeten doorbreken om een "gastheer" te infecteren, kunnen a-specifiek en specifiek zijn. De eerste barrière wordt gevormd door de huid, en bij vissen tevens door schubben en slijm, waarin antibacteriële factoren voorkomen. Deze drempel wordt getypeerd als een a-specifiek uitwendig afweer systeem. Specifieke afweer reacties tegen binnendringende micro-organismen komen voort uit het immuunsysteem: de tweede barrière.

Immuunsystemen van vissen en vogels

Bij mens en dier zijn verschillende organen te onderscheiden die de basis vormen van het immuunsysteem. Voor de vissen (o.a. karper en forel) zijn dat de volgende lymfoïde organen: thymus (zwezerik), milt, kopnier (pronephros) en middennier (mesonephros; zie figuur 2). De middennier fungeert tevens als excretie orgaan. Verder zijn er in de darmwand veel lymfoïde cellen aantoonbaar. Vissen bezitten geen beenmerg, maar aan de nier wordt een vergelijkbare functie toegekend. Hierin bevinden zich de stamcellen van o.a. het immuunsysteem.

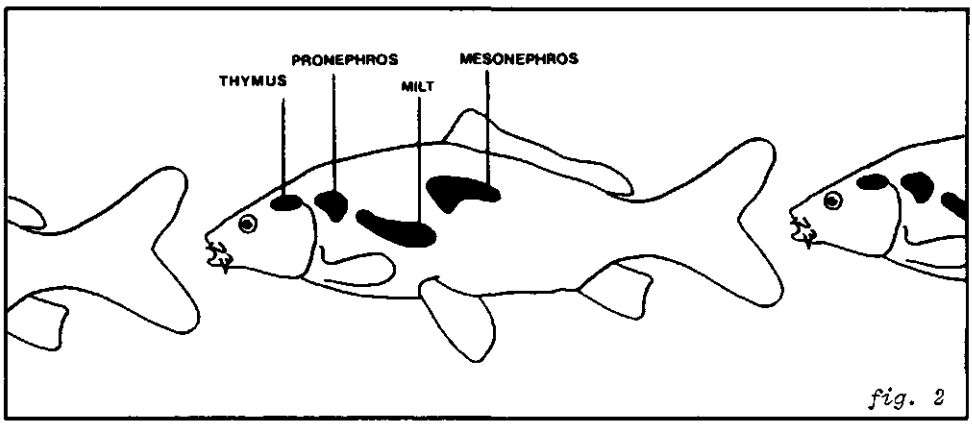


fig. 2

Op grond van een aantal criteria kan men twee typen immuun-reacties onderscheiden: transplantatie-afstoting en antilichaam-productie zijn voorbeelden van respectievelijk a) de cellulaire en b) de humorale respons. Ook bij vissen worden deze reacties waargenomen. Een afstotings-reactie kan worden geïnduceerd door huid of schubben van een vis met een andere genetische achtergrond te transplanteren. De lichaamscellen die voor de cellulaire en humorale reacties verantwoordelijk zijn, worden achtereenvolgens

T- en B-cellen genoemd. De naamgeving van deze lymfoïde cellen is afkomstig van Thymus (T-cellen) en Bursa (B-cellen). Dit zijn beide lymfoïde organen die uitsluitend T- of B-cellen herbergen en waarvan het laatstgenoemde orgaan alleen bij vogels aantoonbaar is. Bij vogels treft men naast thymus, bursa en milt tevens beenmerg aan (zie figuur 3). Voorts zijn er een aantal lymphoide structuren langs de darm.

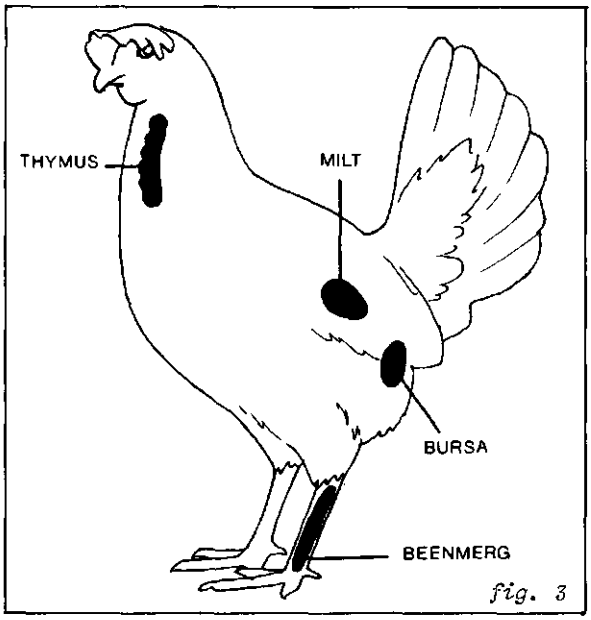


fig. 3

Voor een dier vreemde stoffen (antigenen, o.a. bacteriën) worden verwerkt en bewerkt door bepaalde cellen (macrofagen) en aangeboden aan antigeen gevoelige T- en B-cellen. Vervolgens vermeerderen deze cellen zich door deling en differentiëren tot effector-cellen. Effector-cellen en hun producten (o.a. antilichamen) zorgen voor een snelle eliminatie van het antigeen. Deze immuunrespons is schematisch weergegeven in figuur 4.

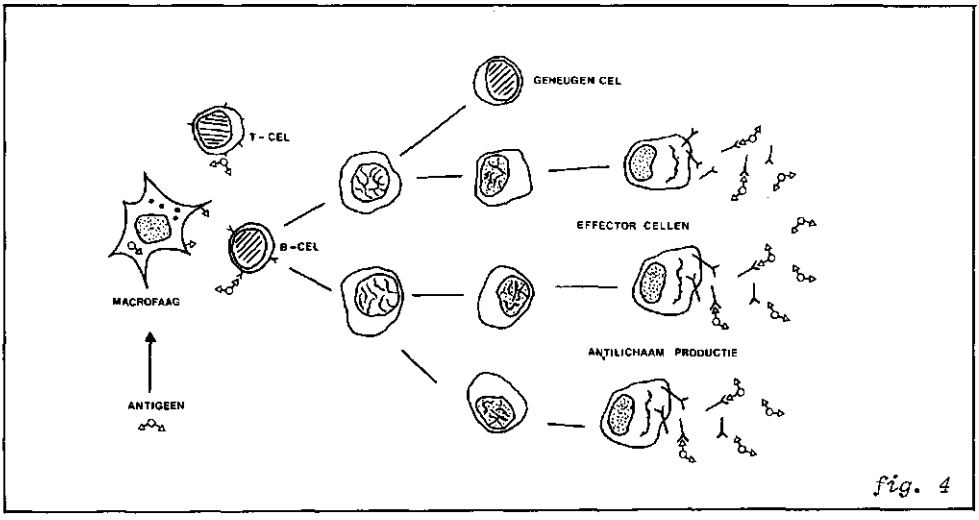


fig. 4

De reactie die volgt op het contact met het antigeen ( ), wordt in hoge mate versterkt door de vermeerdering van het aantal cellen dat in staat is antilichamen ( ) te produceren, die specifiek gericht zijn tegen het antigeen ( ). Tijdens de immuunrespons kunnen enkele cellen differentiëren tot geheugen-cellen. Deze cellen kunnen bij een volgend contact met hetzelfde antigeen snel tot effector-cel differentiëren, waardoor de afweer-reactie kwalitatief en kwantitatief beter verloopt.

Immuunsystemen en antibiotica

Als micro-organismen de verdedigingslinies doorbreken kan ziekte het gevolg zijn (figuur 5). Juist onder deze omstandigheden zijn antibiotica van bijzonder groot nut, althans voor de behandeling van bacteriële infecties. In de dierhouderij worden antibiotica zowel curatief als preventief toegepast. Naast het gebruik van deze middelen voor preventieve doeleinden en het onder controle houden van ziekten, worden enkele antibacteriële producten toegepast vanwege hun groeibevorderende effect.

In het algemeen remmen antibiotica de groei van de bacteriën. Hierdoor wordt het immuunsysteem in staat gesteld de micro-organismen op te ruimen. Op deze wijze werken antibiotica en afweer-mechanisme samen. Het is echter ook bekend dat bepaalde middelen interfereren met het immuunsysteem. Onderzoeken bij zoogdieren, vogels en vissen hebben aangetoond dat enkele antibiotica de immuunrespons kunnen beïnvloeden, positief dan wel negatief. Vooral het laatste effect kan ernstige gevolgen hebben voor het resultaat van een behandeling.

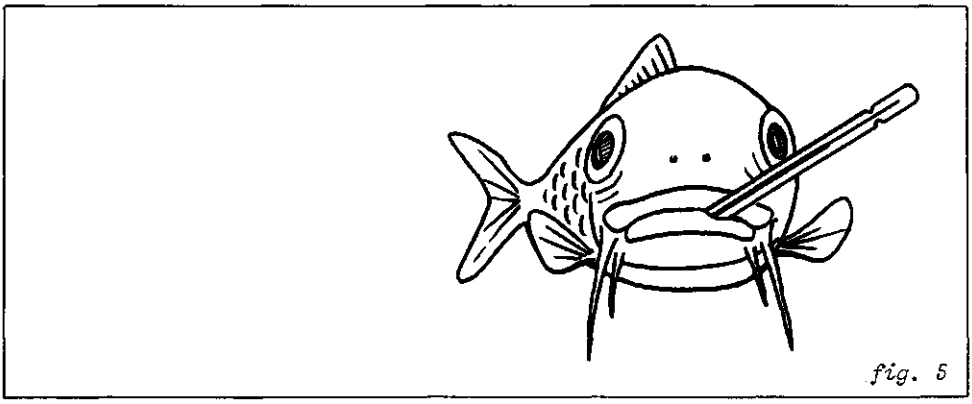


fig. 5

Immuunsystemen en farmacokinetiek

Antibiotica worden toegepast bij een groot aantal diersoorten. Toch blijkt dat voor bepaalde soorten nauwelijks of geen gegevens beschikbaar zijn t.a.v. het gedrag van antibiotica in het lichaam van het desbetreffende dier. Gegevens omtrent concentratie-verloop in het bloed, weefselverdeling en biologische halfwaarde-tijd (farmacokinetische gegevens) kunnen aanzienlijke verschillen te zien geven bij vergelijking van de diersoorten (fig.6).

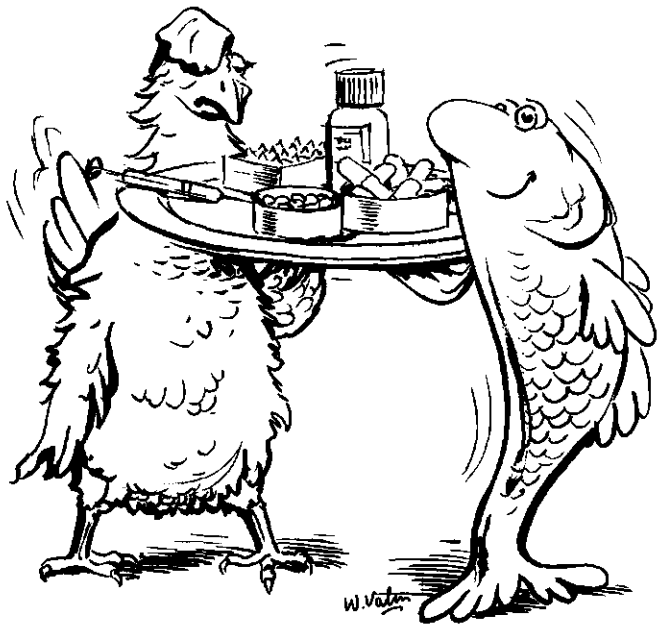


fig. 6

De relatief lage omgevings-temperatuur bij vissen en de hoge lichaamstemperatuur bij kippen zal gevolgen hebben voor de biochemische machinerie. De fysiologische aanpassingen bij deze dieren bepalen niet alleen het farmacokinetische gedrag van een antibioticum, maar kan ook de ontvankelijkheid van immunologische processen voor bijwerkingen sterk beïnvloeden.

In figuur 7 wordt schematisch een aantal factoren aangegeven die het effect van een therapeutische behandeling kunnen beïnvloeden. Na opname van een antibioticum zal het zich verdelen over het lichaam. In eerste instantie zal het bloed via het vaatstelsel hierbij als transportmiddel fungeren, daarna zal het antibioticum door diffusie over de weefsels worden verdeeld. Ter plekke van de infectie zal het ongebonden antibioticum actief kunnen zijn en de groei van de bacteriën remmen. Het afweersysteem is dan al actief en tracht de micro-organismen onschadelijk te maken. Samenwerking tussen antibioticum en immuunsysteem maken een effectief optreden tegen de infectie mogelijk.

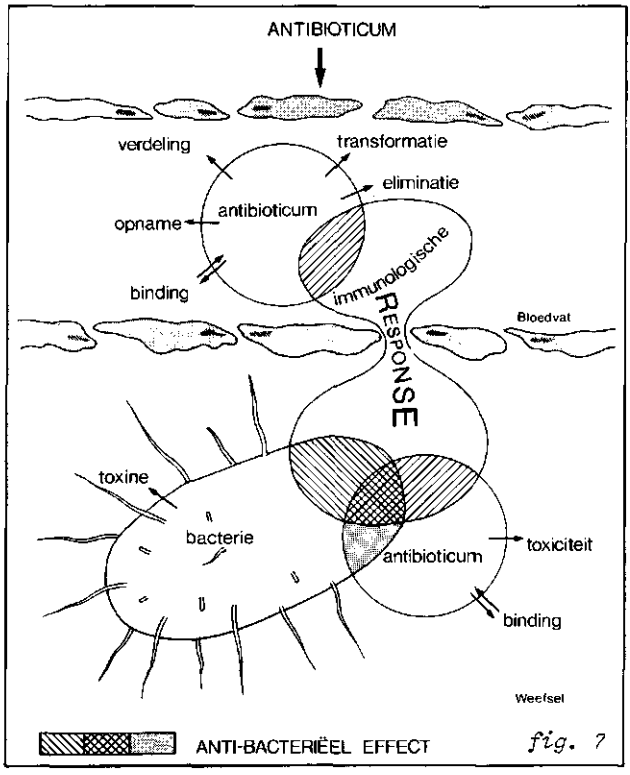


fig. 7

De effectiviteit van een antibioticum in het lichaam wordt mede bepaald door de mate waarin het immuunsysteem in staat is te opereren. Onderzoek dat gericht is op het zoeken naar antibacteriële middelen die het afweermechanisme ondersteunen en/of versterken is veelbelovend.

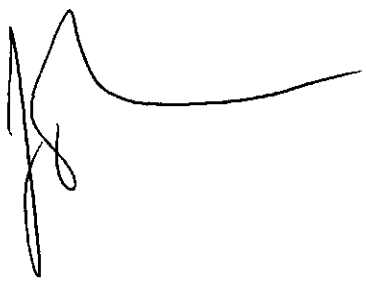
Tegenwoordig staat ons een groot aantal gevoelige methoden ter beschikking bij het immunologisch onderzoek. Dergelijk onderzoek zal in nauwe samenhang met farmacologische studies uitgevoerd moeten worden om het management in de dierhouderij effectief te kunnen ondersteunen.

DANKWOORD

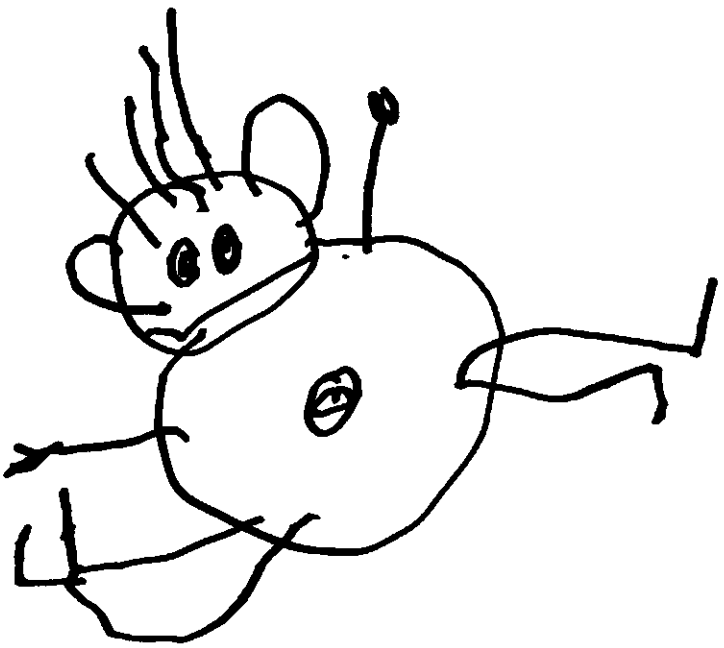
Het moge duidelijk zijn dat de totstandkoming van dit proefschrift mogelijk werd door de directe, maar ook indirecte bijdrage van vele mensen.

Op deze plaats wil ik een ieder voor haar of zijn aandeel, op welke wijze dan ook geleverd, oprecht danken.

Dank jullie wel



CURRICULUM VITAE



CURRICULUM VITAE

* 29 januari 1952	Geboren te Amsterdam
* 1971	Eindexamen HBS-B te Utrecht
* 1980	Doctoraal examen Medische Biologie (Cum laude) Rijksuniversiteit Utrecht Hoofdvak : Klinische Immunologie; I. Prof. Dr. R.E. Ballieux, Dr. E.O. Rijke, RU Utrecht; II. Prof. Dr. W. Leene, Dr. M.L. Kapsenberg, Universiteit van Amsterdam. Bijvakken : I. Scheikundige Dierfysiologie; Prof. Dr. D.I. Zandee, Dr. J.H.F.M. Kluytmans, RU Utrecht. II. Celbiologie; Prof. Dr. W.A. de Voogd van der Straaten, RU Utrecht.
* 1980 - 1981	Wetenschappelijk assistent bij het Interfacultair Instituut voor Moleculaire Biologie, afd. Moleculaire Neurobiologie, te Utrecht (Prof. Dr. W.H. Gispen).
* 1981 - 1984	Wetenschappelijk assistent bij de vakgroepen Experimentele Diermorfologie & Celbiologie, sectie Celbiologie (Prof. Dr. W.B. van Muiswinkel) en Veehouderij, sectie Gezondheidsleer (Prof. Dr. C.C. Oosterlee) aan de Landbouwuniversiteit te Wageningen.
* 1984 - heden	Wetenschappelijk medewerker bij de vakgroep Experimentele Diermorfologie & Celbiologie, sectie Celbiologie aan de Landbouwuniversiteit te Wageningen.

LIST OF PUBLICATIONS

Grondel, J.L. and H.J.A.M. Boesten, 1982.
The influence of antibiotics on the immune system. I. Inhibition of the mitogenic leukocyte response in vitro by oxytetracycline. Dev. Comp. Immunol., Suppl. 2, pp. 211-216.

Van Muiswinkel, W.B., J.L. Grondel and D.P. Anderson, 1982.
The immunosuppressive effect of antibiotics. Int. J. Immunopharmacology, 4: 364.

Grondel, J.L., E. Egberts and W.B. van Muiswinkel, 1983.
In vitro assays to determine immunological side effects of antibiotics. Research report 14C. Developments in molecular and cell biological research for the improvement of animal production, NRLO. The Hague, The Netherlands, pp. 89-94.

Grondel, J.L. and E.G.M. Harmsen, 1984.
Do fish have interleukins ? Fish immunology and fish health. Fish Immunology, M.J. Manning & M.F. Tatner, eds., Proceedings of the Plymouth Meeting, 1983, Academic Press, London.

Grondel, J.L. and E.G.M. Harmsen, 1984.
Phylogeny of interleukins : Growth factors produced by leukocytes of the cyprinid fish, Cyprinus carpio. Immunology, 52: 477-482.

Grondel, J.L., A.G.M. Gloudemans and W.B. van Muiswinkel, 1985.
The influence of antibiotics on the immune system. II. Modulation of fish leukocyte responses in culture. Vet. Immunol. Immunopathol., 9: 251-260.

Grondel, J.L., G.C. Angenent and E. Egberts, 1985.
The influence of antibiotics on the immune system. III. Investigations on the cellular functions of chicken leukocytes in vitro. Vet. Immunol. Immunopathol., 10: 307-316.

Grondel, J.L., J.F.M. Nouws and W.B. van Muiswinkel, 1985.
Immune systems and antibiotics. Aquaculture, PAO, Utrecht.

Grondel, J.L. and W.B. van Muiswinkel, 1986.
Immunological defence mechanisms as a target for antibiotics.
A review. In : Van Miert (Ed.); Comparative Veterinary Pharmacology, Toxicology and Therapy. 3rd EAVPT Congress, Ghent, Belgium. MPT Press Ltd., Lancaster, in press.

Nouws, J.F.M., T.B. Vree, H.J. Breukink, A.S.J.P.A.M. van Miert and J.L. Grondel, 1986.
Pharmacokinetics, hydroxylation and acetylation of sulphadimidine in man, ruminants, horses, pigs and fish. In : Van Miert (Ed.); Comp. Vet. Pharm., Tox. and Ther.. 3rd EAVPT Congress, Ghent, Belgium. MPT Press Ltd., Lancaster, in press.

Boon, J.H., J.L. Grondel, J.G.A. Hemmer and G.H.R. Booms, 1986.
Relationship between cytologic changes in broncho-alveolar lavage fluid and weight gain in calves with gastro-intestinal nematodes and lungworms.
Veterinary parasitology, in press.

Nouws, J.F.M., T.B. Vree, M.M.L. Aerts and J.L. Grondel, 1986.
Pharmacokinetics and residues of sulphadimidine, its N_4-acetyl- and hydroxy-metabolites in food producing animals. Arch. fur Lebensmittelhyg., 37: 57-84.

Geertsma, M.F., J.F.M. Nouws, J.L. Grondel, M.M.L. Aerts, T.B. Vree and C.A. Kan, 1986.
Residues of sulphadimidine and its metabolites in eggs following oral sulphadimidine medication.
The Veterinary Quarterly, in press.

Nouws, J.F.M., M.F. Geertsma, J.L. Grondel, M.M.L. Aerts, T.B. Vree and C.A. Kan, 1986.
Plasma disposition and renal clearance of sulphadimidine and its metabolites in laying-hens.
Research in Veterinary Science, in press.

Grondel, J.L., J.F.M. Nouws and O.L.M. Haenen, 1986.
Fish and Antibiotics. Pharmacokinetics of sulphadimidine in carp (Cyprinus carpio). Vet. Immunol. Immunopathol., 12: 281-286.

Van Muiswinkel, W.B., J.L. Grondel, C.N. Pourreau and D.P. Anderson, 1986. Fish immunology and fish health. Proc. IVth Int. Symp. Vet. Lab. Diag., pp 410-412.

Grondel, J.L., J.H. Boon and L.T.N. Heinsbroek, 1986.
Effect of in vivo temperature and temperature changes on mitogenic stimulation of carp (Cyprinus carpio) lymphocytes. Proc. IVth Int. Symp. Vet. Lab. Diag., pp 413-416.

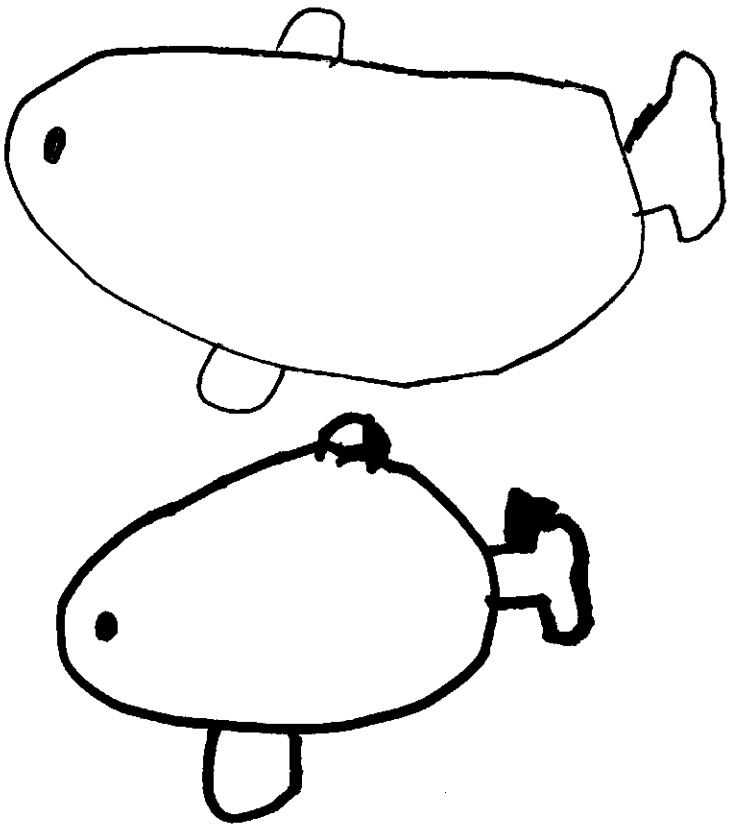
Grondel, J.L., J.F.M. Nouws and W.B. van Muiswinkel, 1986.
The influence of antibiotics on the immune system. IV.
Immuno-pharmacokinetic investigations on the primary anti-SRBC response in carp (Cyprinus carpio) after oxytetracycline injection. J. Fish Diseases, in press.

Egberts, E., N. Cohen, J.L. Grondel, B.M.T. Burgering, A.G.M. Gloudemans and W.B. van Muiswinkel, 1986.
Carp peripheral blood leukocyte culture system and its application to the analysis of teleost histocompatibility system. In prep.

Grondel, J.L., J.F.M. Nouws, M. De Jong, A.R. Schutte and F. Driessens, 1986.
Pharmacokinetics and tissue distribution of oxytetracycline in carp (Cyprinus carpio) following different routes of administration.
J. Fish Diseases, accepted for publication.

Grondel, J.L., J.F.M. Nouws, A.R. Schutte and F. Driessens, 1986.
Comparative pharmacokinetic investigations in rainbow trout (Salmo gairdneri) and African catfish (Clarias gariepinus) following oxytetracycline administration. J. Vet. Pharm. Ther. (accepted).

GLOSSARY



GLOSSARY

Accessory cells, Non-lymphoid cells predominantly of the monocyte and macrophage lineage which cooperate with T and B lymphocytes in immune reactions.

Activated lymphocytes, Lymphocytes which have been stimulated by antigen or mitogen.

Agglutination, An antigen-antibody reaction in which a solid or particulate antigen forms a lattice with antibodies.

Antibiotics, Substances produced by micro-organisms which have an antagonistic effect on the growth or life of other microbes in high dilution.

Antibody, A protein that is produced by plasma cells as a result of the introduction of an antigen and which has the ability to combine specifically with the same antigen that stimulated this response.

Antigen, A substance which can induce an immune response when introduced into an animal.

Apparent volume of distribution, That volume of fluid which would be required to contain the amount of drug in the body if it were uniformly distributed at a concentration equal to that in the plasma.

Bactericidal, Antibiotic which kills or lyses invading bacteria.

Bacteriostatic, Antibiotic which merely inhibits bacterial growth and replication.

Bacteriolysis, The disintegration of bacteria.

B cell, A bursa-derived cell in avian species and, by analogy, bursa-equivalent derived cells in nonavian species. B cells are the precursors of plasma cells which produce antibody.

Bioavailability, Refers to both the rate of drug absorption and to the extent (or completeness) of absorption. By extent of absorption is meant the fraction (F) of the oral or i.m. dosage form which reaches the systemic circulation intact.

Blast cell, A large lymphocyte containing a nucleus with loosely packed chromatin, a large amount of cytoplasm with numerous polyribosomes.

Body clearance, The volume of blood cleared of the drug by the various elimination processes (biotransformation and excretion) per unit of

time.

Bursa of Fabricius, The hindgut organ located near the cloaca of birds which controls the ontogeny of B cells.

Cell-mediated immunity, Immunity in which the participation of T-lymphocytes and macrophages is predominant.

Central lymphoid organs, Lymphoid organs which are essential for the development of the immune system, i.e., thymus and bursa (equivalent).

Classical complement pathway, A series of enzyme-substrate and protein-protein interactions which ultimately leads to biologically active complement enzymes. The first component (C1) binds with an antibody-antigen complex. This process proceeds sequentially C1, 423, 567, 89.

Complement, An enzymatic system of serum proteins that is activated by many antigen-antibody reactions, which is essential for lysis or death of cellular antigens and several other biological reactions.

Concanavalin A, A lectin which is derived from the jack bean and which stimulates predominantly T cells.

Degranulation, A process whereby cytoplasmic granules of phagocytic cells fuse with phagosomes and discharge their contents into the phagolysosomes.

Delayed hypersensitivity, A cell-mediated immune reaction which can be elicited by subcutaneous injection of antigen, with a subsequent cellular infiltrate and edema which are maximal between 24 and 48 hours after antigen challenge in mammals.

Diapedesis, The outward passage of cells through intact vessel walls.

Effector cells, Usually denotes T cells capable of mediating cytotoxicity, suppression or helper function.

Endocytosis, The process whereby external material is internalized by a particular cell. It consists of pinocytosis and phagocytosis.

Endotoxins, Lipopolysaccharides derived from the cell walls of gram-negative micro-organisms sharing toxic and pyrogenic effects when injected.

Enteral administration, A drug is placed directly in the gastro-intestinal tract.

Exotoxins, Diffusible toxins produced by certain gram-negative microorganisms.

Fc fragment, Crystallizable fragment obtained by papain digestion of Ig molecules.

Fc receptor, A receptor present on various subclasses of lymphocytes or mononuclear cells for the Fc fragment of immunoglobulins.

Gamma globulins, Serum proteins with gamma mobility in electrophoresis which comprise the majority of mammalian immunoglobulins.

Germinal centers, A collection of metabolically active lymphoblasts and macrophages which appears within the primary follicle of lymphoid tissues following antigenic stimulation

Half-life of a drug, The time required for the body to eliminate one-half of the particular drug.

Helper T cells, A subtype of T cells which cooperate with B cells in antibody formation.

Hemagglutination, The clumping of red cells by antibody.

Humoral, Pertaining to soluble molecules in body fluids, particularly antibody and complement.

Ia antigens, Antigens which are controlled by Ir genes and are present on various tissues.

Immune complexes, Antigen-antibody complexes.

Immune response (Ir) genes, Genes which control immune responses to specific antigens.

Immunogen, A substance which, when introduced into an animal, stimulates the immune response.

Immunoglobulin, Serum glycoprotein with antibody activity.

Immunization, The administration of antigen to an animal with the intention to induce an immune response.

Interleukins, Glycoproteins derived from macrophages or T helper cells which exert regulatory effects on other cells.

Lectin, Proteins, usually of plant origin, that bind to specific monosaccharides. Many lectins stimulate lymphocyte proliferation.

Lipopolysaccharide, A compound derived from a variety of gram-negative

enteric bacteria which have various biological functions including mitogenic activity for B cells.

Lymphocyte, Small and round white cells originating in bone marrow or other organs mediating immune responses.

Lymphocyte activation, A treatment by antigen or mitogen or mitogen inducing metabolic activity in lymphocytes.

Lymphokines, Lymphocyte derived glycoproteins that exert a regulatory effect on other cells.

Lysosomes, Granules containing hydrolytic enzymes, which are present in the cytoplasm of many cells.

Macrophage, Mononuclear phagocytic cells derived from the bone marrow and subserve accessory roles in immunity.

Major histocompatibility complex, A gene complex containing the genes that code for the major histocompatibility antigens, immune response genes, and genes coding for complement components.

Migration inhibition factor, A lymphokine which is capable of inhibiting the migration of macrophages.

Minimum inhibitory concentration, The lowest antibiotic concentration that will inhibit the growth of a specific micro-organism.

Mitogens, A substance that stimulates cells to divide.

Mixed leucocyte culture, An *in vitro* test for cellular immunity in which lymphocytes or leucocytes from genetically dissimilar individuals are mixed and mutually stimulate DNA synthesis.

Monokines, Glycoproteins derived from monocytes/macrophages that exert a regulatory effect on other cells.

Neutralization, The process by which antibodies block the biological activities of enzymes, toxins or viruses.

O antigens, Somatic antigens from gram-negative bacteria.

Opsonin, A factor that facilitates phagocytosis by binding to the foreign particles.

Parenteral administration, Implies that the gastro-intestinal tract is bypassed, e.g., i.m., i.v. and i.p.

Peripheral lymphoid organs, Lymphoid organs which are important for the immune response but not essential for the ontogeny of immune system,

i.e., the spleen, lymphnodes, tonsils, and Peyer's patches.

Peritoneal exudate cells, Inflammatory cells present in the peritoneal cavity of animals injected with an inflammatory agent.

Phagocytes, Cells which are capable of ingesting particulate matter.

Phagocytosis, The engulfment of micro-organisms or other particulate material.

Pharmacokinetics, The mathematical description of concentration changes of drugs within the body.

Phytohemagglutinin, A lectin which is derived from the red kidney bean and which stimulates predominantly T cells.

Pinocytosis, The ingestion of soluble material by cells.

Plaque-forming cells, Antibody producing cells capable of forming a hemolytic plaque in the presence of complement and antigenic erythrocytes.

Plasma cells, Fully differentiated antibody synthesizing cells derived from B cells.

Pokeweed mitogen, A lectin which is derived from pokeweed and stimulates both T and B cells.

Polyclonal mitogens, Mitogens which activate different clones of lymphocytes.

Primary immune response, The response of an unsensitized animal to antigen.

Primary lymphoid organs, Lymphoid organs which serve as either a source of lymphocytes or as a site of lymphocyte maturation.

Secondary lymphoid organs, The organs in which effector lymphocytes are located.

Secondary response, The response of a sensitized animal to foreign antigen.

Serology, The study of diagnostic tests utilizing serum antibodies.

Serum, The clear yellow fluid expressed when blood has clotted and the clot is permitted to retract.

Suppressor cells, Cells, which may be either T cells or macrophages, capable of exerting a suppressive effect on immune responses.

Syngeneic, Genetically identical.

T cell, A thymus derived cell which participates in a variety of cell-mediated immune reactions.

Thymus, The central lymphoid organ which controls the ontogeny of T cells.

Thymus-dependent antigen, Antigen which depends on T cell interaction with B cells for antibody synthesis, e.g., erythrocytes and serum proteins.

Titer, A measure for the amount of specific antibody.

Toxoid, A toxin which is chemically modified in order to remove its toxicity, but not its immunogenicity.

Vaccination, The administration of antigen (vaccine) in order to stimulate protective immunity.

Virulence, The disease-producing power of a micro-organism.