The Laboratory for Monoclonal Antibodies (LMA)

has been established by collaboration between the

Wageningen Agricultural University, the Agricultural

Research Division and the Directorate for Arable Farming

and Horticulture of the Ministry for Agriculture, Nature Conservation

and Fisheries in the Netherlands

Monoclonal antibodies in agriculture

Proceedings of the symposium 'Perspectives for monoclonal antibodies in agriculture', Laboratory for Monoclonal Antibodies, Wageningen, Netherlands, 30 May 1990

A. Schots (Editor)

Pudoc Wageningen 1990

Abstract

Schots, A. (Editor), 1990. Monoclonal antibodies in agriculture. Proceedings of the symposium 'Perspectives for monoclonal antibodies in agriculture'. Laboratory for Monoclonal Antibodies, Wageningen, Netherlands, 30 May 1990. ISBN 90 - 220 - 1022 - 8, 11 contributions, iv + 94 pp., 15 figs, 13 tables, lit. refs

Different applications are shown of monoclonal antibodies in both plant and animal production. The topics range from detection of plant pathogenic fungi and bacteria and plant viruses with immunoassys to residue analysis of biological samples. Also, some examples are shown of application of monoclonal antibodies in research: the immune system of fish and plant viruses.

Free descriptors: immunoassays, residue analysis, fungi, bacteria, viruses

CIP-data Koninklijke Bibliotheek, Den Haag

ISBN 90 - 220 - 1022 - 8
NUGI 821

Printed in the Netherlands

CONTENTS

PREFACE

After nearly two years of preparation the Laboratory for Monoclonal Antibodies (LMA) was founded on March 7, 1985 as a collaboration between the Wageningen Agricultural University and the Directorates for Agricultural Research and Arable Farming and Horticulture. To commemorate the first lustrum of the LMA on May 30, 1990 a symposium was organised entitled: "Perspectives for Monoclonal Antibodies in Agriculture". During this symposium different aspects of the use and application of monoclonal antibodies in agriculture were elucidated. This book contains the contributions given on this symposium. It clearly shows that monoclonal antibodies have opened new possibilities for serology in both plant and animal production.

A. Schots
(Editor)

INTRODUCTION

One of the characteristics of the immune system is the ability to recognize invading micro-organisms (or antigens) and to mount a specific immune response. Most antigens are complex molecules containing different antigenic determinants. Each of those determinants may stimulate specific lymphocytes to proliferate and develop into clones of antibody forming cells (plasma cells). These clones usually secrete antibodies differing in specificity and affinity. This is the main reason why conventional antisera contain a mixture of different antibodies. Some fifteen years ago, Köhler and Milstein showed how this problem with conventional antibodies could be circumvented. They developed a technique for the *in vitro* fusion of single antibody producing cells with lymphoid tumor cells. The resulting hybrid cell clone could be grown in tissue culture and produced only one type of monospecific antibody. This has several advantages compared with the conventional production of antisera. However, it took some years before most people became aware of the amazing possibilities of the "*monoclonals*". Today, numerous applications are available for applied and fundamental research. Excellent diagnostic tests were developed for medical, veterinary and agricultural purposes. Many intracellular or membrane components can be detected in plant or animal cells. In other words: numerous qualitative and quantitative tests are now based upon the availability of these wonderful monoclonals.

During the last 5 years the Laboratory for Monoclonal Antibodies (LMA) at Wageningen has added a substantial contribution to this field. Several monoclonals detecting plant pathogenic viruses, bacteria and fungi. Moreover, specific antibodies raised against insect hormones, fish muscle cells and leucocytes and liver enzymes in rats etc. became available for different universiy departments and research institutes. In addition to that, the technical facilities of the LMA have been extended and improved. This first lustrum symposium will be a good occasion to provide us with an up-to-date review on current international research and new developments, which can be expected in this fascinating area.

W.B. van Muiswinkel
(Chairman Managing Committee)

A REVIEW ON FIVE YEARS LMA

Arjen Schots & Rikus Pomp
Laboratory for Monoclonal Antibodies, P.O. Box 9060, 6700 GW Wageningen.

1. History and organisation

In 1983 a committee was formed to prepare the foundation of a hybridoma service laboratory for the benefit of agricultural research in the Netherlands. After two years of preparation the Laboratory for Monoclonal Antibodies (LMA) was founded in 1985 as a collaboration between the Wageningen Agricultural University (WAU) and the Directorates for Agricultural Research and Arable Farming and Horticulture of the Ministry of Agriculture. The LMA was originally for 100 % a service laboratory for departments of the WAU and agricultural research institutes. When these institutions required monoclonal antibodies (MAs) they could detach someone at the LMA which had the know-how and equipment available for the preparation of MAs. The LMA staff supervised detached personnel with the preparation of the desired MAs. In this way MAs were developed against many antigens (table 1).

During the first years it became apparent that for an up-to-date application of MAs, research is a pre-requisite. Therefore, a research programme has been developed based on two ideas. First, the LMA should continuously search for new applications of MAs. Along this line research is carried out to develop MAs for the detection and identification of (soil-borne) fungi. Secondly, results obtained in the first few years of the LMA showed that development of monoclonal antibodies for and application in "green" agriculture has been limited by strong cross-reactions with contaminants of the antigen or with antigens from closely related species. To avoid these problems in the future, investigation of the immune response towards these antigens and contaminants is necessary to manipulate the immune response in a way that a more specific immune response is obtained.

2. Monoclonal antibodies developed at the LMA

During the last five years MAs have been developed against plant viruses, plant pathogenic bacteria, plant hormones and other plant compounds, neuropeptides of the Colorado beetle, fish (carp) cells and immunoglobulins and glutathion-S-transferase iso-enzymes. The projects to develop these MAs were always carried out by one of the Departments of the WAU, one of the Agricultural Research Institutes or by one of these authorities in collaboration with the LMA.

In other parts of this book the application and characteristics of some of these MAs is described. Those which are not described will be briefly described in this chapter.

2.1. MAs against viral antigens

- *potato virus A (PVA)*. Two types of MAs were produced. MAs (MA-1) selected in antigen-coated-plate ELISA (ACP-ELISA) and MAs (MA-2) selected in a sandwich-type ELISA (IDAS-ELISA). Both types of MAs reacted with purified virus in both ELISA types. They also both reacted on SDS-PAGE-immunoblots. However, only the MA-2 type antibodies reacted with PVA from infected plants in ELISA and SDS-PAGE-immunoblotting. The MA-1 type antibodies apparently recognize an epitope which was introduced during the purification of the virus. Further studies indicated that oxydation of the virus, during purification and coating to ELISA plates, may be one of the processes responsible for introducing this epitope.

- *potato virus Y (PVY)*. Four MAs were obtained which showed no reaction with the potato viruses A, M, S and X. Three of the MAs reacted with several PVY^o and PVY^c isolate. The fourth MA reacted with none of the PVY^c isolates and with only one PVY^o isolate. One of the MAs reacted also with some other potyviruses. In immunoblotting experiments different MAs showed a different reaction pattern. Some did not react at all, some recognized all three capsid proteins while other MAs only bound to one or two of the capsid proteins.

- *beet necrotic yellow vein virus (BNYVV)*. Mice were immunized with BNYVV purified from infected *Chenopodium quinoa* leaves. From fusion experiments, four MAs were obtained. These MAs differed in their reactivity with purified virus (p-BNYVV), sap from infected *C. quinoa* leaves (c-BNYVV) and sap from infected sugar beet roots (b-BNYVV) (see table 2).

Table 1. Monoclonal antibodies against different antigens isolated at the LMA.

	Antigen	In cooperation with*
1. viral antigens	potato virus A	IPO
	potato virus M	IPO
	potato virus S	IPO
	potato virus Y	IPO
	potato leafroll virus	WAU-virology
	beet necrotic yellow vein virus	IPO/WAU-virology
	potato virus T	IPO
2. bacterial antigens	*Erwinia chrysanthemi*	IPO
	Xanthomonas campestris pv. *campestris*	CRZ
	Xanthomonas campestris pv. *pelargonii*	IPO
	Xanthomonas campestris pv. *begoniae*	IPO
	Rhizobium proteins	WAU-molecular biology
3. plant hormones and compounds	indolyl acetic acid	LBO/CABO
	abscissic acid	LBO/CABO
	solanidin	CPO
	tomatin	CPO
4. insects	neuropeptides of the Colorado beetle	WAU-entomology
5. fish (carp)	myosatellite cells	WAU-exp. animal morphology and cell biology
	mucosal immunoglobulins	WAU-exp. animal morphology and cell biology
	lymphocytes	WAU-exp. animal morphology and cell biology
6. proteins	glutathione S-transferase iso-enzymes	WAU-toxicology

*abbreviations used: CABO: Centre for Agrobiological Research; CPO: Centre for Plant Breeding Research; CRZ: Centre for Variety Research and Seed Technology; IPO: Research Institute for Plant Protection; LBO: Bulb Research Centre; WAU: Wageningen Agricultural University.

In cooperation with Drs. Lesemann and Koenig from the 'Biologische Bundesanstalt' in Braunschweig (Lesemann *et al.*, 1990) the reactivity of the four MAs on the virus was investigated. MAs 3H12 and 4F11 showed a strong and 17G2 a weak coating along the entire length of the virus particles. However, MA 6D8 was bound only at one of the extremeties.

Further studies of Koenig *et al.* (1990) showed that MAs 6D8 and 17G2 react with a discontinuous epitope. These MAs do not react on immunoblots or dotblots. MAs 3H12 and 4F11 do react well on immunoblots and dotblots, their reactivity is not lost after the coat protein was cleaved by V8 protease and they bind to an epitope at the C-terminal part of the coat protein. The latter result was obtained with a hybrid protein containing the aminoacids 104-188 of the BNYVV coat protein at the C-terminus of a truncated cro-lacI-lacZ sequence. This hybrid protein was obtained by cloning the corresponding cDNA sequence of the viral coat protein gene into the truncated derivative of the pEX3 vector (Kocken *et al.*; 1988).
From these experiments one can conclude that the four MAs react with three different classes of epitopes. MAs 6D8 and 17G2 bind to two different discontinous epitopes, MAs 3H12 and 4F11 bind to a continuous epitope at the C-terminal part of the coat protein.

Table 2. Reactivity of the anti-BNYVV MAs with different viral antigen preparations.

Clone	Isotype	p-BNYVV	c-BNYVV	b-BNYVV	IB	V8	aa 104-188	EM
3H12	IgG_3	+	+	+	+	+	+	entire
4F11	IgG_3	+	+	+	+	+	+	entire
6D8	IgG_{2a}	-	±	+	-	-	-	extreme
17G2	IgG_{2a}	-	±	+	-	-	-	entire ±

p-BNYVV: virus purified from C. quinoa leaves; c-BNYVV: sap from infected C. quinoa leaves; b-BNYVV: sap from infected B. vulgaris roots; IB: immunoblotting; V8: proteolytic treatment with Staphylococcus V8 protease; aa 104-188: hybrid protein containing amino acids 104-188 of the coat protein; EM: reactivity in EM (entire: binding over entire length of the virus; entire ±: binding unevenly over entire length of the virus); extreme: binding at one extremity of the virus).

- *potato virus T (PVT)*. PVT is an Andean potato virus which is considered to be a quarantine virus. The virus was purified from *Nicotiana occidentalis* P1 and Balb/c mice were immunized. The first two fusions resulted in five hybridoma clones, while from the third fusion seven clones were isolated. The differences between the last fusion and the first two fusions was that the third one was carried out four days after the last immunization and the others three days after the last injection. The twelve MAs obtained are characterized at present.

2.2. MAs against bacterial antigens

- *Erwinia chrysanthemi*. Mice were immunized with a total cell extract (TCE) of *Erwinia chrysanthemi* (Ech). Screening of the obtained hybridomas was carried out with the TCE, an extract of flagella of Ech and cell

extracts of *Erwinia carotovora* subsp. *atroseptica* and a saprophyte isolated from potato peels. For the latter two antigen preparations it was shown that they both showed a cross reaction with polyclonal rabbit antisera against Ech. These cross-reactions were also observed with the majority of the obtained hybridoma clones. One MA was obtained which produced antibodies specific for proteins from flagella (Boonekamp *et al.* 1988). Two other MAs showed a specific reaction with Ech. However, these MAs were of low affinity and therefore of little value for routine screening purposes. To increase the number of specific MAs two strategies will be used. First, experiments are prepared wherein mice will be used which are tolerant for the two cross-reacting antigen preparations and second, Ech-specific membrane antigens will be purified.

- *Xanthomonas campestris* pvs. *begoniae* and *pelargonii* (Xcb, Xcp). In an attempt to produce highly specific MAs, different antigen preparations (whole cell and two membrane fractions) from Xcb and Xcp were injected into Balb/c and B10.Q mice. The M2 fraction, a mixture of inner- and outer-membranes, responded better serologically than the outer-membrane fraction and was compared with the whole-cell antigen for production of MAs. Balb/c and B10.Q mice responded best to whole cell and M2 antigens. Splenocytes of these mice were used for cell fusion. Resulting hybridoma clones were primarily screened against the antigen preparations using ELISA. MAs with high specificity were observed from the fusion carried out with splenocytes stimulated with Xcb whole-cell preparations, but not from mice stimulated with Xcp M2 or whole- cell preparations.

Xcp whole cells were than injected in Balb/c, C57/Bl, CBA/Rij and CBA/T6 mice. The CBA/Rij mouse responded better serologically than the other three mice. Splenocytes from this mouse were fused, which resulted in three hybridoma clones producing antibodies which reacted strongly with 30 Xcp strains and only weakly with other pathovars of *Xanthomonas campestris* and negatively with saprophytes which reacted positively with polyclonal antisera against Xcp.

From these experiments it was concluded that gram-negative bacteria which have a repeating antigenic determinant (lipopolysaccharide), respond serologically different in mice with a different genetic background (*i.e.* a different H2-haplotype).

2.3. MAs against protein antigens

- *MAs against rat glutathione S-transferase (GST) isoenzymes* (Vos *et al.*, 1989). The glutathione S-transferases (EC 2.5.1.18) are a group of dimeric proteins, important in the biotransformation of xenobiotics. Balb/c mice were immunized with GST isoenzymes 1-1, 2-2, 3-3 and 4-4 respectively. The mice immunized with GST 2-2 and 3-3 responded serologically well, while the mice immunized with one of the other isoenzymes failed to elicit an immune response. Fusion experiments with splenocytes from mice immunized with GST 2-2 and 3-3 resulted in MAs specific for the respective protein antigens. It is known that for protein antigens like myoglobin, insulin and bovine albumin the immune response is under genetic control of the major histocompatibility complex (MHC; Berzofsky, 1978; Kapp *et al.*, 1979; Riley *et al.*, 1982). This can also be the case for the different GST isoenzymes. Therefore, four inbred mice strains with a different H-2 haplotype were immunized with GST 1-1 and 4-4 (Balb/c, H-2^d; CBA/BrARij,H-2^q; CBA/CaHRij-T6, H-2^k and C57Bl/LiARij, H-2^b). Figure 1 shows that GST 1-1 elicited a good immune response in CBA/CaHRij-T6 mice and a reasonable response in CBA/BrARij mice. GST 4-4 elicited a good reponse in CBA/BrARij mice. The high serum-antibody titers observed in these mice offer perspectives for the production of specific monoclonal antibodies against GST 1-1 and 4-4 in the near future.

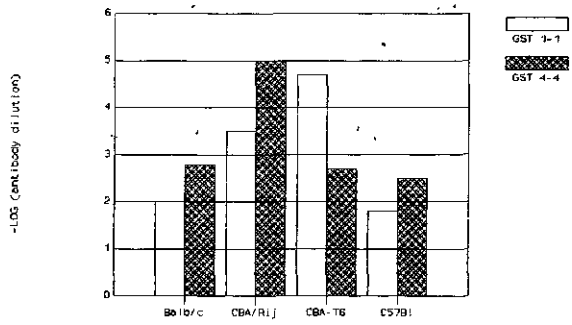

Figure 1. The immune response elicited in four different inbred mice strains by Glutathion-S-transferase iso-enzymes 1-1 and 4-4.

References

Berzofsky, J.A., (1978). Genetic control of the immune response to mammalian myoglobins in mice. I. More than one I-region gene in H-2 controls the antibody response. *J. Immunol.* **120**, 360-369.

Boonekamp, P.M., Kaan, A.M., Van Overbeek, L.S., & Zilverentant, J.F. (1988). Monoclonal antibodies against *Erwinia chrysanthemi* (E.c.). Abstracts of Papers 5th International Congress of Plant Pathology, Kyoto, August 20-27, 1988.

Kapp, J.A., Strayer, D.S., Robbins, P.F., & Perlmutter, R.M. (1979). Insulin-specific antibodies of limited heterogeneity. I. Genetic control of spectrotypes. J. Immunol. **123**, 109-114.

Kocken, C.H.M., Scheer, J.M.J., Welling, G.W., & Welling-Wester, S., (1988). Purification of fusion proteins expressed by pEX3 and a truncate pEX3 derivative. *FEBS letters* **236**, 132-134.

Koenig, R., Commandeur , U., Lesemann, D.-E., Bürgermeister, W., Torrance, L., Grassi, G., Alric, M., Kollerhof, J., & Schots, A. (1990). Antigenic analysis of the coat protein of beet necrotic yellow vein virus by means of monoclonal antibodies. Manuscript submitted.

Lesemann, D.E., Koenig, R., Torrance, L., Buxton, G., Boonekamp, P.M., Peters, D. & Schots, A. (1990). Electron microscopical demonstration of different binding sites for monoclonal antibodies on particles of beet necrotic yellow vein virus. *J. Gen. Virol.* **71**, 731-733.

Riley, R.L., Wilson, L.D., Germain, R.N., & Benjamin, D.C. (1982). Immune responses to complex protein antigens. I. MHC control of immune responses to bovine albumin. *J. Immunol.* **129**, 1553-1558.

Vos, R.M.E., Lutke Schipholt, I.J., Boonekamp, P.M., Schots, A., Pomp, H., Van Bladeren, P.J., & Koeman, J.H. (1989). Monoclonal antibodies against rat glutathione S-transferase isoenzymes 2-2 and 3-3. *Hybridoma* **8**, 475-480.

DEVELOPMENT OF MODERN DIAGNOSTIC TESTS AND BENEFITS TO THE FARMER

Sally A. Miller, James H. Rittenburg, Frank P. Petersen & G. David Grothaus
Agri-Diagnostics Associates, 2611 Branch Pike, Cinnaminson, NJ 08077, U.S.A.

1. Introduction

The benefits of the use of monoclonal antibodies in diagnosis of disease in clinical medicine and agriculture have often been acknowledged (Halk & DeBoer, 1985; Miller & Martin, 1988; Chu *et al.*, 1989). While polyclonal antisera are still the active ingredient in many useful immunoassays, the complexity of some target organisms does not permit their use without encountering extensive cross-reactivity with non-target organisms. This is particularly true for many plant pathogenic fungi, where monoclonal antibodies have permitted the development of sensitive, specific pathogen detection systems. Worldwide, fungi cause the greatest amount of crop damage by plant pathogens and require the highest expenditures by farmers for their control. In addition to the monetary cost of control, farmers must now also face growing concerns about the environmental impact of fungicide use. It is therefore becoming more and more critical that farmers have available to them the means of rapid, reliable diagnosis of plant disease caused by fungi, to enable them to make informed crop management decisions. Immunoassays can provide accurate disease diagnoses, as well as information on the presence and level of particular fungi in the absence of disease symptoms.

We have developed monoclonal antibodies, immunoassays, and crop applications systems for a number of fungal plant pathogens, including species of *Phytophthora, Pythium, Rhizoctonia* and *Sclerotinia* (Miller *et al.*, 1988; Petersen *et al.*, 1989[a,b]). This paper will focus on the development and application of immunoassays for detection of *Phytophthora* spp. in crops and soil. *Phytophthora* spp. are worldwide in distribution and cause economic loss in a wide range of annual and perennial crops.

Diagnosis of *Phytophthora* based on symptoms is often unreliable, and isolation and identification by traditional laboratory methods are time-consuming and require a high level of technical expertise. Enumeration of some *Phytophthora* spp. in soil is possible by dilution plating methods, but these are also time-consuming and may give highly variable results. Laboratory and rapid, on-site enzyme-linked immunosorbent assays (ELISA), based on monoclonal antibodies, for *Phytophthora* detection have been developed and applied to a number of crops.

2. Specificity of monoclonal antibodies

Monoclonal antibodies were developed by standard methods (Petersen *et al.*, 1989[a,b]) using soluble extracts of mycelia of *Phytophthora megasperma* f. sp. *glycinea* (Pmg). Antibodies were initially selected based on reactivity with the target isolate of Pmg in an indirect enzyme-linked immunosorbent assay. Selected hybridomas were subcloned and scaled up, followed by additional screening of monoclonal antibody-containing culture supernatants against an extensive panel of isolates of related and unrelated fungi. Of eighteen hybridomas secreting monoclonal antibodies that reacted strongly with isolates of *Phytophthora* spp. but not with non-target fungi, two were selected for further scale up and evaluation. Affinity-purified polyclonal antibodies raised in sheep were also screened for reactivity with these isolates.

The *Phytophthora* spp. tested were: *P. cactorum*, *P. cambivora*, *P. capsici*, *P. cinnamomi*, *P. citricola*, *P. citrophthora*, *P. cryptogea*, *P. drechsleri*, *P. erythroseptica*, *P. fragariae*, *P. infestans*, *P. megasperma* f. sp. *glycinea*, *P. megasperma* f. sp. *medicaginis*, *P. megasperma* f. sp. *trifolii*, and *P. parasitica*. Polyclonal antisera reacted strongly with all of the *Phytophthora* spp. tested (absorbance values >1.5), and did not react with any non-Pythiaceous fungi. However, the antisera cross-reacted with *Pythium aphanidermatum*, *P. coloratum*, *P. dissotochum*, *P. irregulare*, *P. rostratum*, *P. torulosum*, *P. ultimum* and *P. vexans* (absorbance values 0.25 - 1.8).

Monoclonal antibody PH4830 was highly reactive with all of the *Phytophthora* spp. tested except *P. cinnamomi*; reactivity with the four

isolates of this species tested was weak (absorbance values 0.4 - 0.7). Reactivity with *P. vexans*, a known transitional species between *Phytophthora* and *Pythium*, *P. dissotochum* and *P. coloratum* was observed, but cross-reactivity with other *Pythium* spp. was low or negligible. The reactivity profile of monoclonal antibody PH3812 was similar to that of PH4830, except that PH3812 reacted positively with isolates of *P. cinnamomi*.

Eighteen monoclonal antibodies, including PH3812 and PH4831, and the sheep polyconal antisera were also tested for reactivity with a soluble extract of broken, purified oospores of Pmg. Five of the 18 monoclonal antibodies, including PH 3812 and PH 4830, and the polyclonal antiserum, reacted positively with the extracts. Immunofluoresence studies indicated that PH3812 reacted strongly with inner oospore walls and cytoplasm, while PH4830 reacted primarily with mycelial and antheridial components.

3. Immunoassays for pathogen detection in plant tissue

A double antibody multiwell immunoassay was developed and optimized utilizing affinity-purified sheep polyclonal capture antibody and a monoclonal antibody cocktail consisting of a mixture of PH4830 and PH3812, each conjugated with horseradish peroxidase. The combination of monoclonal antibodies provides specificity toward *Phytophthora* species, including *P. cinnamomi*, and reactivity toward both mycelia and oospores. As few as 10 oospores per well, and 25 ng protein /ml Pmg mycelial extract, can be detected using this immunoassay. The assay can be carried out in a simple laboratory in less than one hour.

The same antibodies were formatted in a simple, rapid, on-site double antibody ELISA that utilizes "flow-through" technology (Miller *et al.*, 1988). Antibodies immobilized on the surface of the detector "capture" specific antigens as an extract containing the pathogen flows through the detection device. Capture of the antigens is visualized using peroxidase-conjugated antibodies and a precipitating substrate. The amount of pathogen present can be quantified using a simple, hand-held reflectometer. The sensitivity of this assay is nearly equivalent to that of the multiwell immunoassay described above, but can be carried out in the field in approximately 10 minutes.

Plant tissue (root, stem, leaf, fruit, etc.) is easily extracted by grinding it between two abrasive pads. One of the pads is removed from its backing and placed in extraction buffer. In this way, a measured amount of plant tissue is extracted for each assay. The same extraction method can be used for multiwell and on-site assays.

Both types of immunoassay have proven to be effective in detecting low levels of *Phytophthora* in a wide range of field crops, vegetables, fruit trees, small fruits, and ornamental nursery crops. They are at least as sensitive as standard isolation techniques in detecting *Phytophthora* spp. in plant tissue, and have the additional advantages of detection of the pathogen in poor samples and quantitation of pathogen levels. In an experimental system, 1% infection of chrysanthemum roots by *P. cryptogea* was reliably ($P = 0.05$) detected by the multiwell ELISA (MacDonald *et al.*, 1990). We have detected *P. cinnamomi* and *P. cactorum* in artificially-inoculated roots of rhododendrons, azaleas and junipers by both assays within 2 - 3 weeks after inoculation, well before expression of symptoms above the soil line.

Multiwell assays can be carried out in a minimally equipped laboratory with relatively inexpensive equipment. It is particularly suited for diagnostic laboratories where numerous samples are tested, but it has also been used in the United States by managers of large nurseries where the volume of sample testing may be high. Alternatively, for many growers, the ability to diagnose disease and quantify pathogen levels rapidly in the field is important. The on-site format allows growers, farm advisors, and others involved in crop management, who are not experienced in ELISA techniques, to carry out an immunoassay and determine the presence and level of *Phytophthora*.

4. Detection of *Phytophthora* spp. in soil

The multiwell assay described above has been used successfully to detect *Phytophthora* spp. in soil samples (Miller *et al.*, 1989[a]). *Phytophthora* propagules are concentrated and recovered from dried soil samples using a simple flotation technique, ground thoroughly and tested by immunoassay. A standard curve is derived from extracts of known concentrations of oospores tested each time the immunoassay is carried out. Immunoasssay

values for individual samples can then be compared to the standard curve, resulting in an estimate of the level of *Phytophthora* present in the sample. The assay has been validated for detection of Pmg in soybean field soils (Miller *et al.*, 1989[b]). In soils collected in the midwestern United States, the equivalent of 1.5 -2.0 *Phytophthora* "units" (one unit corresponds to one laboratory-prepared Pmg oospore) per g of dried soil can be reliably detected. In preliminary studies, *Phytophthora* spp. have also been detected in soil samples from alfalfa, pepper, and tomato using this assay system.

5. Benefits to the farmer

There are clear advantages to the grower in the ability to detect and monitor *Phytophthora* spp. in crop tissue at an early stage of disease development: 1) disease control measures, including sanitation and fungicide application, can be initiated when they are likely to be most effective, 2) the correct fungicide can be chosen, 3) cost savings may result from more rational use of fungicides, and 4) product quality can be improved by reduction of disease. This is particularly important for root diseases, which may remain hidden to the grower until environmental stress or other factors cause extensive above-ground symptoms to develop. Root pathogens may also cause significant yield reductions that are never identified with the pathogens because above-ground symptoms do not develop. Immunoassays, carried out in the field or laboratory, provide farmers with the information they need to manage diseases and optimize yield and quality of their crops.

The ability to detect and quantify *Phytophthora* spp. in soil can increase the farmer's options for disease control prior to planting. Should *Phytophthora* levels be shown by ELISA to be high, the farmer may consider the use of a disease-resistant variety, an at-plant fungicide application, or cultural practices that limit disease severity. For many *Phytophthora* spp., the ELISA method is the only method available to provide this type of information. The assay we have developed to detect Pmg in field soil should prove in the future to be applicable to a number of crops. Both the soil and tissue assays should be viewed as tools of plant disease management, providing data to farmers that can help them produce crops more profitably, while minimizing environmental risks.

Acknowledgements

Phytophthora monoclonal antibodies and immunoassays were developed with the support of CIBA-GEIGY Corporation, Greensboro, NC, U.S.A.

References

Chu, P. W. G., Waterhouse, P. M., Martin, R. R., & Gerlach, W. L. (1989). New approaches to the detection of microbial plant pathogens. *Biotechnol. Genet. Eng. Rev.* **7**, 45-111.

Halk, E. L., & DeBoer, S. H. (1985). Monoclonal antibodies in plant disease research. *Ann. Rev. Phytopathol.* **23**, 321-350.

MacDonald, J. D., Stites, J., & Kabashima, J. (1990). Comparison of serological and culture plate methods for detecting species of *Phytophthora*, *Pythium* and *Rhizoctonia* in ornamental plants. *Plant Disease* **74** (in press).

Miller, S. M., Petersen, F. P., Miller, S. A., Rittenburg, J. H., Wood, S. C., & Grothaus, G. D. (1989). Development of a direct immunoassay to detect *Phytophthora megasperma* f. sp. *glycinea* in soil. *Phytopathology* **79**, 1139 (abstr.).

Miller, S. A., Korjagin, V., Miller, S. M., Petersen, F. P., Klopmeyer, M., Lankow, R. K., & Grothaus, G. D. 1989. Detection and quantitation of *Phytophthora megasperma* f. sp. *glycinea* in field soil by immunoassay. *Phytopathology* **79**, 1139 (abstr.).

Miller, S. A., & Martin, R. R. (1988). Molecular diagnosis of plant disease. *Ann. Rev. Phytopathol.* **26**, 409-432.

Miller, S. A., Rittenburg, J. H., Petersen, F. P., & Grothaus, G. D. (1988). Application of rapid, field-usable immunoassays for the diagnosis and monitoring of fungal pathogens in plants. In: *Proceedings of the British Crop Protection Conference - Pests and Diseases 1988, Vol. 2.* Surrey, BCPC Registered Office, pp. 795-803.

Petersen, F. P., Maybroda, A. M., & Miller, S. A. (1989). Monoclonal antibodies to *Sclerotinia homoeocarpa. United States Patent Number* **4,803,155.**

Petersen, F. P., Maybroda, A. M., Grothaus, G. D., & Miller, S. A. (1989). Monoclonal antibodies and methods for fungal pathogen detection. *United States Patent Number* **4,845,197.**

THE USE OF MONOCLONAL ANTIBODIES TO DETECT PLANT INVADING FUNGI

Frances M. Dewey
Department of Plant Sciences, University of Oxford, South Parks Road, Oxford. OX1 3RA. U.K.

The development of immunodiagnostic techniques for the detection of fungi in diseased plants has been slowl. Progress in this area contrasts markedly with the relatively rapid development of both polyclonal and monoclonal antibody (MAb) assays for plant viruses. Probably the most significant reason for the lack of progress has been the difficulty in raising antisera that are specific. Antisera raised against mycelial fragments, extracts from lyophilized mycelia, surface washings of solid cultures or culture filtrates cross-react widely. They recognize, when tested by immunofluorescence, enzyme-linked-immunosorbent-assay (ELISA) or immunoelectrophoresis, related and unrelated fungi and host tissues or extracts (Aldwell *et al.*, 1985; Chard *et al.*, 1985 [a,b]; Dewey, 1988; Mohan, 1989). Attempts to improve specificity by diluting out non-specific antibodies or cross-absorbing antisera with related fungi have rarely proved satisfactory(Musgrave, 1984; Gerik *et al.*, 1987). However, the presence of specific antibodies in such antisera has been demonstrated by immunodiffusion and immunoelectrophoresis (Dewey, 1984; Chard *et al.*, 1985 [a,b]). Thus, the use of hybridoma technology is particularly important for fungi. It allows us to tease out and grow in bulk those cell lines that secrete antibodies that are specific.

Most of the MAbs raised against fungi have not been raised for diagnostic purposes. However, some, such as the species specific MAbs raised against *Phytophthora cinnamomi* (Hardham *et al.*, 1986), *P. megasperma* var. *glycinea* (Wycoff *et al.*, 1987) and *Pythium aphanidermatum* (Estradia-Garcia *et al.*, 1989), clearly have diagnostic potential. It is unfortunate that the MAbs for *P. cinnamomi* and *P. aphanidermatum* raised by Hardham *et al.*(1986) and Estradia-Garcia *et al.* (1989) respectively, only differenntiate species on the basis of zoospores. Specific assays

for the detection of mycelia in infected plants or soil would be more useful.

Mitchell & Sunderland (1988) and Wright *et al.* (1987) have successfully raised and used MAbs in ELISA and BLOT assays for the detection of the seed-borne fungus *Sirococcus strobilinus* and sporocarps of the vesicular-arbuscular fungus *Glomus occultum*. Wong *et al.* (19?) have raised a MAb to the banana wilt fungus, *Fusarium oxysporum f.sp. cubense*, that will differentiate by immunofluoresence the thick-walled chlamydospores of strain 4 from those of strains 1 and 2.

Our own experiences in raising MAbs that are specific to various species of fungi have been mixed. Development of diagnostic assays for the pathogen causing Dutch Elm disease, *Ophiostoma ulmi* (Dewey, 1988) and two fungi, that are involved in post-harvest yellowing of rice grains, *Humicola lanuginosa* (Dewey *et al.*, 1989[a]) and *Penicillium islandicum* (Musgrave, 1984) was relatively straight forward and quick. In contrast, we have had a lot of difficulty in raising MAbs that are specific, even at the genus level for the Eyespot pathogen of cereals *Pseudocercosporella herpotrichoides* (Dewey, 1988). The degree of difficulty appears to be related to the immunogenicity of the different fungi which in turn, probably reflects both the levels of soluble proteins and the presence of non-specific carbohydrates or glycoproteins that induce a non-T cell stimulated responses. The site and nature of species-and sub-species-specific antigens is not generally known but in the case of *P. islandicum* we have shown (unpublished) that the antigens recognized by our specific MAb are present in the walls and cross walls of the hyphae but not the spores and that some of this same antigen can be removed by gently washing the surface of a solid slant culture with phosphate buffered saline (PBS). There is no consensus about the best source of immunogen or the effectiveness of Freund's adjuvant in stimulating a specific response (Dewey, 1990[b]).

We raised a panel of MAbs to *Ophiostoma ulmi* using mycelial homogenates with Freund's adjuvant (Dewey *et al.*, 1989[b]). Hybridoma supernatants were screened by ELISA against soluble antigens of the pathogen but of the 20 cell lines that secreted MAbs recognizing the pathogen, only 3 proved to be specific. Some MAbs, like the polyclonal antisera, were non-specific,

cross-reacting widely with species from other genera. The MAbs that were species-speciofic were all IgGs belonging to the sub-classes IgG_1 and IgG_{2a} whereas the genus-specific antibodies were mostly IgM antibodies. When all the MAbs were tested against micro-titre wells coated with extracts from diseased tissue (1 in 30 w/v), no correlation was found between fungal specificity and the ability to distinguish infected from non-infected plant material. For example one of the species-specific MAbs clearly distinguished between extracts from diseased and healthy tissue whereas another species-specific MAb cross-reacted with healthy tissue giving higher readings with these extracts than with diseased extracts. Another MAb that recognized species of both *Ophiostoma* and *Ceratocystis* gave low absorbance valves when tested against antigens produced *in vitro* but high readings when tested against extracts from diseased plants. It did not cross-react with extracts from healthy tissue.

In the development of the assays to detect *P. islandicum* and *H. lanuginosa*, cell-free surface washings of the fungi were used directly as the immunogen without concentration or freezing or the addition of Freund's adjuvant ((Dewey *et al.*, 1989[a], 1990[a]). This simple method of antigen preparation was very effective. In both cases antisera titres were high and only one fusion was needed to identify cell lines secreting MAbs with the specificity needed to develop diagnostic assays. However, using exactly the same technique with *Aspergillus flavus* (unpublished), a fungus that is involved in post-harvest deterioration of copra, we have so far only obtained an antibody that detects a limited number of both *Aspergilli* and *Penicillia*.

In trying to raise a MAb suitable for the detection of the Eyespot pathogen we have used several different immunogens including surface washings, mycelial fragments, soluble extracts and protein and carobhydrate fractions (Dewey, 1988). All these immunogens were tried with and without Freund's adjuvant. The general response to these different immunogens has been weak and non-specific. Antisera from mice immunized with surface washings or hyphal fragments had low titres; dilution end points for antisera tested by ELISA against surface washings were 1 in 50,000 compared with 1 in 200,000 for *H. lanuginosa*. From 16 fusions, yielding 1750 cell lines, we have identified many cell lines

secreting non-specific antibodies but only three with the specificity required for a diagnostic assay.

Most of the MAb diagnostic assays developed for fungi are, with the exception of the few commercial assays, all indirect assays. They involve direct coating of micro-titre wells or membranes with the mixture of antigens present in extracts from the infected plants followed by incubation with the specific MAb and the use of a commercial secondary antibody-enzyme or gold conjugate as the reporter antibody (Mitchell & Sutherland, 1986; Mitchell, 1988; Wright *et al.*, 1987; Wong *et al.*, 19?, Dewey *et al.*, 1989[a], 1990[a]). We have found, (as did Gleason *et al.* (1987) using polyclonal antisera[20]), that these assays work particularly well where the fungus is present on or near the surface of the infected tissue and where passive release, by overnight soaking, is sufficient to enable detection at very low levels. This method has proved invaluable in detection of *H. lanuginosa* and *P. islandicum* in rice grains (Dewey *et al.*, 1989[a], 1990[a]). Grains are soaked, individually, overnight in micro-titre wells and removed the next day. The wells are then processed as usual. This method has enabled us to determine if there is a low level of infection in all grains or if only a few individual grains are infected. We have also developed a "user-friendly" Dip-stick assay that has proved successful under field conditions. It has enabled my colleagues to prove that significant growth of *H. lanuginosa* takes place in Paddy heaps, in situ (i.e. in the Philippines) within 5 days of harvest. The dip-sticks were made of a new Millipore membrane 'Immobilon P'. Fungal diffusates from grains soaked individually, in PBS, in Eppendorfs were allowed to coat the surface of the Dip-sticks overnight. The Dip-sticks were then incubated with hybridoma supernatants followed by commercial immunogold conjugates and the signal amplified by exposure to a commercial silver enhancing solution.

The specific fungal antigens that we have worked with bind strongly to both the new Millipore membrane and to micro-titre wells. We have also found that the specific antigens are recognized rapidily to their respective antibodies. More than 95% of the total MAb that binds to the antigen is bound within 30 seconds. The nature of most specific antigens is not known but we have shown that in the case of *P. islandicum* and *H.*

lanuginosa that the specific antigens are glycoproteins(Dewey *et al.*, 1989[a], 1990[a]).

Only a few MAb double antibody sandwich assays (DAS-ELISA) have been developed for detection purposes. Most of these are commercial and have been developed for detection of turf grass diseases by Miller *et al.* (1988) for Agri-Diagnostics. They employ both MAbs and polyclonal antisera.

References

Aldwell, F.E.B., Hall, I.R., & Smith, J.M.B. (1985). *Trans. Brit. Mycol. Soc.*, **84**, 399-402.

Chard, J.M., Gray, T.R.G., & Frankland, J.C. (1985)[a]. *Trans. Brit. Mycol. Soc.* **84**, 235-241.

Chard, J.M., Gray, T.R.G., & Frankland, J.C. (1985)[b]. *Trans. Brit. Mycol. Soc.* **84**, 243-249.

Dewey, F.M., Barrett, D.K., Vose, I.R., & Lamb, C.J. (1984). *Phytopathology* **74**, 291-296.

Dewey, F. M. (1988). *Proceedings of 1988 Brighton Conference - Pests and diseases* pp. 777-786.

Dewey, F.M., MacDonald, M.M., & Phillips, S.I. (1989)[a]. *J. Gen. Microbiol.* **135**, 361-374.

Dewey, F.M., Munday, C.J., & Brasier, C.M. (1989)[b]. *Plant Pathology*, **38**, 9-20.

Dewey, F.M., MacDonald, M.M., Phillips, S.I., & Priestley, R.A. (1990)[a]. *J. Gen. Microbiol.* In press.

Dewey, F.M. (1990)[b]. In: *Techniques for Rapid Detection and diagnosis of Plant disease*. Ed. Duncan, J.M., & Torrance, L. Oxford: Blackwell Scientific Publications.

Estradia-Garcia, M.T., Green, J.R. Booth, J.M. White, G., & Callow, J.A. (1989). *Exp. Mycol.* **13**, 348-356.

Gleason, M.L., Ghabrial, S.A., & Ferris, R.S. (1987). *Phytopathology* **77**, 371-375

Hardham, A.R., Suzaki, E., & Perkin, J.L. (1986). *Can. J. Bot.* **64**, 311-321.

Miller, S.A, Rittenburg, J.H., Petersen, F.P.,, & Grothaus, G.D. (1988) *Proceedings of 1988 Brighton Conference - Pests and diseases* pp. 795-803.

Mitchell, L.A. (1988). *Plant Disease* **72**, 664-667.

Mitchell, L.A., & Sutherland, J.K. (1986). *Can. J. Forest Res.* **16**, 945-948.

Mohan, S.B. (1989). *Plant Pathol.*, **38**, 352-363.

Gerik, J.S., Lommel, S.A., & Huisman, O.C. (1987). *Phytopathology*, **77**, 261-266.

Musgrave, D.R.(1984). *New Zeal. J. Agric. Res.* **27**, 283-288.

Wong, W.C., White, M., & Wright, I.G. *Lett. Appl. Microbiol.* **6**, 39-42.

Wright, S.F., Morton, J.B., & Sworbuk, J.E. (1987). *Appl. Environ. Microbiol.* **53**, 2222-2225.

Wycoff, K.L., Jellison, J., & Ayers, A.R. *Plant Physiol.* **85**, 508-515 (1987).

PRODUCTION, CHARACTERIZATION AND APPLICATION OF MONOCLONAL ANTIBODIES FOR DETECTING *XANTHOMONAS CAMPESTRIS* PV. *CAMPESTRIS* IN CABBAGE SEEDS

A.A.J.M. Franken
Centre for Variety Research and Seed Technology (CRZ), P.O. Box 32, 6700 AA Wageningen, the Netherlands

Introduction

Xanthomonas campestris pv. *campestris*, the causal agent of black rot in crucifers, is generally considered the most important disease of crucifers in the world (Williams, 1980). The use of healthy seeds is important to prevent the black rot disease. Methods to detect the bacterium in the seedare thus needed. Plating assays are available and were summarized by Schaad (1989). However, the success of a plating assay very much depends on theseed lot and media used (Schaad, 1989). For routine assays it is impossibleto use all the media published for this pathogen. This makes it hard to givea reliable result with respect to presence or absence of this bacterium in aparticular seed lot. Therefore, other test, e.g. serological tests using monoclonal antibodies, should be used to obtain the needed information.

Monoclonal antibodies for *X.* c. pv. *campestris* were produced and evaluated by Alvarez *et al.* (1985) and Yuen *et al.* (1987). These antibodies were specifically tested for application in a radioimmunoassay (RIA) and an enzyme-linked immunosorbent assay (ELISA) and for identification ofisolates. The aim of our study was to produce and evaluate monoclonal antibodies for direct detection of *X.* c. pv. *campestris* in cabbage seed lots by immunofluorescence microscopy (IF). IF is regarded as the most sensitive serological method to detect seed-borne bacteria (Franken and Van Vuurde, 1990).

Material and methods

Production of monoclonal antibodies. Monoclonal antibodies were produced against crude flagella extracts of *X.* c. pv. *campestris*, prepared

according to Martin and Savage (1985), or outer membrane extracts, prepared according to Lugtenberg *et al.* (1975), with some modifications. For production of monoclonal antibodies BALB/c mice were used. For hybridization, spleen cells were fused with SP2/0 myeloma cells. The cells were cultured in a selective
medium containing hypoxanthine and azaserine.

Selection of monoclonal antibodies. Antibodies of hybridoma lines were first screened in ELISA with extracts (outer membrane extracts and/or flagella extracts) of the homologous strain and afterwards with whole cells of a selected number of strains of relevant bacteria (three strains of *X. c.* pv. *campestris*, one strain of respectively *X. c.* pv. *phaseoli*, *X. c.* pv. *phaseoli* var. *fuscans*, *X. c.* pv. *vesicatoria*, *Pseudomonas syringae* pv. *maculicola*, *P. s.* pv. *phaseolicola* and *Clavibacter michiganensis* subsp. *michiganensis* and a saprophyte isolated from cabbage seeds). A third screening was done with antibodies of selected hybridoma lines in ELISA, a dot-blot immunoassay and IF using boiled cells (15 minutes 100 °C) of (a wider range of) isolates of various bacterial genera.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The stacking and running gels contained 4.0% and 12.5% (w/v) acrylamide, respectively. Proteinase K treatment of samples was done according to De Weger *et al.* (1987). Gels were stained with Coomassie brilliant blue.

ELISA. Polystyrene microplates were coated for 30 minutes at 21 °C with poly-l-lysine, 0.1 mg/ml, in carbonate buffer, pH 9.6. Washing in between steps was done using PBS with 0.1% Tween 20. Antigen incubation was done overnight at 4 °C. Antibodies were added after blocking and incubation was done for 3 h at 4 °C. Incubation with goat-anti-mouse alkaline phosphatase- conjugate (GAM-AP) took place at 37 °C (2 hours). Incubation with substrate and reading of absorbance values were done as described by Clark and Adams (1977).

Dot-blot immunoassay and development of other blots. For dot-blotting, one microliter of antigen suspension (either whole cells or extracts) were spotted onto nitrocellulose membranes. After blocking and washing, hybridoma supernatants or diluted antibodies were added and incubated for

2 h at 21 °C. The washing step was repeated and GAM-AP was added (incubation for 1 h at 21 °C). Finally blots were developed with nitroblue tetrazolium to which 5-bromo-4-chloro-3-indolyl phosphate (BCIP) solution was added. Development of blots of SDS-polyacrylamide gels was done as with dot-blot immunoassays.

Immunofluorescence microscopy and dilution-plating. Indirect and direct IF was done as described by Van Vuurde *et al*. (1983). Plating was done on the media NSCA and NSCAA, as described by Schaad (1989).

Results

Production of monoclonal antibodies. Monoclonal antibodies were prepared against crude flagella extracts or outer membrane extracts. After screening tests, using whole cells of 10 isolates of several bacterial genera, 14 stable hybridoma cell lines producing antibodies to *X. c.* pv. *campestris* were selected (table 1). For the next screening, cells were boiled to diminish endogenous phosphatase activity. Some of the results are shown in table 2. Considerable differences were found between the serological assays used. Antibodies of e.g. hybridoma line 10H12 gave no reaction in ELISA. However, in the dot-blot immunoassay this line proved to be relatively specific for the *Xanthomonas* pathovars tested. For other hybridoma lines like 11B6,
crossreactions were obtained with all isolates, not belonging to one of the pathovars of *Xanthomonas* in ELISA and the dot-blot immunoassay. However, in IF this line gave relatively specific reactions. Generally, the greater part of the tested isolates of *X. c.* pv. *campestris* reacted in the dot-blot immunoassay whereas the reaction in IF and ELISA varied per hybridoma line. In IF, antibodies of hybridoma line 20H6 gave very brilliantly stained cells. This staining was superior to that of other antibodies. No hybridoma line could be obtained which produced antibodies reacting with all isolates of *X. c.* pv. *campestris* isolates, and not with isolates of other pathovars of *X. campestris*. Additional tests also showed that antibodies of hybridoma lines 10C5 and 20H6 reacted both with the 2 tested strains of *X. c.* pv. *amoraciae*, line 17C12 did not react with these isolates. Other lines were not tested with *X. c.* pv. *amoraciae*.

Table 1. Selected antibodies of hybridoma supernatants prepared against antigens of *Xanthomonas campestris* pv. *campestris*.

hybridoma cell line	homologous[1] antigen	antibody isotype
12F7	FE 367	IgM
10C5	FE 367	IgM
14G11	FE 367	IgM
20H6	FE 367	IgG1
16B5	FE 367	IgG2a
17C12	FE 367	IgG2a
2F4	FE 367	IgG3
18G12	FE 367	IgG3
6F12	OM 671	IgM
7H2	OM 671	IgM
9H4	OM 671	IgG3
10H12	OM 671	IgG3
11B6	OM 671	IgM
11E4	OM 671	-[2]

[1]Designation: FE=crude flagella extract, followed by the strain number of *Xanthomonas campestris* pv. *campestris*: strain number 367 (National Culture Collection for Plant Pathogenic Bacteria, Harpenden, England, no. 1645) and strain number 671 (donated by dr. N.W. Schaad as strain no. B24).
[2]- = unknown.

Reaction of monoclonal antibodies in SDS-PAGE-immunoblotting. Purified antibodies of the hybridoma lines 20H6, 10C5, 16B5, 2F4 and 18G12, which were the most specific on the basis of the first screening tests, were used for immunodetection on blots of SDS-polyacrylamide gels (SDS-PAGE-immunoblotting) with total bacterial extracts of several isolates of some *X. campestris* pathovars, an isolate of *Pseudomonas syringae* pv. *maculicola* and a saprophyte isolated from cabbage, which, often crossreacted with polyclonal antisera. The extracts were or were not treated with proteinase K to determine whether a reaction occurs with the lipopolysaccharide (LPS). The treatment of total bacterial extracts with proteinase K showed in SDS-PAGE-immunoblotting that some of the tested antibodies reacted stongest with the LPS of the homologous strain 367 of *X. c.* pv. *campestris*, except for antibodies of hybridoma line 16B5.
Antibodies of some lines reacted in SDS-PAGE immunoblotting specifically with the LPS (e.g. antibodies of hybridoma line 20H6 and 18G12) or with a ± 35 kDa protein band (e.g. antibodies of hybridoma line 16B5). The ± 35 kDa protein band was also present in other pathovars of *Xanthomonas campestris*. The "LPS-antibodies" also reacted with the LPS of *X. c.* pv. *vesicatoria*, but not as strong as with the LPS of the homologous strain.

Table 2. Reaction of antibodies of some hybridoma supernatants prepared against antigens of *Xanthomonas campestris* pv. *campestris* with boiled cells of various isolates.

Strain[1]	Hybridoma supernatant	Strains positive (no.)/Strains tested (no.)		
		ELISA	Dot-blot immunoassay	IF[2,3]
Xcc	10C5	33/37	26/37	28/37 (13/37)
Xcv		2/2	2/2	2/2 (2/2)
Xcp		0/2	0/2	0/2
Xcpf		0/2	0/2	0/2
other		0/15	0/15	0/15
Xcc	20H6	27/37	27/37	21/37 (15/37)
Xcv		2/2	1/2	1/2 (1/2)
Xcp		0/2	0/2	1/2 (0/2)
Xcpf		0/2	0/2	0/2
other		0/15	0/15	0/15
Xcc	16B5	18/37	37/37	20/37 (20/37)
Xcv		1/2	2/2	1/2 (0/2)
Xcp		1/2	2/2	1/2 (1/2)
Xcpf		2/2	2/2	2/2 (2/2)
other		0/15	0/15	0/15
Xcc	17C12	24/37	37/37	23/37 (18/37)
Xcv		1/2	2/2	0/2
Xcp		2/2	2/2	1/2 (1/2)
Xcpf		2/2	2/2	2/2 (2/2)
other		0/15	0/15	0/15
Xcc	10H12	0/37	33/37	18/37 (8/37)
Xcv		0/2	2/2	0/2
Xcp		0/2	2/2	1/2 (0/2)
Xcpf		0/2	2/2	2/2 (0/2)
other		0/15	0/15	0/15
Xcc	11B6	18/37	37/37	16/37 (4/37)
Xcv		1/2	2/2	0/2
Xcp		1/2	2/2	0/2
Xcpf		2/2	2/2	1/2 (0/2)
other		15/15	15/15	1/15 (0/15)

[1]Xcc = *X. c.* pv. *campestris*, Xcp = *X. c.* pv. *phaseoli*, Xcpf = *X. c.* pv. *phaseoli* var. *fuscans*, Xcv = *X. c.* pv. *vesicatoria*. Other isolates are: *Pseudomonas syringae* pv. *phaseolicola*, *P. s.* pv. *pisi*, *P. s.* pv. *syringae*, *P. s.* pv. *lachrymans*, *P. s.* pv. *maculicola*, *P. viridiflava*, *P. aeruginosa*, *Curtobacterium flaccumfaciens* subsp. *flaccumfaciens*, *Clavibacter michiganensis* subsp. *michiganensis*, *Cl. m.* subsp. *insidiosus* and a saprophyte, isolated from cabbage seed.

[2]The number of isolates reacting with the antibodies in IF is indicated in front of the slant line.

[3]Between brackets, the number of isolates which showed a good staining of the complete cell wall in IF is indicated in front of the slant line.

Table 3. Evaluation of polyclonal antibodies and monoclonal antibodies for direct detection of *Xanthomonas campestris* pv. *campestris* in seed lots by immunofluorescence microscopy.

Seed lot	Antibodies[1]	Cell counts (mean log cells/ml)		
		Extraction method: 5 min. shaking	2.5h shaking	1.5h soaking
B188	PCA 94	0.62[2]	0.79[2]	1.90[2]
	MCA 20H6	0.78[2]	0.84[2]	1.46[2]
	MCA 16B5	0.32	0.00	0.00
	MCA 17C12	0.00	0.00	0.00
	MCA 10C5	0.00[2]	0.58[2]	0.63[2]
B189	PCA 94	3.10	5.05	2.47
	MCA 20H6	1.74	4.99	2.35
	MCA 16B5	0.00	1.40	0.00
	MCA 17C12	0.00	0.53	0.00
	MCA 10C5	-[2]	-	-
B190	PCA 94	1.90	3.63	2.08
	MCA 20H6	0.53	2.35	1.42
	MCA 16B5	0.00	0.00	0.00
	MCA 17C12	0.00	0.00	0.00
	MCA 10C5	1.79	2.38	1.61
B196	PCA 94	2.69	3.50	2.45
	MCA 20H6	2.42	2.24	1.80
	MCA 16B5	-	-	-
	MCA 17C12	-	-	-
	MCA 10C5	2.17	2.39	1.06
B209	PCA 94	4.83	5.71	5.53
	MCA 20H6	5.06	5.65	5.46
	MCA 16B5	-	-	-
	MCA 17C12	-	-	-
	MCA 10C5	3.97	5.65	5.14
B213	PCA 94	4.66	5.19	4.73
	MCA 20H6	3.68[2]	4.71[2]	2.81[2]
	MCA 16B5	0.26	0.32	0.00
	MCA 17C12	0.88	3.75	1.38
	MCA 10C5	3.35[2]	4.39[2]	2.70[2]
B214	PCA 94	0.53	2.14	0.85
	MCA 20H6	2.54	1.22	0.62
	MCA 16B5	-	-	-
	MCA 17C12	-	-	-
	MCA 10C5	0.26	0.53	0.45
B215	PCA 94	1.21	1.99	1.63
	MCA 20H6	1.95	1.74	1.00
	MCA 16B5	-	-	-
	MCA 17C12	-	-	-
	MCA 10C5	1.04	0.89	0.00

[1]PCA= polyclonal antiserum, MCA=monoclonal antibodies (antibodies of hybridoma supernatants).
[2]n=10, for other treatments n=5; - = not tested.

The use of antibodies of hybridoma supernatants for direct detection of Xanthomonas campestris pv. campestris in seed lots by immunofluorescence microscopy. Antibodies of four hybridoma supernatants, viz. 20H6, 17C12, 16B5, 10C5 and polyclonal antiserum (PCA) 94 were used in IF to index seed lots for *X. c.* pv. *campestris*. The results, summarized in table 3 show that PCA 94 and antibodies of hybridoma lines 20H6 and 10C5 gave generally the highest number of cells. Antibodies of lines 16B5 and 17C12 gave for the tested seed lots generally low cell counts. Significant differences were found between PCA 94, and antibodies of line 20H6 and 10C5 for each extraction method (B188, B190, B196, B209, B213, B214 and B215 tested at 95% probability using Friedman's two-way analysis of variances). However, these differences were less for 5 min shaking and largest for 1.5 h soaking. At 5 min shaking no differences were found between PCA 94 and antibodies of hybridoma line 20H6 for seed lots B188, B190, B196, B209, B213, B214 and B215 (at 95% probability using Wilcoxon matched-pairs signed-ranks test). However, significant differences were found between PCA 94 and antibodies of line 20H6 for extraction methods 2.5 h shaking and 1.5 h shaking.

When comparing the log cells per ml (cell counts by IF, table 3) to the log colony forming units (plate counts by dilution-plating, table 4), the data showed that cells counts were generally higher than the corresponding (= the same seed lot) plate counts for PCA 94, and antibodies of 20H6 and 10C5. Cell counts for antibodies of line 17C12 and 16B5 were often lower than the plate counts for the corresponding seed lots.

Discussion

The most suited antibodies for use in IF were produced against crude flagella extracts of strain 367 of *X. c.* pv. *campestris*. The results presented in table 2 show that great differences can appear between serological tests. A possible explanation is that the major antigens in ELISA are soluble antigens and in IF cell wall antigens. In a dot-blot immunoassay possibly cell wall antigens and soluble antigens can be detected. Antibodies which were initially selected on the basis of a positive reaction with outer membrane extracts or flagella extracts, were sometimes found in negative in ELISA when using whole or boiled cells (e.g. antibodies of line 10H12). Generally, hybridoma lines producing

Table 4. Results of the dilution-plating assay for *Xanthomonas campestris* pv. *campestris*.

Seed lot	Plate counts (mean log colony forming units per ml1)		
Extraction method:	5 min shaking	2.5 h shaking	1.5 h soaking
B188	0.01	0.00	0.30
B189	0.06	1.09	0.33
B190	0.00	0.00	0.56
B196	0.08	0.18	0.01
B209	0.63	1.11	1.72
B213	1.14	1.52	0.57
B214	0.20	0.73	0.35
B215	0.14	0.04	0.01

[1]Means of plating results on the media NSCA and NSCAA; n=120 for B188 and B213, n=60 for other seed lots.

potentially specific antibodies could more sensitively be detected in the ELISA procedure, described here, when testing with outer membrane extracts or flagella extracts than when testing with whole or boiled cells.

Although ELISA may seem the most suited technique to select hybridoma lines producing specific antibodies, our findings suggest that it is necessary to use other serological tests or several variants of one serological test, when the antibodies are needed for other tests than one particular ELISA. Preferably, the antibodies should be selected with the tests they are intended for. Another way to avoid that suitable clones are erroneously discarded may be initial specificity testing with different antigens of the homologous strain, e.g. crude flagella extracts, outer membrane extracts, boiled cells and whole cells.

Although antibodies of hybridoma 16B5 and 17C12 seemed to be the most suited antibodies in IF on the basis of reactions with pure cultures (table 2), IF tests with seed lots showed that cell counts were often far less for these antibodies than cell counts for antibodies of hybridoma lines 20H6 and 10C5 (table 3). This indicates that seed lots may contain strains which are serologically predominant and which do not react with antibodies from line 16B5 and 17C12. This is supported by the fact that several serologically different strains were isolated from one single seed lot (data not shown). For routine application it is important to know whether seed lots may contain one single isolate which does not react in IF with the used monoclonal or polyclonal antibodies and thus does give false negative results in IF. Comparisons of dilution-plating

assays with IF showed that no seed lots were found positive (=infested) in a dilution-plating assay but were found negative in IF, when using antibodies of hybridoma line 20H6 and 10C5. On the other hand, negative results in a dilution-plating assay and positive results in IF were found when using antibodies of 20H6 and 10C5 (table 3 and 4). Further applications of mixtures of monoclonal antibodies need to be investigated.

Differences between PCA 94 and antibodies of the hybridoma lines varied for the used extraction method, indicating that cells may change serologically during longer extraction times. This change may be caused in part by cell division during the extraction. During the cell division the composition of the cell membranes may change. This is supported by our observation that more partially stained cells were found for longer extraction times.

At specific dilutions of antibodies of some hybridoma lines (e.g. 20H6) crossreactions are possible with *X. c.* pv. *vesicatoria* and *X. c.* pv. *amoraciae*. Also Alvarez *et al.* (1985) and Alvarez and Lou (1985) found a serological relationship of *X. c.* pv. *campestris* with *X. c.* pathovars *vesicatoria* and *amoraciae*. Since *X. c.* pv. *vesicatoria* is a pathogen on tomato, this crossreaction is not a problem in the detection of *X. c.* pv. *campestris*. *X. c.* pv. *amoraciae* can affect crucifers. In some pathogenicity tests, the 2 tested strains of pv. *amoraciae* gave symptoms identical to symptoms, caused by some strains of *X. c.* pv. *campestris* on some varieties. Moreover, since weakly virulent or avirulent strains of *X. c.* pv. *campestris* can be found in seed lots it is extremely difficult to distinguish strains of pv. *amoraciae* and pv. *campestris*. Until additional studies clearly demonstrate otherwise, it is believed that all seed lots infested with pathogenic *Xanthomonas* isolates should be considered as potential sources for disease in the field. The significance of pv. *amoraciae* in causing a major disease of crucifers needs to be investigated.

Black rot diagnosis and detection of seed-borne *X. c.* pv. *campestris* will be enhanced with specific monoclonal antibodies. For reliable detection of *X. c.* pv. *campestris* it is advisable to combine IF, using monoclonal antibodies, with dilution-plating (isolation) and additional confirmation tests.

References

Alvarez, A.M., Benedict, A.A., & Mizumoto, C.Y. (1985). Identification of xanthomonads and grouping of strains of *Xanthomonas campestris* pv. *campestris* with monoclonals antibodies. *Phytopathology* **75**: 722-728.

Clark, M.F., & Adams, A.N. (1977). Characteristics of the microplate method of enzyme-linked immunosorbent assay for the detection of plant viruses. *J. Gen. Virol.* **34**: 475-483.

De Weger, L.A., Jann, B., Jann, K., & Lugtenberg, B. (1987). Lipopolysaccharides of *Pseudomonas* spp. that stimulate plant growth: composition and use for strain identification. *J. Bacteriol.* **169**: 1441-1446.

Franken, A.A.J.M., & Van Vuurde, J.W.L. (1990). Problems and new approaches in the use of serology for seed-borne bacteria. *Seed Sci. Technol.* **18**, in press.

Martin, J.H., & Savage, D.C. (1985). Purification and characterization of flagella from *Roseburia cecicola*, an obligately anaerobic bacterium. *J. Gen. Microbiol.* **131**: 2075-2078.

Lugtenberg, B., Meijers, J., Peters, R., Van der Hoek, P., & Van Alphen, L.(1975). Electrophoretic resolution of the "major outer membrane protein"of *Escherichia coli* K12 into four bands. *FEBS Letters* **58**: 254-258.

Schaad, N.W. (1989). Detection of *Xanthomonas campestris* pv. *campestris* in Crucifers. In Detection of bacteria in seed and other planting material (eds. A.W. Saettler, N.W. Schaad and D.A. Roth), pp. 68-75. The American Phytopathological Society, St. Paul, U.S.A.

Van Vuurde, J.W.L., Van den Bovenkamp, G.W., & Birnbaum, Y.E. (1983). Immunofluorescence microscopy and enzyme-linked immunosorbent assay as potential routine tests for the detection of *Pseudomonas syringae* pv. *phaseolicola* and *Xanthomonas campestris* pv. *phaseoli* in bean seed. *Seed Sci. Technol.* **11**: 547-559.

Williams. P.H. (1980). Black rot: a continuing threat to world crucifers. *Plant Disease* **64**: 736-742.

Yuen, G.Y., Alvarez, A.M., Benedict, A.A., & Trotter, K.J. (1987). Use of Monoclonal antibodies to monitor the dissemination of *Xanthomonas campestris* pv. *campestris*. *Phytopathology* **77**: 366-370.

SELECTION OF MONOCLONAL ANTIBODIES TO THE PLANT GROWTH REGULATORS INDOLE-3-ACETIC ACID AND ABSCISIC ACID AFTER IMMUNIZATION WITH CONJUGATES COUPLED THROUGH THE CARBOXYLIC GROUP

P.M. Boonekamp[1,2], H. Pomp[2], E. Davelaar[3], & C.R. Vonk[3]
[1]Bulb Research Centre, Lisse, The Netherlands
[2]Laboratory for Monoclonal Antibodies, Wageningen, The Netherlands
[3]Centre for Agrobiological Research, Wageningen, The Netherlands

Introduction

During the growing season of bulbs, assimilates formed in the leaves are transported to the bulbs and stored.
The new sprout, containing leaf- and flowerprimordia is initiated and in general already completed before or immediately after harvest. This means that the harvested bulb contains already the complete flower for the next season. After harvest bulbs require storage conditions (in general at low temperatures), to break a kind of dormancy. During storage a physiological program is initiated, that enables a rapid growth of the sprout after planting. This program includes the ability of root formation, mobilization of sugars from the bulb scales and transportation to the sprout, stem elongation and the outgrowth of a complete flower. Although only limited information is available studies point to a regulatory role of various classes of plant growth regulators (PGR's) in the programming during storage as well as during the metabolic cascade leading to the outgrowth of the plant.

To study the role of PGR's both sensitive and convenient detection techniques are required, enabling measurements in small pieces of tissue of the flower or the bulb. In this study, antibodies to two classes of PGR's were produced, the auxin indole -3- acetic acid (IAA) and abscisic acid (ABA), to be used in ELISA for quantitative measurements. The production of polyclonal antibodies against ABA and IAA has been reported, using these PGR's bound to a carrier protein for immunization (Weiler, 1979, 1980, 1981; Weiler *et al.*, 1981).

However, in our hands polyclonal antibodies against ABA, produced according to Weiler (1979), did not react well with the naturally occuring ABA. Those against IAA were produced according to Weiler (1982) but were not convenient since a methylation step (of the sample) was required. In this report the production of monoclonal antibodies (MAb's) is presented with high affinity and sensitivity to either free IAA or ABA. The way the PGR-carrier protein complex, was prepared, was essential for a good immune response. In addition an ELISA is presented which allows selection of MAb's against the free naturally occuring growth regulators.

Materials and methods

Plant-growth regulators (Fig. 1). Cis-(±)-ABA, cis-(+)-ABA (naturally occurring enantiomer in plants), trans-(+)-ABA (inactive isomer formed by UV light), cis(±)ABAGE (racemic mixture of the predominant ABA-glucose ester in plants), IAA, and various indole-3 derivatives and precursors as mentioned in the text. For some experiments IAA-or ABA-compounds were methylated using diazomethane as described (Weiler *et al.*, 1982).

Immunization. To prepare antigens for immunization the carboxylic groups (C_1) of either cis-(±)-ABA or IAA were coupled to bovine serum albumine (BSA) using the carbodiimide method (Weiler, 1979) and to porcine thyroglobuline (PTG) or keyhole limpets hemocyanin (KLH) using the mixed anhydride method (Weiler, 1982). The reaction mixture consisted of 100 mg protein and 25-50 mg hapten.

For some experiments the PGR-BSA conjugates were cross-linked with 0.04% glutaraldehyde for 4h at RT, prior to immunization.

Polyclonal antibodies. Rabbits were immunized with IAA or ABA conjugates and antisera were prepared essentially as described for cytokinins, (Vonk *et al.*, 1986).

Monoclonal antibodies. Balb/c mice were immunized intraperitoneally with 100 ug antigen emulsified in Freund's complete adjuvant or in alum, after 3 weeks with the antigen in Freund's incomplete adjuvant and alum respectively, and after more than 8 weeks a third time with the antigen in PBS.

Three days after the last injection spleen cells were fused with SP 2/0-Ag-14 myeloma cells and cultured as described previously (Vos *et al.*, 1989). Media from growing cells were tested in ELISA or RIA for antibody

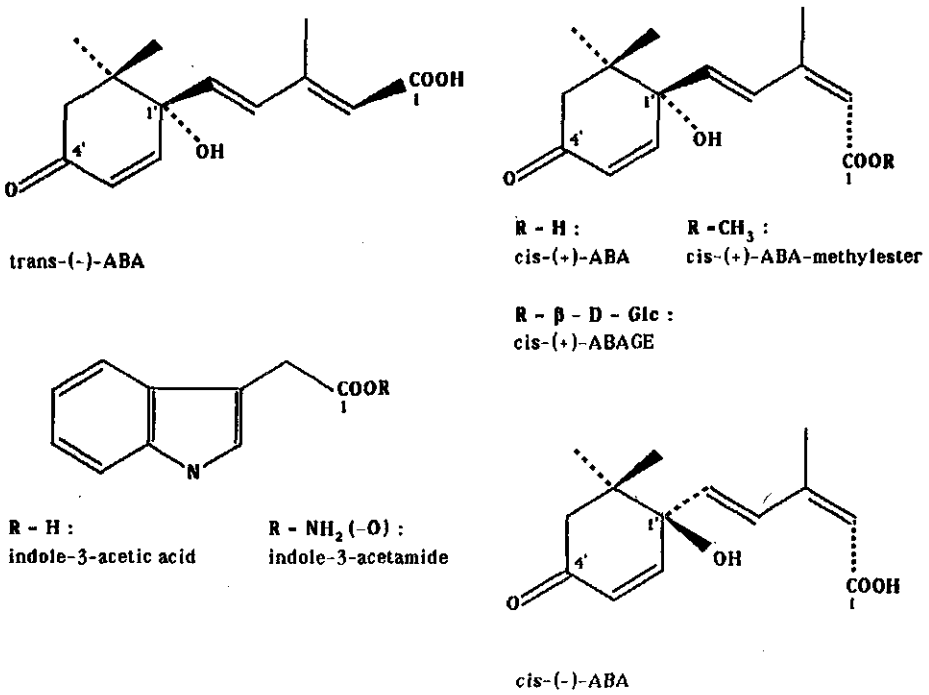

Figure 1. Compounds used for immunization and for assaying cross-reactivity.

activity. The immunoglobulin class of the MAb's was determined in ELISA using a commercial kit (Bio-Rad).

ELISA and RIA. The ELISA procedures were as described (Vonk *et al.*, 1986). In brief:

1) coating with 10 ug/ml of KLH-IAA or KLH-cis-(±)-ABA in bicarbonate buffer (pH 9.6) for 2 hr, 37°C; 2) incubation for 2 hr, 37°C with MAb's-containing media, 1/100-1/1000 diluted in phosphate buffered saline + 0.05% Tween 20, pH 7.4, (PBT) w/o the addition of competing free PGR's (0-10,000 pmol); 3) incubation for 2 hr, 37°C with goat anti-mouse Ig, conjugated with alkaline phosphatase (Sigma); 4) incubation with substrate, reading A405 nm. Maximal MAb binding (Bo) and minimal MAb binding (Bu) were determined using PBT buffer or a large excess of the free PGR's respectively, together with the MAb's in the second incubation step. When only Bo is measured (Bo-ELISA) MAb's are considered to react with bound PGR's. When in addition Bo-Bu is measured ((Bo-Bu)-ELISA) and

positive, MAb's are considered to react with bound as well as with free PGR's. When cross-reactive compounds were added (step 2) in stead of the free PGR's, the percentage of cross-reactivity was calculated as 100. X/Y; X is the amount of PGR (in pmol) and Y is the amount of cross-reacting compound, necessary to obtain 50% of the Bo-value.

RIA was performed as described previously (Weiler, 1979) using methyl 14C-IAA and 3H- cis-(±)-ABA.

Results

In the first fusion experiments (table I, exp. 1-6), BSA was used as a carrier protein for immunization. When alum was used as adjuvant no immunoresponse and no viable hybridoma's were obtained (table I, exp. 1-4). A poor immunoresponse was observed when the antigens were applied in Freund's adjuvant (Table 1, exp. 5,6). Although many viable hybridoma's were obtained, only a few produced MAb's against the bound PGR's (Bo-ELISA data) and none apparently reacted with the free PGR's ((Bo-Bu)-ELISA data). All MAb's were of the IgM-class. In the next fusion experiments (table I, exp. 7,8), PTG was used as a carrier protein.
The immune responses for both PGR's were very good, many hybridoma's were obtained and many of them apparently produced MAb's of the IgG-class against the PGR's.
However, many MAb's reacting with the bound PGR's (Bo-ELISA) did apparently not react with the free PGR's ((Bo-Bu)-ELISA).
The specificity of some selected hybridoma's is shown in table 2 and table 3. Although immunized with cis-(±)-ABA conjugate the rabbit antiserum primarily reacted with the methylated form of cis-(±)-ABA, and not with the naturally occurring enantomer cis-(+)-ABA (table 2). One type of MAb's reacted similarly but others could be selected that reacted preferentially with cis-(+)-ABA. MAb-7-364 showed the highest sensitivity for cis-(+)-ABA, but cross-reacted strongly with the racemic mixture of the naturally occurring conjugate cis-(+)-ABAGE (table 2). For IAA the rabbit antiserum reacted also primarily with the methyl ester of IAA although an IAA conjugate was used for immunization. One type of MAb's showed a similar specificity, but others reacted preferentially with the non-methylated IAA which was used for immunization. All selected MAb's cross-reacted strongly with indole-3-acetamide a precursor of IAA.

Table 1. Reactions of mouse sera and hybridoma's immuniced with conjugates of IAA and ABA;

fusion	antigen for immunization	adjuvant	reaction of of mouseserа in Bo-ELISA	number of hybridoma's	number of hybridoma's positive in Bo-ELISA	(Bo-Bu)ELISA	RIA
1/2	ABA-BSA/IAA-BSA	alum	0	0	0	-	-
3/4	ABA-BSA/IAA-BSA[a]	alum	0	0	0	-	-
5	ABA-BSA	Freund	<0.1	800	5	0	0
6	IAA-BSA	Freund	<0.2	700	4	0	0
7	ABA-PTG	Freund	>1.0 (<0.05[b])	700	690	260	95
8	IAA-PTG	Freund	>1.0 (<0.05[b])	900	145	40	15

- = not performed;
a) = cross-linked by glutaraldehyde treatment;
b) = reaction with KLH alone

Table 2. Specificity of a rabbit antiserum and some selected monoclonal antibodies from fusion 7 with ABA-like compounds. Data are expressed as percentages of crossreactivity; the values of x in pmoles are given in parenthesis.

compound	percentages of cross-reactivity of rabbit anti-ABA serum	7-3G4	7-3A8	7-10A8
cis-(+)-ABA	<0.1	100 (2.5)	100 (20.0)	0
cis-(±)-ABA	5.0	50	50	-
cis-(+)-ABA methyl ester	2.5	-	70	2.4
cis-(±)-ABA methyl ester	100 (1.0)	55	35	100 (0.6)
cis-(±)-ABAGE	-	130	25	0.6
trans-(-)-ABA	-	0.9	-	-

- = not performed

Table 3. Specificity of a rabbit antiserum and some selected monoclonal antibodies from fusion 8 with IAA-like compounds. Data are expressed as percentages of cross-reactivity; the values of x in pmoles are given in parenthesis.

compound	percentages of cross-reactivity of rabbit anti-IAA serum	8-1C10	8-2A3	8-2B3	8-4C8
Indole-3-acetic acid	0.1	100 (8.0)	100 (4.0)	0	100 (50.0)
Indole-3-acetamide	-	470	200	320	250
Indole-3-pyruvic acid	-	47	50	10	50
Indole-3-methyl ester	100 (1.0)	1.4	0	100 (50)	0
Indole-3-ethyl ester	-	3.2	-	-	-
Indole-3-butyric acid	-	0	-	-	-
Indole-3-propionic acid	-	0	-	-	-
Indole-3-acetyl-B-D glucosyl ester	-	0	-	-	-
Indole-3-lactic acid	-	0	-	-	-
5-hydroxy-indole-3-acetic acid	-	0.6	-	-	-
4-chloro-indole-3acetic acid	-	0.2	-	-	-
D,L tryptophan	-	0	-	-	-

MAb-8-1 C10 showed the highest sensitivity for the naturally occuring IAA. The cross-reactivity with most Indole-derivatives was low and was abscent with the IAA-precursor tryptophan.

Discussion

Immunogenicity of carrier-PGR conjugates. In our hands cis-(±)- ABA or IAA conjugated to BSA with the carbodiimide method were good immunogenic in rabbits. However rabbits immunized with cis-(±)-ABA-BSA preferentially produced antibodies tot cis-(-)-ABA and not against the naturally occurring cis-(+)-ABA. This was also observed by others (Walton *et al.*, 1979), when cis-(±)-ABA was coupled through the carboxylic group (C_1). For both IAA and ABA, we found that the antisera reacted better with the methylated PGR's than with the non-methylated forms used for immunization. This was also observed by others (Weiler, 1979; Weiler *et al.*, 1981).

We observed that cis-(±)- ABA or IAA conjugated to BSA with the carbodiimide method were poorly immunogenic in mice. The few MAb's obtained reacted only with bound and none with free PGR's, and the MAb's were all of the IgM type. It might be that either BSA is a poor immunogenic carrier protein in Balb/c mice or that the bound PGR do not induce a good immune response. For IAA it has been reported (Mertens *et al.*, 1985) that IAA-BSA was immunogenic in mice and that MAb's against IAA could be selected. Since these MAb's reacted only with methylated IAA and we have screened with non-methylated IAA, we may have missed such MAb's. For ABA it has been reported (Mertens *et al.*, 1982) that cis-(±)-ABA bound through C'_4 to BSA was immunogenic in mice, and that MAb's could be obtained against cis-(+)-ABA. No reports are available on the (lack of) immunogenicity in mice of C_1 bound cis-(±)-ABA-BSA conjugates.

With PTG as a carrier we found that IAA and C^1-coupled cis-(±)-ABA appeared to be very immunogenic and that MAb's of the IgG type against free cis-(+)-ABA and IAA could be obtained. This strongly suggests that PTG is a better immunogenic carrier protein for IAA and ABA than BSA. On the other hand it might be that the PGR's in the PTG conjugates were better exposed than in the BSA conjugates, since together with the use of PTG the coupling procedure was changed from the carbodiimide method to the mixed-anhydride method.

It was reported by Weiler (1980) that rabbits did only produce antibodies

to cis-(-)-ABA and not to cis-(+)-ABA, when immunized with C^1 -coupled cis-(±)-ABA.

This was explained by assuming that the hydroxyl group at C'_1 played an important role in immunogenicity: after C_1 coupling this group might be exposed in cis-(-)-ABA but shielded in cis-(+)-ABA (Weiler, 1980). On the other hand Weiler reported that antibodies against cis-(+)-ABA could be obtained if cis(+)-ABA was used for immunization, coupled through C_1 to BSA with either carbodiimide or with the mixed anhydride methode (Weiler, 1980, 1982). This indicates that if the hydroxyl group at C'_1 is essential for immunogenicity of cis-(+)-ABA, it is similarly exposed after conjugation with the mixed anhydride method as with the carbodiimide method. Therefore our data may be explained by assuming that PTG is a much better immunogenic carrier in Balb/c mice than BSA.

Screening-assay. MAb's against IAA (Mertens *et al.*, 1985) and ABA (Mertens *et al.*, 1982) were selected with free hormones in RIA. Our data show that when B_o-ELISA was used for screening, the majority of the selected MAb's do not react with the free hormones. This is also observed by others (Quarrie & Galfre, 1985). Therefore, for obtaining MAb's against free hormones, either RIA or the more sensitive (Bo-Bu)-ELISA should be used for screening and not the Bo-ELISA. With these methods MAb's against cis-(+)-ABA could be selected, even if C_1 -bound cis-(±)-ABA was used for immunization.

Selectivity. It has been reported that cis-(+)-ABA specific antibodies, were obtained when rabbits were immunized with cis-(±)-ABA coupled through C'_4 (Weiler, 1980). Indeed the use of this conjugate was successful to produce a few MAb's that react with free cis-(+)-ABA in RIA (Mertens *et al.*, 1982). In a detailed study in rats it was found that most MAb's obtained with a similar procedure react with bound ABA and not with free ABA (Quarrie & Galfre, 1985). One MAb can be purchased commercially (Idetek Inc., 1057 Sheath Lan, San Bruno, CA; Phytodek Product Bulletin, 1988) which binds cis-(+)-ABA in a (Bo-Bu)-ELISA, and does not cross-react with cis-(+) methyl ABA, cis-(+)-ABAGE and other ABA derivatives (Norman *et al.*, 1988).

On the other hand with cis-(±)-ABA coupled through C_1, we obtained a high number of MAb's, which reacted with different specificity with free ABA. Due to coupling through C_1, some MAb's showed a high cross-reactivity with

naturally occurring ABA-conjugates, as already implicated by others (Weiler, 1980; Quarrie & Galfre, 1985). Using C_1-coupled IAA for immunization, only antisera could be produced reacting with methylated IAA (Weiler *et al.*, 1982). With N-coupled IAA, antisera against non-methylated IAA could be raised, but the antibody titre was low (Pence & Caruso, 1987). However, in our hands immunization with C_1-coupled IAA was very succesful in producing MAb's against non-methylated IAA, if the Bo-Bu ELISA was used for screening. Previously produced MAb's only reacted with indole-3-methyl ester (Mertens *et al.*, 1985). Those and the presently described MAb's both show cross-reactivity with some naturally occurring IAA conjugates, which means that such compounds have to be removed from the sample before assaying.

Applications. MAb's 7-3G_4 and 8-1C_{10} are routineously used now for assaying ABA and IAA respectively, in extracts from various plants including bulbs. The presented competitive ELISA is very sensitive and a range of 1 pmol to 100 pmol of hormone in plant extract can be detected. Cross-reactive compounds could be reliably removed from the samples (Franssen, pers. com.). The MAb's have a very high affinity and MAb's from tissue culture medium can be directly used in ELISA in dilutions from up to 1000 times. This means that no ascites production and no purification is required. In comparative studies, the present MAb's are equally useful as those commercially available against IAA and ABA (Idetek) with the advantage that for assaying IAA, methylation of the sample is no longer required.

Acknowledgements

Dr. J.M. Franssen, Bulb Research Centre, Lisse, The Netherlands is greatly acknowledged for helpful discussions and providing unpublished information.

References

Mertens, R., Deus-Neumann, B., & Weiler, E.W. (1982). Monoclonal antibodies for the detection and quantitation of the endogenous plant growth regulator, abscisic acid. *FEBS lett.* **160**, 269-272.

Mertens, R., Eberle, J., Arnscheidt, A., Ledebur, A., & Weiler, E.W. (1985). Monoclonal antibodies to plant growth regulators. II. Indole-3-acetic acid. *Planta* **166**, 389-393.

Norman, S.M., Poling, S.M., & Maier, V.P. (1988).An indirect enzyme-linked immunosorbent assay for (+)- abscisic acid in *Citrus*, *Ricinus*, and *Xanthium* leaves. *J. Agric. Food Chem.* **36**, 225-231.

Pence, V.C., & Caruso, J.L. (1987). ELISA determination of IAA using antibodies against ring-linked IAA. *Phytochemistry* **26**, 1251-1255.

Quarrie, S.A., & Galfre, G. (1985). Use of different hapten-protein conjugates immobulized on nitrocellulose to screen monoclonal antibodies to abscisic acid. *Anal. Biochem.* **151**, 389-399.

Vonk, C.R., Davelaar, E., & Ribot, S.A. (1986). The role of cytokinins in relation to flower-bud blasting in *Iris* cv. Ideal: cytokinin determination by an improved enzyme-linked immunosorbent assay. *Plant Growth Regulation* **4**, 65-74.

Vos, R.M.E., Lutkeschipholt, I.J., Boonekamp, P.M., Schots, A.J., Pomp, H., van Bladeren, P.J., & Koeman, J. (1989). Monoclonal antibodies against at glutathione S-transferase isoenzymes 2-2 and 3-3. *Hybridoma* **8**, 475-480.

Walton, D., Dashek, W., & Galson, E. (1979). A radioimmunoassay for abscisic acid. *Planta* **146**, 139-145.

Weiler, E.W. (1979). Radioimmunoassay for the determination of free and conjugated abscisic acid. *Planta* **144**, 255-263.

Weiler, E.W. (1980). Radioimmunoassays for the differential & direct analysis of free and conjugated abscisic acid in plant extracts. *Planta* **148**, 262-272.

Weiler, E.W. (1982) An enzyme-immunoassay for cis-(+)-abscisic acid. *Physiol. Plant. Pathol.* **54**, 510-514.

Weiler, E.W., Jourdan, P.S., & Conrad, W. (1981). Levels of indole-3-acetic acid in intact and decapitated coleoptiles as determined by a specific and highly sensitive solid-phase enzyme immunoassay. *Planta* **153**, 561-571.

ETIOLOGICAL STUDIES AND DIAGNOSTIC OF GRAPEVINE LEAFROLL DISEASE IMPROVED BY MONOCLONAL ANTIBODIES

P. Gugerli, B. Rosciglione, J.J. Brugger, Ss. Bonnard, M.E. Ramel & F. Tremea

Federal Agricultural Research Station of Changins, CH-1260 Nyon Switzerland

Introduction

Leafroll is a major virus-like disease of grapevine which occurs worldwide and affects the grape production qualitatively and quantitatively. The main symptoms are downward rolling and interveinal discoloration (reddening or yellowing) of the leaf blade and irregular ripening of the grapes (Fig. 1). Severe infections also reduce the growth and the grafting success rate. Mealybugs (*Planococcus ficus*) were shown to transmit the disease (Rosciglione *et al.* 1983, Rosciglione & Gugerli, 1987) but it is not yet proven that these insects are important vectors in the vineyard. There is in fact little or no natural spread in the field. A viral etiology was assigned to this disease because of the characteristic symptoms, the transmissibility by graft-inoculation and the possible therapy by heat treatment (see Hoeffert and Gifford 1967, Bovey *et al.* 1980). The experimental proof is however still lacking. Grapevine viruses are indeed difficult to study since they generally occur in very low concentration in this host plant The extraction of viral nucleoprotein is also difficult because of the presence of large amounts of inactivating tannins. Traditional techniques such as mechanical virus transmission, ordinary electron microscopy and simple serology are therefore not reliable. Several viruses were nevertheless identified in thin tissue sections or in crude extracts of leafroll diseased grapevine (see Martelli 1986). The association of closterovirus-like particles to grapevine leafroll was substantiated when we found better conditions for their extraction, directly from grapevine leaf tissue, as well as for their purification and electron microscopy. This also allowed us to produce the first specific polyclonal antibodies

Figure 1. Gamay grapevine with leafroll symptoms: atypical downward rolling and early interveinal reddening of the older leaves.

required for more extended etiological studies (Gugerli *et al.*,1984). Diseased grapevine was often found infected by several viruses which might have contributed to the disease or simply coexisted as latent pathogens. Polyclonal antisera made by immunization of experimental animals with enriched extracts from such plants could therefore recognize several viral entities which were not necessarily distinct enough to be identified by techniques such as immuno-electron microscopy. In the case of grapevine leafroll, we found in fact at least 4 associated closteroviruses (GLRaV I, II, III and IV) which were physically nearly identical except in the average maximum particle length (1800and 2200 nm) (Rosciglione & Gugerli, 1986 and Gugerli, unpublished results). The different GLRaV's were however likely to be biologically variable since

they were associated to distinct symptoms (Gugerli *et al.* 1990). Two serotypes of an other closterovirus with shorter particles (800 nm), the grapevine virus A (GVA I and II), and a non-identified virus with isometrical particles were also frequently present in extracts from leafroll diseased grapevines (Gugerli, unpublished results). Consequently, polyclonal antisera yielded sometimes conflicting results in epidemiological surveys as well as in analytical applications. Monospecific antibodies were therefore a prerequisite for further progress in the characterization of these new viruses and in the understanding of this complex disease.

Production of monoclonal antibodies to GLRaV I, III and GVA I

Monoclonal antibodies to three grapevine closteroviruses were produced principally by using the conditions described for the development of monoclonal antibodies to potato virus Y (Gugerli & Fries, 1983). Immunization of Balb/c mice was done with partially purified virus preparations obtained from leaves of either diseased Rauschling (GLRaV I), Frapatto (GLRaV III) or *Nicotiana clevelandii* (GVA I). Six injections of a few micrograms of viral nucleoprotein each were applied over a period of at least 10 weeks including the final booster 4 days before harvesting the spleen cells. The spleen cells from the immunized mice and X63/0 myeloma cells were fused at a ratio of 2 to 5 using polyethyleneglycol. The selection of specific antibody producing hybridoma was done by testing the cell supernatants by indirect ELISA using crude plant extracts with the viral antigen bound either by direct coating or by polyclonal antibody (rabbit) mediated trapping. The indirect antigen binding procedure generally yielded better results when sufficiently specific antisera were used. In fact, several attempts of production of monoclonal antibodies against GLRaV II failed, probably due to the lack of a proper screening system, e.g. the availability of a suitable polyclonal antiserum, but also due to the difficulty in the enrichment and purification of this particular antigen for efficient immunization. For GLRaV I, III and GVA I several cell lines producing specific antibodies were isolated after various passages of subcloning by limiting dilution. Their specificity was defined by reacting them with several hundred samples of leafroll diseased grapevine from Switzerland and over

Figure 2. Immuno-electronmicroscopy of grapevine leafroll associated closterovirus particles: nondecorated (left) and decorated with Mab 2-4 to GLRaV I (right).

a dozen other countries. Three suitable monoclonal antibodies, Mab 2-4 to GLRaV I, Mab 8-n to GLRaV III and Mab 14-9 to GVA I, were selected for general diagnostic purposes. They reacted with as many or more isolates than the polyclonal antibodies and were therefore not restricted in their specificity. As seen by electron microscopy, they precipitated and decorated the homologous closterovirus particles (Fig. 2). They stained a single protein, the putative virus coat protein, on electroblots obtained by SDS-polyacrylamide gel electrophoresis from crude extracts of diseased plants (Fig. 3).

Mab 2-4 to GLRaV I and Mab 14-9 to GVA are commercially distributed by Bioreba Ltd, Gempenstrasse 8, CH-4008 Basel since 1988.

Application of monoclonal antibodies

Etiology. The new monoclonal antibodies were first used by ELISA, immuno-electron microscopy (Fig. 2) or immuno-staining of electroblots of electrophoretically fractionated plant extracts (Fig. 3) to select grapevine clones with distinct viral infections and symptoms. The plants originated from a collection which was characterized earlier by symptoms, indexing and by serology using less specific polyclonal antibodies

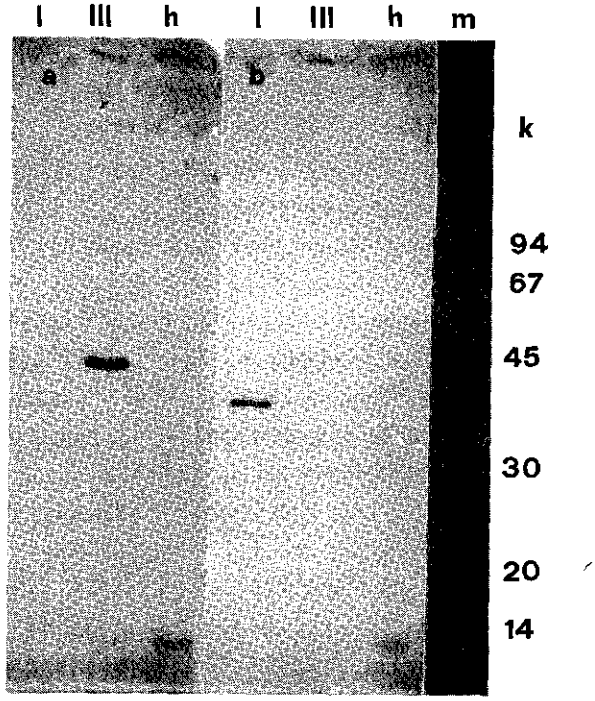

Figure 3. Immuno-stained electroblot of enriched crude Gamay leaf extracts separated on a 12 % SDS polyacrylamide gel.
(I) leafroll diseased Gamay infected by GLRaV I; (III) leafroll diseased Gamay infected by GLRaV III; (h) healthy Gamay; (m) molecular weight marker proteins from Biorad: phosporylase b: 94'000, bovine serum albumin 67'000, hen egg white ovalbumin 30'000, bovine carbonic anhydrase 30'000, soybean trypsin inhibitor 20'100 and hen egg white lysozyme 14'400.
(a) Stained with Mab 2-4 to GLRaV I; (b) stained with Mab 8-n to GLRaV III.

(Gugerli et al, 1984, Rosciglione & Gugerli, 1986). The characterized plants can now be used for further etiological studies. The new immunochemical reagents allow us also to follow precisely the fate of the virus particles during heat therapy or graft-transmission experiments in order to confirm the consistency and specificity of the association of these particles with the grapevine leafroll disease.

The characterized grapevine clones are also used to reconstitute mixed infections in order to reveal the possible synergic interactions between different closteroviruses in this complex disease. Preliminary results indicate that single infections by either GLRaV I, II and III are

associated with clearly distinct symptoms and that mixed infections by GLRaV I and III cause more severe symptoms than single infections.

Coat protein characterization. The specific monoclonal antibodies are not only optimal tools for tracing the new viruses in the diseased plants but they are also useful for the determination of some physical properties such as the molecular weight of the structural proteins. Since it is extremely difficult to purify the viral nucleoprotein from grapevine tissue for physical analysis, we have submitted partially purified and enriched preparations to SDS-polyacrylamide electrophoresis, electroblotting onto nitrocellulose filters and subsequent immunostaining with the homologous antibodies. Due to the specificity of the monoclonal antibodies, only the coat proteins were stained. The relative electrophoretical mobilities of these proteins were then compared to those of marker proteins and coat proteins of well characterized plant viruses such as potato virus Y. By this approach, we found apparent molecular weights of GLRaV I and III coat proteins of approximately 38000 and 43000. The most likely estimate for GLRaV, II coat protein was about 36000. However, since this was established with a poor polyclonal antiserum, which stained several other bands, this estimation needs to be confirmed when specific monoclonal antibodies will also be available. A molecular weight of about 22000, was determined for the coat protein of GVA, in accordance with Conti *et al.* (1980). The clearly distinct molecular weights of the coat proteins corroborate the serological and biological differences between the GLRaV's. Electrophoresis of crude extracts can now be used for the analysis of mixed infections.

Diagnostic. Grapevine leafroll disease can be controlled by rigorous sanitary selection, since there is little natural spread but transmission and dissemination through grafting. This is achieved by checking carefully the mother-plants used for grapevine propagation. In the past, the necessary virological controls could only be done by indexing. The suspected material was grafted onto indicator vines. In the field, the indicator plants had to be checked at least during two seasons. At present the ELISA technique can be used which reduces the time needed for the testing to a few hours. This is a very considerable simplification of the sanitary selection in viticulture. We obtained good results by testing wood or leaf samples from European grapevines. The former are

taken during the rest period and the latter in summer or autumn from the lower part of the vines. The monoclonal antibodies yield an increased specific signal to noise ratio in ELISA compared to the reaction obtained with polyclonal antibodies. Further improvement of the detectability is nevertheless needed for the detection of latently occurring GLRaV's in some American rootstock vines. Large-scale diagnostic surveys, carried out in collaboration with numerous European grapevine breeding and research institutes, revealed that the different GLRaV's were not evenly distributed. GLRaV I was shown to be predominant in grapevine cultivars grown in the Northern European vine producing areas (Germany and Switzerland) whereas GLRaV III prevails in the Mediterranean area (France, Italy, Portugal, Spain, Turkey, Yugoslavia). GLRaV III appears to occur more frequently worldwide (Europe, South America, Northern Africa).

Conclusions

As far as grapevine leafroll is concerned, monoclonal antibodies are powerful new tools for (1) the identification of viruses associated to this complex disease as well as the detection of new hidden viruses, (2) the physical characterization of these new viruses, and (3) the improvement of the sanitary selection of grapevine.

REFERENCES

Bovey, R., Gaertel, W., Hewitt, W.B., Martelli, G.P., & Vuittenez, A. (1980). Virus and virus-like diseases of grapevines. Editions Payot Lausanne.

Conti, M. Milne, R.G., Luisoni, E. & Boccardo, G. (1980). A closterovirus from a stem-pitting diseased grapevine. *Phytopathology* **70**, 394-399.

Faoro, F, Tornaghi, R. Fortusini, E. & Belli, G. (1981). Association of a possible closterovirus with grapevine leafroll in Northern Italy. *Rivista di Patologie Vegetale* **17**, 183-189.

Gugerli, P., Brugger, J.-J., & Bovey, R. (1984). L'enroulement de la vigne: mise en évidence de particules virales et développement d'une méthode immunoenzymatique pour le diagnostic rapide. *Rev. Suisse Vitic.Arboric. Hortic.* **16**, 299-304.

Gugerli, P. & Fries, P. (1983). Characterization of monoclonal antibodies to potato virus Y and their use for virus detection. *J. Gen. Virol.* **64**, 2471-2477.

Gugerli, P., Cazelles, O, Ryser, J.-P, Beuret, E. & Murisier, F. (1990). Maladies, carences et accidents de la vigne. *Rev. Suisse Vitic. Arboric. Hortic.* **22**, 33-48.

Hoefert L.L & Gifford, E.M.(1967). Grapevine leafroll virus-history and anatomic effects. *Hilgardia* **38**, 403-426.

Martelli, G.P. (1986). Virus and virus-like diseases of the grapevine in the Mediterranean area. *FAO Plant Prot. Bull.* **34**, 25-42.

Rosciglione, B, Castellano, M.A. & Martelli, G.P. (1983). Mealybug transmission of grapevine virus A. *Vitis*, **22**, 331-347.

Rosciglione, B. & Gugerli, P. (1986). Maladies de l'enroulement et du bois strié de la vigne: analyse microscopique et sérologique. *Rev. Suisse Viric. Arboric. Hortic.* **18**, 207-211.

Rosciglione, B & Gugerli, P. (1987). Transmission of grapevine leafroll disease and an associated closterovirus to healthy grapevine by the mealybug *Planococcus ficus* (Syn). In: *Proc. Ninth Meeting of the International Council for the Study of Viruses and Virus Diseases of the Grapevine*. Kiryat-Anavim, Israel, September 6-11, p. 13-14 (abstract).

MONOCLONAL ANTIBODIES IN POTATO LEAFROLL VIRUS RESEARCH

J.F.J.M. van den Heuvel & D. Peters
Department of Virology, Wageningen Agricultural University, Binnenhaven 11, 6709 PD Wageningen, the Netherlands

Potato leafroll virus (PLRV), a member of the luteovirus group is persistently transmitted by aphids. Studying the interactions between virus, vector, and plant, the use of monoclonal antibodies (McAbs) as compared with polyclonal antibodies allows various approaches as to detection and localization of viral antigen and the functional characterization of epitopes present on the coat protein of the virus.

A panel of ten stable hybridoma cell lines secreting monoclonal antibodies (McAbs) specific for PLRV antigen was produced in two fusion experiments with murine splenic and myeloma cells. The McAbs were tested in different ELISA procedures, in Western blotting, and in immunogold labelling experiments to evaluate their usefulness in different immunological detection techniques and to determine the nature of the epitopes on the viral coat protein.

Nine McAbs were identified as being directed against different epitopes on PLRV coat protein by a competitive binding assay, in which biotinylated McAbs were challenged with a dilution series of unlabelled McAbs for binding to the PLRV coat protein.

Testing the McAbs in ELISA to other luteoviruses viz. tomato yellow top virus (TYTV), beet western yellows virus, beet mild yellowing virus, bean leafroll virus and three strains of barley yellow dwarf virus, revealed that most McAbs displayed heterologous reactivity. Only three McAbs were found to detect solely PLRV and TYTV. New serological relationships among luteoviruses were disclosed.

A functional characterization of the epitopes was initiated after we found that only four McAbs detected poorly a PLRV strain from tomato which was inefficiently transmitted by *Myzus persicae*. These McAbs may detect epitopes involved in initial recognition of the virus within its vector aphid.

Materials and Methods

Virus purification and immunization. PLRV-Wageningen was purified from infected *Physalis floridana* Rydb. leaves using a modified enzyme-assisted purification procedure (Van den Heuvel *et al*., 1990). Two female BALB/c mice, designated A and B, were intraperitoneally (i.p.) injected with an emulsion of 50 µg purified PLRV and Freund's complete adjuvant. Mouse A received a second injection of 25 µg PLRV intravenously (i.v.) 7 weeks later. Mouse B was i.p. injected with 25 µg PLRV emulsified with Freund's incomplete adjuvant on day 32, and i.v. with 25 µg PLRV 92 days later.

Hybridoma production and screening. Hybridomas were derived from fusion experiments between splenocytes of mouse A and B, and the mouse myeloma line SP 2/0-Ag 14. The fusions were carried out with polyethylene glycol 4000 GA as fusion agent. The spleen cells of mouse A and B were fused with the myelomas four days after the last injection. Cell culture and cloning was done following methods described by Schots (1989). A triple antibody sandwich (TAS) ELISA was applied to detect the presence of PLRV specific antibodies produced by the fusion products from the spleen of donor mouse A. The fusion products of the spleen of donor mouse B were analyzed by TAS-ELISA and by another indirect ELISA, in which the virus was coated under alkaline conditions to the plates (ACP-ELISA), resulting in particle disruption (Massalski & Harrison, 1987).

Isotyping. The isotypes of the McAbs were determined in a sandwich ELISA based of rat McAbs to the mouse isotypes IgA, IgG1, IgG2a, IgG3 and IgM, using hybridoma culture supernatants.

Production of McAbs. Ascitic fluid was raised in adult BALB/c mice and the McAbs were partially purified by precipitation with 45% saturated ammonium sulphate. After centrifugation, the pellets were resuspended in 0.01 M phosphate buffered saline, pH 7.4 (PBS) containing 0.05% sodium azide. The protein concentration in stock suspensions was adjusted to 2 mg/ml prior to use.

Biotinylation. Biotinyl N-hydroxysuccinimide ester (0.2 mg) was added dropwise to 1 mg purified antibodies. The mixtures were incubated while gently shaking for 3 h at room temperature. The reaction was stopped by adding 10 µl 1 M ammoniumchloride per ml (Zrein *et al*., 1986), and

dialyzed extensively versus PBS.

Competitive binding assay. Microtitre plates were coated with polyclonal antisera and incubated with antigen. Subsequently, a mixture of a 10,000-fold diluted biotinylated antibody and a 10-fold dilution series of the unlabelled antibodies to compete for binding was added to the wells, and incubated for 3 h at 37 °C. The immobilized biotin was detected by streptavidin alkaline phosphatase conjugate and the presence of alkaline phosphatase was in the last step visualized by the addition of para-nitrophenyl phosphate disodium salt. Substrate conversion was measured at 405 nm. The maximum amount of bound biotinylated antibody was determined in the presence of anti-thyroglobulin antibodies. Incubation with biotinylated antibodies treated with healthy *P. floridana* leaf material served as negative controls.

Virus isolates. BWYV and beet mild yellowing virus (BMYV) derived from infected sugar beets and were maintained at our laboratory. Bean leafroll virus (BLRV) was obtained from Mr. N. Huyberts (Institute for Plant Protection (IPO), Wageningen, the Netherlands), and from Miss L. Katul (FBRC, Braunschweig, FRG). Tomato yellow top virus (TYTV) on *Lycopersicon esculentum* L. was supplied by Mr. A. Dusi (National Center for Horticultural Research (EMBRAPA), Brasilia, Brazil). Desiccated leaf material containing BYDV strain MAV and PAV was provided by Dr. W. Huth (FBRC, Braunschweig, FRG). BYDV strains MAV, PAV and RPV from Canada were provided by Dr. S. Haber (Agriculture Canada, Winnipeg).

Polyclonal antibodies. Rabbit anti-PLRV, anti-BWYV, anti-BLRV, and anti-BYDV sera were kindly provided by Mr. D.Z. Maat (IPO, Wageningen, the Netherlands), Dr. S. Marco (Vulcani Center, Bet Dagan, Israel), Miss L. Katul, and Dr. W. Huth and Dr. S. Haber, respectively. Rabbit anti-BMYV serum was prepared in our department.

Results and Discussion

A panel of 10 stable hybridoma cell lines producing McAbs specific for PLRV antigen was generated. Eight cell lines derived from fusion A and two from fusion B. They were assigned WAU-Ax and WAU-Bx, respectively (Table 1).

Table 1. The isotypes, and the reactivity of the monoclonal antibodies directed against potato leafroll virus in ELISA and Western blotting.

Designation	Isotype	Titre[a] in ELISA: Biotinylated McAbs DAS[b]	ACP[c]	Western blotting
WAU-A2	IgG1	> 160,000	80,000	-
WAU-A5	IgG1	5,000	5,000	-
WAU-A6	IgG1	40,000	20,000	-
WAU-A7	IgG1	20,000	10,000	-
WAU-A12	IgG1	> 160,000	1,000	-
WAU-A13	IgG2a	40,000	10,000	-
WAU-A24	IgG2b	20,000	1,000	-
WAU-A47	IgG2a	> 160,000	> 160,000	-
WAU-B9	IgG2b	> 160,000	1,000	-
WAU-B10	IgM	100	10,000	+

[a] ELISA titre represents the dilution of the McAbs in the different ELISA formats that yielded an A_{405} of at least 0.10 (control < 0.010) after one hour of colour development at room temperature.
[b] ELISA format: polyclonal rabbit anti PLRV - antigen - biotinylated McAbs - streptavidin conjugate - pNPP.
[c] ELISA format: antigen - biotinylated McAbs - streptavidin conjugate - pNPP.

Testing the McAbs in different immunological detection assays. The panel could be split up into three different groups of McAbs. The McAbs WAU-A2, -A5, -A6, -A7, -A13, and -A47 of the first group reacted with comparable strength with intact virus (DAS-ELISA) and with virus which was partially dissociated under alkaline conditions (ACP-ELISA). Furthermore, they did not detect viral antigen in Western blotting (Table 1). Therefore, it is suggested that they are directed against discontinuous epitopes present on subunits of the coat protein which remain intact after alkaline dissociation of the virus. A second group consists of the McAbs WAU-A12, -A24, and -B9, which reacted only strongly in DAS-ELISA, but at low titres when the virus was bound to the microtitre plates. Also the McAbs of this group did not react in Western blotting (Table 1). They are probably directed against discontinuous epitopes formed by the quartenary

protein structure or on the coat protein subunit configuration sensitive for alkaline dissociation. The third group comprises only WAU-B10. This McAb only detected viral antigen in Western blotting and in ACP-ELISA (Table 1). Presumably, WAU-B10 is directed to a continuous epitope exposed on the surface of the viral coat protein subunit that is not accessible when the virus is intact.

Some McAbs proved their usefulness for *in situ* localization with immunogold labelling and silver enhancement of PLRV antigen in sections of infected leaf material. However, this staining could only be observed after production of the McAbs in ascitic fluids. The fetal calf serum in the cell culture medium inhibited the binding of the antibodies to the antigen in the immunogold labelling procedure.

Competitive binding assays. In the competitive binding assays, the biotinylated McAbs had to compete for binding to the antigen with unlabelled McAbs. Failure of unlabelled antibody to reduce binding of labelled antibody provides evidence that the antibodies bind to distinct determinants on the antigen (Yewdell & Gerhard, 1981). The effect of 10-fold diluted stock suspension of the unlabelled antibodies on the maximum biotin bound is presented (Figure 1). Three pairs of the McAbs reacting in both ELISA systems competed strongly with each other in their reciprocal tests, viz. WAU-A2 with WAU-A47, WAU-A5 with WAU-A13, and WAU-A6 with WAU-A7. Based on the reaction profiles in reciprocal tests with the unlabelled McAbs at lower concentrations, WAU-A47 could be distinguished from WAU-A2, and WAU-A5 from WAU-A13. WAU-A6 and WAU-A7 displayed almost identical competition profiles, indicating that they might react with the same epitope (van den Heuvel *et al.*, 1990).

The McAbs from the second group which reacted at high titres in DAS-ELISA but not in ACP-ELISA, did not compete with each other in the reciprocal tests. For this reason it is assumed that they are all directed against different epitopes. Within this group, a positive cooperative binding among the antibodies was consistently observed. Apparently, antibody binding results in conformational changes in the protein molecules which favour the binding of the biotinylated antibodies through increased accessibility or better fit.

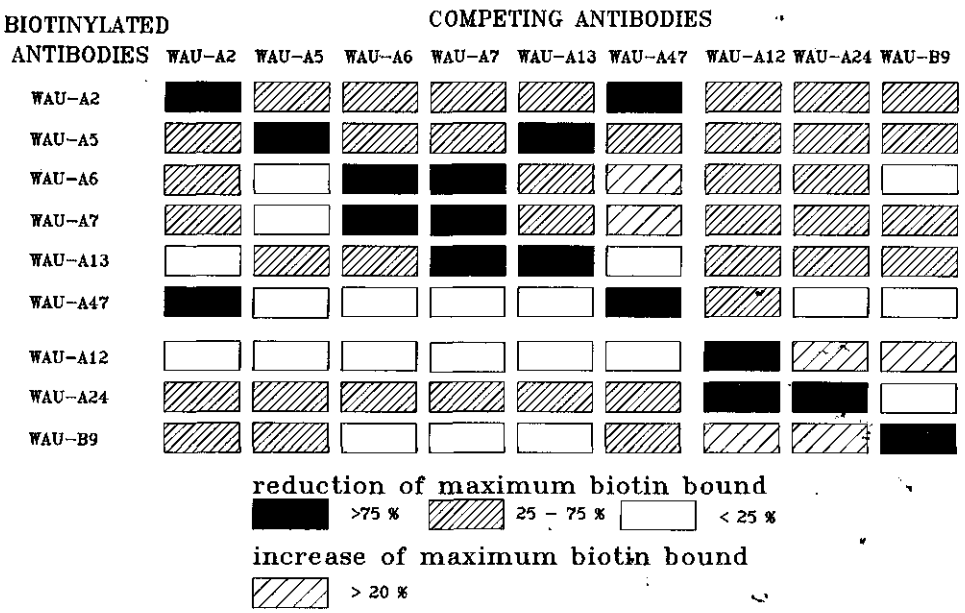

Figure 1. Effect on the maximum biotin bound in the competitive binding assay using the panel of monoclonal antibodies to PLRV.

When testing the McAbs of the two groups in reciprocal tests, it appeared that almost all unlabelled antibodies when present at high concentration inhibited the binding of the biotinylated antibodies of the other group for 25 - 75% (Figure 1). This may indicate that most of the epitopes recognized by the McAbs in the panel will partially overlap each other. WAU-B10 was not included in these experiments since it did not react in DAS-ELISA. In total, a minimum number of nine different epitopes (including WAU-B10) located on the PLRV coat protein was established using our panel of ten McAbs.

Reactivity of McAbs with luteoviruses. In tests with other luteoviruses and luteovirus strains, the antigen was caught onto the solid phase by their homologous polyclonal antisera. The McAbs were used as detecting antibodies. The McAbs WAU-A6 and -A7, and WAU-A2, -A47 and -B9 gave identical reaction patterns. All McAbs reacting with PLRV also did with TYTV; an observation which corroborates the classification of TYTV as a PLRV strain infecting tomatoes (e.g. D'Arcy *et al.*, 1989). WAU-A13

differentiated BMYV from BWYV. Since WAU-A5 and WAU-A24 were reactive to BLRV it can be concluded that PLRV and BLRV have epitopes in common, which has not been reported before.

The differentiation of McAbs, produced against luteoviruses, has so far been made solely in ELISA tests in which different virus isolates or strains were used (D'Arcy *et al.*, 1989; Massalski & Harrison, 1987). Following this approach, these authors distinguished five different reaction patterns and thus discriminated five epitopes on PLRV coat protein using panels of 27 and 10 McAbs, respectively. The competitive binding assays presented in this abstract indicated conclusively that differentiating McAbs on base of reactivity with other viruses results in an underestimation of the minimum number of epitopes present on the viral coat protein.

Functional characterization of the epitopes. Luteoviruses circulate through the bodies of their vectors, passing from the gut to the haemocoele and then into cells of the accessory salivary glands and saliva (Harrison & Robinson, 1988). Two hypothetical models for transcellular transport in the luteovirus group are suggested. The first one is that vector specificity is mainly determined by the ability of virus particles to pass from the haemocoele into 'coated pits' within salivary gland cells. Particles of non-transmitted isolates are found in the basal lamina of the salivary gland but apparently do not enter them (Gildow & Rochow, 1980). In the second model, vector specificity is determined by luteovirus transport through the hindgut into the haemocoele. Attachment of the virus particle to the apical plasmalemma of the epithelium could induce endocytosis of the virus into a coated pit. In both models, the vector-virus specificity is probably determined by attachment of the virus to the membranes by luteovirus-recognizing receptor molecules embedded in it (Adam *et al.*, 1979; Gildow, 1987), which suggests that one or more epitopes in the particle protein should play a key role in determining aphid transmissibility (Massalski & Harrison, 1987).

In ELISA we tested PLRV-Wageningen and TYTV as to their reactivity with the panel of McAbs. PLRV-Wageningen is readily transmitted by *M. persicae*, whereas TYTV is poorly transmitted by this aphid. It was shown that WAU-A5 and -A13 failed to detect TYTV, and that WAU-A6 and -A7 displayed reduced reactivity with TYTV as compared with PLRV-Wageningen.

From the competitive binding studies we learnt that WAU-A5 and -A13 are directed to strongly overlapping epitopes, and that WAU-A6 and -A7 are probably directed against the same epitope (Figure 1). The involvement of these epitopes in determining the aphid transmissibility of PLRV is now further investigated.

References

Adam, G., Sander, E., and Shepherd, R.J., 1979. Structural differences between pea enation mosaic virus strains affecting transmissibility by *Acyrthosiphon pisum* (Harris). *Virology* **92**, 1-14.

D'arcy, C.J., Torrance, L. & Martin, R.R. (1989). Discrimination among luteoviruses and their strains by monoclonal antibodies and identification of common epitopes. *Phytopathology* **79**, 869-873.

Gildow, F.E. (1987). Virus-membrane interactions involved in circulative transmission of luteoviruses by aphids. In: *Current topics in vector research*, Vol. **4**. 93-120. K.F. Harris, ed. Springer-Verlag New York Inc.

Gildow, F.E. & Rochow, W.F. (1980). Role of accessory salivary glands in aphid transmission of barley yellow dwarf virus. *Virology* **104**, 97-108.

Harrison, B.D. & Robinson, D.J. (1988). Molecular variation in vector-borne plant viruses: epidemiological significance. In: *The epidemiology and ecology of infectious disease agents*. p. 121-136. R.M. Anderson & J.M. Thresh, eds. London, The Royal Society, 1988.

Massalski, P.R. & Harrison, B.D. (1987). Properties of monoclonal antibodies to potato leafroll luteovirus and their use to distinguish virus isolates differing in aphid transmissibility. *J. Gen. Virol.* **68**, 1813-1821.

Schots, A. (1989). A serological approach to the identification of potato cyst nematodes. Ph.D. thesis, Wageningen Agricultural University, the Netherlands.

Van den Heuvel, J.F.J.M., De Blank, C.M., Goldbach, R.W. & Peters, D. (1990). Localization and characterization of epitopes present on potato leafroll virus coat protein. submitted.

Yewdell, J.W. & Gerhard, W. (1981). Antigenic characterization of viruses by monoclonal antibodies. *Ann. Rev. Microbiol.* **35**, 185-206.

Zrein, M. Burckard, J. & Van Regenmortel, M.H.V. (1986). Use of the biotin-avidin system for detecting a broad range of serologically related plant viruses by ELISA. *J. Virol. Methods* **13**, 121-128.

THE USE OF ANTIBODIES FOR RESIDUE ANALYSIS IN BIOLOGICAL SAMPLES.

A.R.M. Hamers & R.J.A. Paulussen
Department of Biopharmaceutical Analysis, State Institute for Quality Control of Agricultural Products (RIKILT), Bornsesteeg 45, 6708 PD Wageningen, The Netherlands

Introduction

The development of methods for residue analysis in agricultural products is one of the fields of interest of RIKILT. At the Department of Biopharmaceutical Analysis attention is focused on the detection of growth promoting agents in livestock production.

The composition of feeds is an important factor in optimization of the growth of farm animals. A number of substances are known to influence the feed conversion, often through effects on the intestinal bacterial flora. Furthermore, growth and carcass composition may be influenced by the use of anabolic substances: steroid hormones, repartitioning agents (β_2-agonists), thyreostatics and somatotropins. The use of the first group, often used as food additives, is permitted, provided that a withdrawal period is respected. EC (directive 86/469) and national regulations prohibit the use of the second group of compounds to improve growth of livestock (including the administration of naturally occurring substances belonging to this group). EC member states have to execute control programs (the National Residue Monitoring Programs), to detect the illegal use of these growth stimulants.

Control of the illegal use of growth promoters

As many of these substances already exert their pharmacological activity at relatively low concentrations, and are often readily metabolized, only very low concentrations of residues and metabolites (ppb to ppt levels) are available for analysis in edible tissues or in the more easily obtainable matrices that are mostly used for control purposes (urine, blood, faeces). Therefore, highly sensitive methods are needed to trace

illegal use of growth stimulators. Although these low detection limits can be achieved with several techniques, mostly GC-MS (gas chromatography in combination with mass spectrometry) methods are required, to assure adequate legal evidence of the use of forbidden growth promoters.

Especially during the last decade, the number of available substances with growth promoting properties has increased markedly. For many types of growth promoters, there is a collection of chemically closely related compounds, all with similar pharmacological effects. The highly sophisticated GC-MS procedures are only able to detect those substances to which they are optimally adjusted, which leads to a rising number of analyses to be performed, and consequently to disproportionally high control costs.

To ensure an efficient detection of illegal use of growth promoters, these costs have to be maintained at an acceptable level. This may be achieved by using a two-stage control procedure, consisting of a screening- and a confirmation-phase. In the latter, only those samples found positive in the screening are analyzed with the time and cost-expensive GC-MS procedures. The applied screening methods should be fast and reliable (no false-negative, and a minimal number of false-positive results), and be able to handle a large number of samples at a low price per analysis.

Biospecific interactions of molecules already find many diagnostic applications in modern medicine and biochemistry. Carrier proteins, receptors or antibodies are used in isolation and purification procedures, where they, covalently linked to a solid support, selectively adsorb substances from complex mixtures. The same molecules can be used in (quantitative) binding assays, that are well suited for the screening of large numbers of samples. The antibody-based immunoassays have already shown their applicability to routine diagnostics in clinical chemistry. Especially the developments of new, sensitive non-radioactive labelling techniques have opened the possibility of a more widespread use of these assays. Major advantages of immunoassays are the low detection limit, high sample capacity, short assay time, and low costs. Immunoassays may be directly performed on bloodserum or urine samples, mostly requiring no more pretreatment than a pH adjustment of the sample. However, the possibility of cross-reactions of the applied antibodies with other components of the sample infers a serious risk of false-positive results. Therefore, confirmation of positive samples by a separate technique is

always required.

Immunoassays are particularly suited for the determination of compounds that do not occur naturally in the sample. The same antibodies can, however, also be used in immuno-affinity chromatography. With this technique, antibodies are covalently immobilized on a column, and used to selectively retain the desired analyte(s) from a sample. The bound molecules are eluted and used for further analysis by e.g. HPLC, giving more detailed information. Detection limits can be decreased by increasing the applied sample volume. A further advantage is the possibility of a direct GC-MS confirmation on fractions collected from the HPLC column. In cooperation with the Free University of Amsterdam (Dept. Analytical Chemistry), we developed a fully automated system for the determination of the anabolic steroid 17β,19-nortestosterone (β-19-NT) in cattle urine by immuno-affinity chromatography coupled on-line to HPLC analysis with UV-detection (Farjam *et al.*, 1988). The hormone can also be assessed in bloodserum, bile and extracts of animal tissues with the same system (Haasnoot *et al.*, 1989). Immuno-affinity chromatography can also be used (off-line) as a simple and efficient sample clean-up procedure for GC-MS or other analyses.

Antibodies

The growth promoters mentioned above (except somatotropins), have molecular weights of just several hundreds of daltons. To elicit an immune response against such small molecules, they have to be conjugated to a high molecular mass carrier, e.g. bovine serum albumin (BSA). Often, a bridge is incorporated between the hapten and the protein to obtain more specific antibodies. The site of conjugation on the molecule is very important, with regard to the specificity of the antibodies.

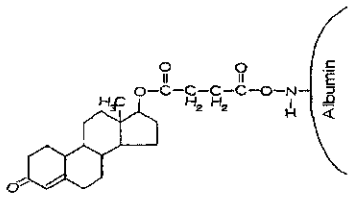

Figure 1. 19-nortestosterone, 17β-hemisuccinate-albumin

17β,19-Nortestosterone is an anabolic steroid, that occurs endogenously in male horses and pigs (Benoit *et al.*, 1985; Maghuin-Rogister *et al.*, 1988), but is not present in cattle, where it can be (illegally) used as growth modifier.

Table I. Schedule of automated analysis of urine samples.

Event	Valve positions 1	2	3
rinse affinity column with 15 ml water	A	A	A
rinse capillaries with sample	B	A	A
apply sample to affinity column	A	A	A
rinse capillaries with water	B	A	A
rinse affinity column with 15 ml water	A	A	A
rinse capillaries with water	B	A	A
rinse C_{18} precolumn with 5 ml water	B	A	B
rinse capillaries with desorbant (190 µg NG/l, 5% acetonitrile)	B	B	B
affinity column and C_{18} precolumn in series, elute bound analyte with 33 ml desorbant	A	B	B
transfer analyte from C_{18} precolumn to analytical column	A	B	A
rinse affinity column with 20 ml methanol-water (70:30)	A	A	A

NG = norgestrel
note: position A of each valve corresponds to the position in Fig. 3.

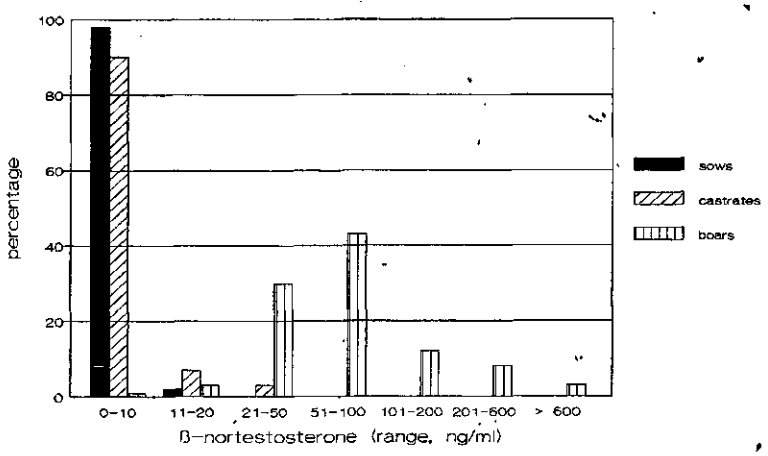

Figure 2. 17β,19-Nortestosterone concentration in pig urine samples determined by enzyme immunoassay. Bars represent the percentages of the total number of samples analyzed of each class.

We produced both polyclonal and monoclonal antibodies against 19-nortestosterone, conjugated to albumin through a hemisuccinate bridge at the 17-position (Fig. 1). To optimize the immune response, a conjugate with a high steroid/protein ratio was used (31 mol/mol). The obtained antibodies were applied in enzyme immunoassays and in immuno-affinity chromatography.

Immunoassays

A competitive enzyme immunoassay was developed for the quantitative determination of nortestosterone in urine, using a 19-nortestosterone-17β hemisuccinate-horseradish peroxidase conjugate as label. 96-Well microtitre plates were coated with anti-rabbit or -mouse Ig. The incubation mixture contained anti-nortestosterone, nortestosterone-peroxidase and standard or sample. O-phenylene diamine was used as substrate to assess bound peroxidase. The lower detection limit of the assay was 1 ng/ml (using 50 μl samples), directly in urine (adjusted to pH 7.5).

Cross-reactivities of the antibodies with related steroid hormones were determined at a 50% inhibition level (Table II).

The assay was used to determine the nortestosterone content of urine samples from cattle and pigs. With this assay the endogenous presence of 17β,19-nortestosterone in male pigs could clearly be established (Fig. 2) (Haasnoot *et al.*, manuscript in preparation).

Table II. Cross-reactions of anti-nortestosterone antibodies with various steroid hormones.

Steroid	antibody				
	14D12 14E6 19F7	21C11 23H2	20D1	20G8	poly-clonal
17ß,19-nortestosterone	100	100	100	100	100
17α,19-nortestosterone	25	54	8	86	46
norethindrone	15	65	7	65	36
norgestrel	5	12	4	74	21
17ß-trenbolone	5	11	11	13	6
5α-dihydrotestosterone	9	0.05	0.3	4	< 0.5
19-nor,4-androstene-3,17ß dione	174	25	1539	55	n.d.
19-nor,4-androstene-6ß ol-3,17 dione	5	1	30	3	n.d.
19-nor,4-androstene-15α ol-3,17 dione	4	1	30	41	n.d.
methyltestosterone	1	0.2	< 0.05	14	< 0.5
progesterone	34	0.1	< 0.05	11	< 0.5
17α-testosterone	1	0.1	< 0.05	8	n.d.
17ß-testosterone	0.6	< 0.05	< 0.05	1	< 0.5
17α-estradiol	< 0.05	< 0.05	< 0.05	< 0.05	< 0.5
17ß-estradiol	< 0.05	< 0.05	< 0.05	< 0.05	< 0.5
5α-androstane-3α,17ß diol	0.05	< 0.05	< 0.05	< 0.05	n.d.
estrone	0.4	< 0.05	< 0.05	< 0.05	n.d.
estriol	< 0.05	< 0.05	< 0.05	< 0.05	< 0.5
zeranol	< 0.05	< 0.05	< 0.05	< 0.05	< 0.5
ethinyl-estradiol	0.2	< 0.05	< 0.05	< 0.05	< 0.5
5α-estrane-3ß,17α diol	1	0.1	< 0.05	< 0.05	n.d.

Cross-reactions were determined at 50% inhibition of the binding of 17ß,19-nortestosterone-peroxidase. Values are means of 3 independent experiments, expressed as percentages.

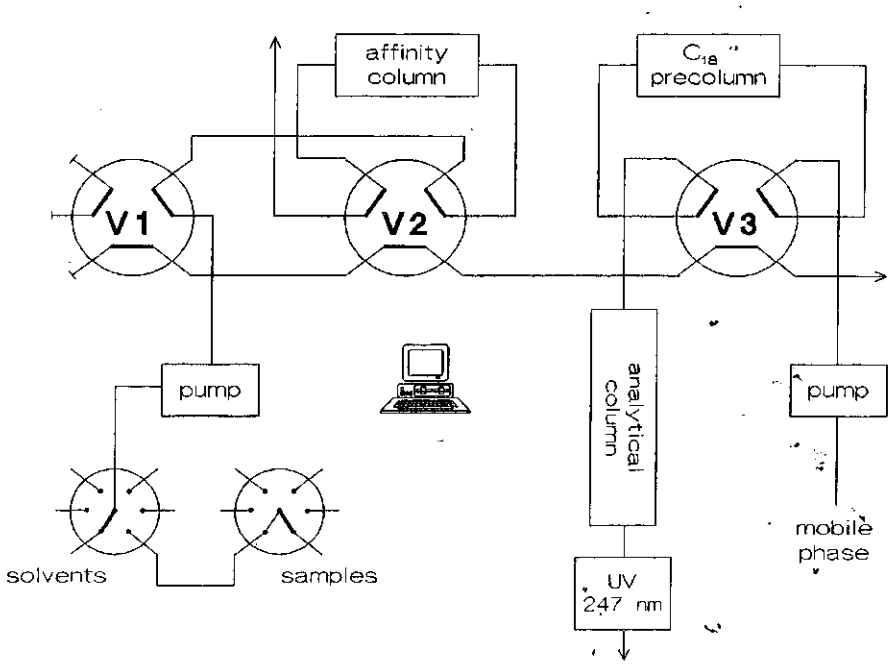

Figure 3. Design of the automated immuno-affinity HPLC system.

Affinity chromatography

A fully automated column switching system was developed that combines an immuno-affinity column (10 x 4 mm) for selective preconcentration of nortestosterone from urine samples, with a 5 μm C_{18} reversed-phase analytical HPLC column (100 x 3 mm).

The affinity column contains tresyl-Sepharose immobilized anti-nortestosterone IgG (approximately 8 mg/ml). Sepharose was chosen because of its low non-specific interactions with components of biological samples. To prevent damage of the Sepharose affinity column from pressure shocks from the analytical column, a precolumn with low back-pressure (40 μm C_{18}; 10 x 2 mm) was placed in between. The computer-controlled system (Fig. 3) comprises two HPLC pumps, three switching valves and two solvent select valves (of which the second is used as autosampler for sample volumes of more than 25 ml). The operating procedure of the system is summarized in Table I. Routinely, samples (extracts) are diluted with water and loaded onto the affinity column. Bound analytes are desorbed

with a competitive steroid (norgestrel, NG) in 5% acetonitrile, that can be well separated from nortestosterone on the analytical column (Fig. 4). Prior to analysis, glucuronide and sulphate conjugates of the steroids are enzymatically hydrolysed. Nortestosterone can efficiently be determined in urine with UV detection at 247 nm (Fig. 4), with a lower detection limit of 0.05 μg/l (with 26.5 ml urine samples). The columns proved to be very stable, (at least) several hundreds of samples can be analyzed.

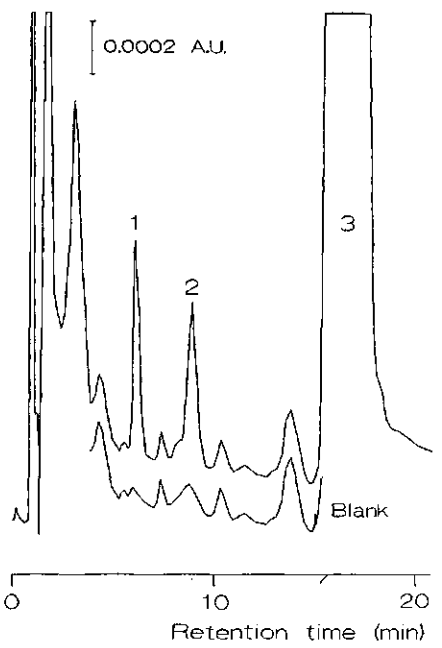

Figure 4. Immuno-affinity HPLC analysis of a calf urine sample spiked with 300 ng/l each of 17α and 17β,19-nortestosterone, with corresponding blank. 26.5 ml urine samples were analyzed. 1: 17β-NT; 2: 17α-NT; 3: NG

The nortestosterone content was determined of urine samples from calves treated with 19-nortestosterone,17β-laurate (intramuscular). The maximum concentration of β-19-NT was found 7 days after administration (1.3 μg/l).

The concentration of the main metabolite in cattle, 17α,19-nortestosterone(α-19-NT), was higher in all samples (maximum 3.1 μg/l). Bile samples of the same animals contained only α-19-NT (55 μg/l), in muscle only the β-form was detectable (0.1-1.6 μg/kg), and in liver low concentrations of both isomers were observed (0.5-0.9 μg α-19-NT, and 0.05-0.1 μg β-19-NT per kg) (Haasnoot *et al.*, 1989).

Recently, the same system was applied to the analysis of the β_2-agonist clenbuterol, with an affinity column containing polyclonal antibodies against clenbuterol. Only minor changes had to be made to obtain a functioning system (Haasnoot *et al.*, 1990).

Monoclonal antibodies

To ensure a continuous availability of defined antibodies against 19-nortestosterone, a conjugate of the 17-hemisuccinate derivative of the steroid with rabbit serum albumin was used to produce monoclonal antibodies. A nortestosterone-BSA conjugate was used as antigen to screen the obtained clones after fusion of the mouse spleen cells and SP 2/0 myeloma cells. A parallel screening was carried out with addition of an excess free β-19-NT. Finally, 9 cell lines were obtained that produced the desired antibodies. With large scale in vitro culture in spinner-flasks, 7 out of 9 lines abundantly produced immunoglobulins. The cross-reactivities with a group of related hormones of these antibodies were determined with enzyme immunoassays set up as described above. The obtained data (Table II) showed that 4 different antibody types could be defined. Experimental work is now in action to assess the properties of these 4 lines in immuno-affinity chromatography.

Acknowledgements

The investigations described here were carried out in cooperation with Gerard van Bruchem, Monique Ploum, Christine van Boxtel, Willem Haasnoot and Robert Schilt.

References

Benoit, E., Garnier, F., Courtotand, D. & Delatour, P. (1985). Ann.Rech.Vét. **16**, 379-383

Farjam, A., De Jong, G.J., Frei R.W., Brinkman U.A.Th, Haasnoot W, Hamers, A.R.M., Schilt, R. & Huf, F.A. (1988). *J.Chromatogr.* **452**, 419-433

Haasnoot, W., Schilt, R., Hamers, A.R.M., Huf, F.A., Farjam, A., Frei, R.W. & Brinkman, U.A.Th. (1989). *J.Chromatogr.* **489**, 157-171

Haasnoot, W., Schilt, R., Van Bruchem, G.D., & Paulussen, R.J.A., manuscript in preparation.

Haasnoot, W., Ploum, M.E., Paulussen, R.J.A., Schilt, R. & Huf, F.A. (1990). submitted for publication in *J.Chromatogr.*

Maghuin-Rogister, G., Bosseloir, A., Gaspar P., Pasnois, C. & Pelzer, G. (1988). *Ann.Rech.Vét.* **132**, 437-440

NOVEL RANGE OF PEPTIDE MESSENGERS IN INSECT NERVOUS SYSTEM DETECTED BY MONOCLONAL ANTIBODIES

H. Schooneveld & H.M. Smid
Department of Entomology, Agricultural University, Wageningen, the Netherlands

Introduction: The use of monoclonal antibodies

The Colorado potato beetle, *Leptinotarsa decemlineata* (Say), has been studied in our laboratory as a physiological model for over 30 years. The insect is not causing major problems in Dutch agriculture because it lives here at its northernmost distribution area in Europe. In Eastern Europe and in the Northern America's, however, the beetle is representing an increasingly important pest due to its acquired resistance to common insecticides. Possibilities for alternative biological control strategies are limited (Hare, 1990) and the possibilities for hormonal control by substances with insect hormone or antihormone action are limited as well (Schooneveld *et al.*, 1979). It seems that novel control strategies, for instance those based on hormonally disturbed life functions, are badly wanted. Our research is currently focussed on insect neuropeptides, because peptide hormones are candidates for future pest control chemicals (Keeley and Hayes, 1987).

We are still in the first phase and developed and utilized monoclonal antibodies ("Mab's") for making an inventory of neuropeptides in the central nervous system and the associated neuroendocrine system (CNS/NES). We describe here the route we followed to generate and evaluate the Mabs and to exploit them for the detection and characterization of novel neuropeptides by immunohistochemical means. Part of the work has been published earlier (Schooneveld and Smid 1989a,b; Schooneveld *et al.*, 1989; Schooneveld, 1990) . We hope and expect that the principles developed here will be useful for other insect species as well.

Approach

a. Raising Mab's against unknown peptide antigens. Our problem was that research on insect neuropeptides is still in its infancy and relatively few peptides (approximately 30 at this time) have so far been characterized (Holman *et al.*, 1990). No peptides from *L. decemlineata* have been chemically identified although immunohistochemical methods employing polyclonal antibodies against a variety of neuropeptides of vertebrate and invertebrate origin clearly indicated that the CNS of this species contains a wide variety of peptides in different locations (Veenstra *et al.*, 1985).

Instead of extracting peptides in sufficient quantities to raise polyclonal antibodies, we followed the suggestion of Denburg *et al.*, (1986) to produce monoclonal antibodies against antigens in crude homogenates of the CNS. The idea is based on the finding that certain peptide antigens seem to be very immunogenic in mouse if the crude homogenate is properly prepared. In other words, the mouse is able to recognize relatively scarce antigens in a mixture and reacts by producing antibodies. It is the experimenter's task to identify and isolate the hybridomas producing these Mab's and put the Mab's to work for specific immunohistochemical and chemical purposes.

We found that the following factors influenced the frequency and quality of Mab's obtained (Table 1):

- Physiological state of the beetles from which antigens are extracted: Age and nutritional state of the adult beetle influenced the degree of accumulation of secretory peptides in certain regions of the CNS/NES, as followed from pilot experiments (Schooneveld *et al.* 1989). Animals were therefore starved for a few days before being sacrificed at an age of 2 weeks.

- Preparation of antigens: Homogenates were prepared in different ways, such as to influence the content of large molecules versus the low-MW peptides in the sample and couples of mice were immunized with each of the 4 different samples made. The variation in procedures included: No, low, or high-speed centrifugation of the original samples; the extraction in methanol to eliminate high MW proteins; the amount of fresh starting tissue; the addition of glutaraldehyde to favour the cross-linking of peptides to enhance their immunogenicity; and the number of booster injections.

Table 1. Summary of Mab's obtained after immunizing mice with antigens processed according to 4 different protocols. Classes of immunoglobulin are indicated. Antibodies are referred to as 'MAC': monoclonals anti-Colorado potato beetle.

Mab	Ig subclass	Staining characteristics[1]
MAC-1	nd	CA cells and sediment from the blood
MAC-2	IgG1	Peptidergic and some other types of neurons in most parts of the nervous system
MAC-3	IgM	'FMRFamide-neurons' in C-type NSC, OL and SOG
MAC-4	nd	'FMRFamide-neurons' in SOG and FG
MAC-5	nd	Type of grouped interneurones in PC, TC, and SOG
MAC-6	nd	CA cells, oenocytes and specific neuroglia in DC neuropil
MAC-7	IgM	Medial NSC, SOG neurons, ESC, FG, bipolar minineurons in SOG
MAC-8	IgM	Lateral and medial NSC, ESC
MAC-9	IgM	As MAC-8; in addition ISC, FG and SOG neurons
MAC-10	nd	Motorneurons in SOG and FG; sediment in CC
MAC-11	IgM	SOG neurons and FG, weak medial NSC
MAC-12	IgM	ESC, FG, SOG, lateral NSC
MAC-13	IgM	A_1-type NSC in PI, 2+2 neurons in ventral PI, 10 in TC, 12 in OL, FG, SOG. In addition: cells of defence glands, male accessory glands, and epidermal cells in penis.
MAC-14	IgM	SOG: 4+6 neurons
MAC-15	nd	Medial NSC, unusual groups (4+4) in SOG
MAC-16	IgG1	ISC in CC and 4 neurons on OL
MAC-18	nd	Medial NSC (B?), lateral NSC, ESC, CA, SOG, FG (?). In addition: endocrine cells in male ejaculatory duct, chitinous structures in abdomen.

[1] *Abbreviations*: DC, deutocerebrum; CA, corpus allatum; CC, corpus cardiacum; EC, endocrine cells; ESC, extrinsic glandular cells in the CC; FG, frontal ganglion; ISC, intrinsic secretory cells in the CC; nd, not determined; NSC, neurosecretory cells; OL, optic lobe; PI, pars intercerebralis; SOG, suboesophageal ganglion; TC, tritocerebrum.

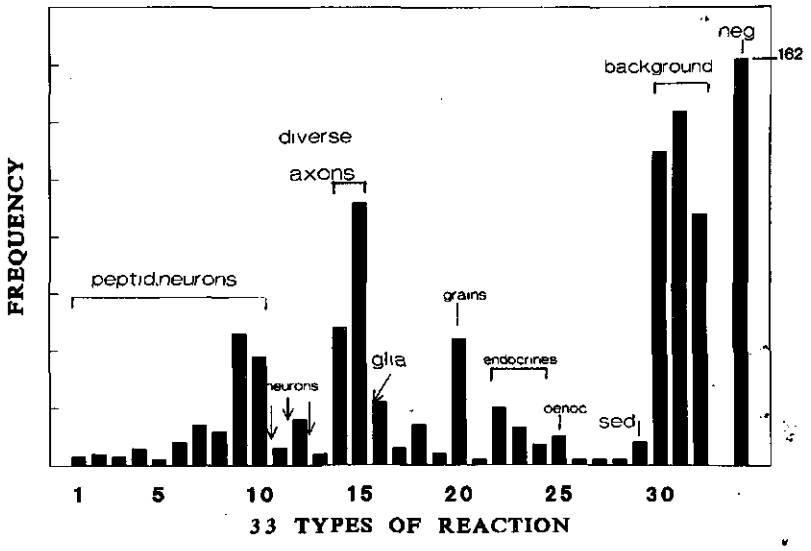

Figure 1. Frequency-distribution of hybridoma's producing Mab's which recognize one of 33 types of reactions observed during the immunization experiment with crudest immunogen preparation (n=665 tests). Major types of reactions are given over groups of columns.

The conclusion of these experiments was that the proportion of Mab's recovered after each procedure did not greatly influence the "rendement" of searching. Crude homogenates produce much immunological "noise" in the form of Mab's irrelevant for the present purposes (Fig. 1). Purified and conjugated immunogens produced a larger proportion of peptide-specific Mab's, but in a rather low yield.

b. Testing of hybridomas. The screening of hybridomas was done on paraffin serial sections of the entire CNS/NES in the head. We did not know where neuropeptides were located and every section of the thousands of slides had to be inspected for the binding of primary antibody. This binding could be made visible by the generation of a dark brown colour due to the oxidation of a reagent (DAB) by the enzyme horseradish peroxidase that was conjugated to a secondary antibody directed against mouse immunoglobulins that recognized the first mouse antibody.
Many different microscopically visible structures in the head were labelled in the course of the experiments but our interest was raised

only if specific neurons were labelled after the incubation with supernatant of primary hybridomas, namely the potentially peptidergic types of neuron. Such neurons are specialized in the production of neuropeptide for communication with other neurons or with target organs outside the CNS/NES. Peptidergic neurons are usually larger (but not always) than "ordinary" neurons and some of them have long axons through which the cell product (often peptide hormones) are transported to neurohaemal organs for the release of product into the blood. "Positive" hybridomas were subcloned immediately, that is within one day after the initiation of the test.

For a rapid screening of large numbers of hybridomas or subclones, we applied successfully the technique of Verhaert *et al*, (1986). This technique consisted of the testing of supernatants on single sections through the neurohaemal organs (corpora cardiaca -CC) in which several of the peptidergic neurons have their axon terminals. The sections are called "bulk sections" and because they are glued to the bottom of a certain brand of ELISA plates, they can be processed immunohistochemically with high efficiency. Testing of a few of these bulk sections for each supernatant gives most of the information needed to decide whether hybridomas are worth being kept or rejected. A speedy testing of large numbers of samples takes a fraction of the time needed for complete serial sections but a small loss of information (neurons lacking axons in the CC are not detected) should be accepted. Both methods of testing are applied in case promising hybridomas are to be evaluated in more detail.

c. *Types of Mab's obtained*. The immunization with less-purified antigens induced Mab's that showed a wide range of affinities although only a minority of all hybridomas produced antibody (Fig. 1). Most antibodies in this series bound to various tissues in the slides without any degree of specificity (columns 30-32 in Fig.1). Other Mab's showed affinity for various tissues and altogether 29 types of reaction were scored. Of interest to us were those Mab's which had a high affinity for peptidergic neurons (columns 1-10) or other compartments of the endocrine system such as the intrinsic glandular cells of the CC or the glandular cells of the corpus allatum (columns 22-24). Several subtypes of peptidergic neurons in brain, SOG and other ventral ganglia were stained and some Mab's had affinity for neurons in the stomatogastric nervous system.

The immunization with the more purified antigens gave partly similar and partly new Mab's. Several of them revealed neurons in the ventral nerve chain and one even reacted with what we call 'peripheral' neurons, i.e. neurons attached to nerves emanating from the thoracic ganglia. Other Mab's revealed immunoreactive cells in midgut and epithelium of the male accessory sex gland.

Twelve of the hybridomas withstood repeated subculturing and produced useful amounts of Mab's with separate and specific affinity for secretions in different subsets of neurons.

d. Types of putative peptidergic neurons distinguished. We recognized 6 different classes of peptidergic neurons; their locations are given in Fig. 2 and 3:

1. Most conspicuous are neurons in brain and suboesophageal ganglion that are specialized in the synthesis of large amounts of neuropeptides and release of peptides into the blood via the corpora cardiaca (CC). Several clusters of such neurons were detected, some had not seen before by polyclonal antisera in earlier screening programs (Veenstra *et al.* 1985). For details, see Table 1. The products of these neurons have hormonal functions.

2. Other neurons in the CNS did not seem to release their product into the blood. Their axon branchings remain within the CNS and may have a synaptic function.

3. Neurons in frontal and thoracic ganglia innervate target organs via a direct nervous link. Targets are, for instance, muscles of several organs.

4. Large dispersed ("diffuse") neurons are located on major nerves in the ventral region and have elaborately branched processes for the release of cell product.

5. Intrinsic secretory cells of the CC produce the adipokinetic hormone.

6. Both the midgut and the male accessory reproductive gland have epithelia containing dispersed slender peptidergic cells with presumed local hormone function.

Several of the antigens recognized by our panel of Mab's are localized in more than one type of neuron. On the other hand, we found in a few instances that antigens reacting with different Mab's may be colocalized in the same neurons in brain, thoracic and frontal ganglia. Electron microscope work is in progress that shows that antigens are even coloca-

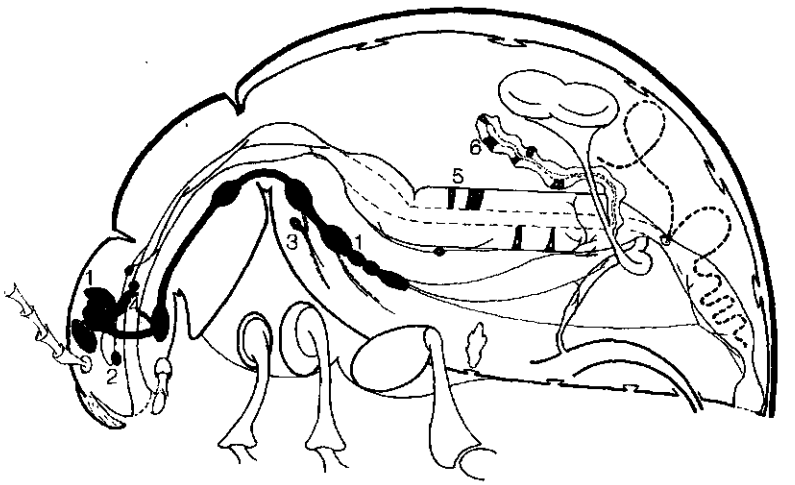

Figure 2. Diagram of Colorado potato beetle showing in black the major parts of the nervous system and the other major locations of peptidergic neurons revealed by Mab's. 1, central nervous system; 2, visceral nervous system; 3, peripheral neurons; 4, peptidergic endocrine gland cells in corpus cardiacum; 5, endocrine cells in midgut; 6, endocrine cells in male accessory gland.

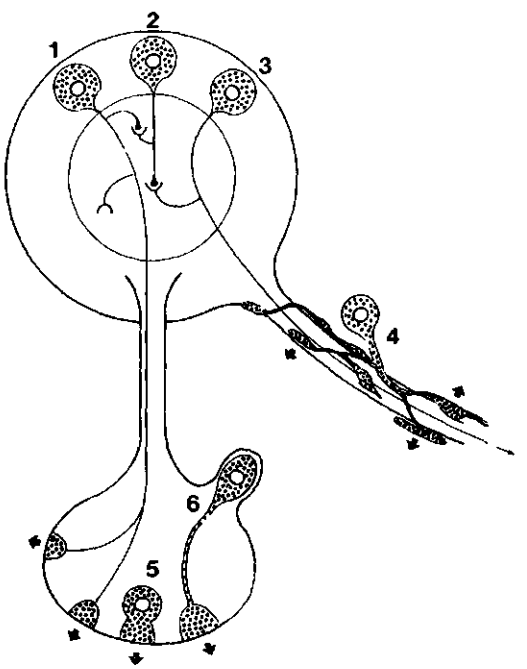

Figure 3. Diagram of ganglion (top), neurohaemal organ (bottom), and afferent nerve (right) showing classes of peptidergic neurons with secretory materials revealed by Mab's, and forms of association of such neurons with the nervous system. Neurons 1 through 4 represent neurosecretory -, inter -, motor -, and peripheral neurons, respectively. Cell 5 is an intrinsic glandular cell; cell 6, an extrinsic secretory cell of the corpus cardiacum. Thick arrows indicate zones where release of secretions into the blood takes place. Thin arrow indicates course of immunoreactive axon of motorneuron to remote target organ.

lized in the same secretory peptide granules (Schooneveld *et al.*, in preparation).

Discussion

We have established a number of hybridoma cell lines which stably produce Mab's recognizing antigens in a variety of neurons in different compartments of the CNS/NES of *Leptinotarsa decemlineata*. We have to present a few considerations before speculating on how the Mab's can be used for experimental purposes. Firstly, we realize that we only collected Mab's resulting from a restricted immunization plan. Continued immunizations with homogenates of different origin or made from animals in different physiological condition might have increased the number of useful antibodies. Secondly, we may have missed a number of potentially interesting Mab's by not testing supernatants at all tissues of interest to us. There may also be the integrity of tissues providing the antigens and the integrity of tissue in the microscopic slides on which Mab's were assayed. Homogenates may or may not be made with linker molecules, such as glutaraldehyde, which modifies the 3-dimensional structures of the antigen molecule. On the other hand, such antigens may have undergone a very different denaturation process in the course of tissue processing for immunohistochemistry. It quite possible that there is a certain mismatch between antigen and antibody, leading to the situation that Mab's are discarded that may have been recognized as useful if other assays like RIA or ELISA were employed. It seems desirable, for that reason, that immunogen preparation and antigen preservation should be conducted as consistent as possible.

Can we put our Mab's to work for pest control purposes? The answer is: not yet. We have so far only identified substances in neurons, probably neuropeptides, of potential interest and we have seen a perhaps small proportion of the wealth of different peptides present in the CNS/NES of the Colorado potato beetle. We have to establish by biochemical means that those substances really are peptides rather than representing uninteresting cell constituents or peptide precursors. Immunohistochemistry with immunogold on the electron microscope level demonstrated that we are looking at the content of secretion granules (Schooneveld *et al.* 1989). There is a fair chance, therefore that our Mab's indicate the presence of secretory peptides.

As mentioned, it is important that we determine the chemical nature of these peptides and the physiological processes controlled by them. We plan to direct our research on these two questions and we will adopt the following strategy:

1. Purification of the immunoreactive products by HPLC and PAGE whereby the separated peptide is traced and spotted with immunoblotting and -dot techniques.
2. Affinity chromatographic purification of peptide fractions with immobilized antibodies.
3. Developing bioassay systems for monitoring hormone action.
4. Use of molecular biology techniques, implicating the detection of peptides as gene products in the screening programs for in vitro RNA translation systems.

This basic approach is necessary before peptides can be identified that may be candidates for some form of 4th-generation biological control. It would be necessary also to find out what aspect of insect life is most efficiently deranged to achieve effective insect control. We must also know whether this effect can be achieved through the experimental application of peptides or peptide analogues, or through an inhibition of normal peptide functions by deregulating peptide synthesis, breakdown, or action on target organs. Most challenging, however, is the problem how to get the peptide into the body of the pest insect. In due time the decision should be made whether we will work with the actual peptide compounds or by the introduction of genes coding for peptides through biotechnological procedures. We need much more basic knowledge before such applications can be planned for practical insect control. We have made only the first steps.

Acknowledgement

We are indebted to our collegues at the Laboratory for Monoclonal Antibodies in Wageningen for use of their facilities, their expert advice and help in several phases of the work.

References

Denburg J.L., R.T. Caldwell, & J.A.M. Marner (1986). Monoclonal antibodies to the cockroach nervous system. *J. Comp. Neurol.* **245**, 123-136.

Hare J.D. (1990). Ecology and management of the Colorado potato beetle. *Ann.Rev. Entomol.* **35**, 81-100.

Holman G.M., Nachman, R.J., & Wright M.S., (1990). Insect neuropeptides. *Ann. Rev. Entomol.* **35**, 201-217.

Keeley L.L., & Hayes T.K. (1987). Speculations on biotechnology applications for insect neuroendocrine research. *Insect Biochem.* **17**, 639-651.

Schooneveld H. (1990) Analysis of neural and neuroendocrine centers in the Colorado potato beetle with monoclonal antibodies. *Proc. Exp. Appl. Entomol.* **1**, 131-136.

Schooneveld H., Kramer, S.J., Privee, H.M., & van Huis A. (1979). Evidence of controlled corpus allatum activity in the adult Colorado potato beetle. *J. Insect Physiol.* **25**, 449-453.

Schooneveld H., Smid, H.M., Boonekamp, P.M., & van Haeften T. (1989). Peptidergic neurons in the Colorado potato beetle, *Leptinotarsa decemlineata* (Say),identified immunocytochemically by monoclonal antibodies against homogenates of neuroendocrine tissue. *Cell Tissue Res.* **257**, 29-39.

Schooneveld H., & Smid H.M. (1989a). Monoclonal antibodies obtained through brain homogenates: Tools for cell-specific neuroanatomical studies in the Colorado potato beetle. *Entomol. Exp. Appl.* **257**, 283-296.

Schooneveld H., & Smid H.M. (1989b). Monoclonal antibodies for neuroanatomical purposes: Antigens in brain homogenates of the Colorado potato beetle. *Med. Fac. Landbouwwet. Gent* **54**, 753-758.

Veenstra J., Romberg-Privee, H.M., Schooneveld, H., & Polak J.M. (1985). Immunocytochemical localization of peptidergic neurons and neurosecretory cells in the neuroendocrine system of the Colorado potato beetle with antisera to vertebrate regulatory peptides. *Histochem.* **82**, 9-18.

Verhaert P., de Loof, A., Huybrechts, R., Delang, I., Theunis, W., Clottens, F., Schoofs, L., Swinnen, K., & Vandesande F. (1986). A new alternative for simultaneous immunohistochemical screening of 96 hybridoma clones for tissue-specific antibody production selects a monoclonal antibody to insect corpus cardiacum. *J. Neurosci. Meth.* **17**, 261-268.

ANALYSIS OF THE IMMUNE SYSTEM OF FISH WITH MONOCLONAL ANTIBODIES.

J.H.W.M. Rombout, E. Egberts, N. Taverne & J.C.E. Van Diepen.
Department of Experimental Animal Morphology and Cell Biology, Agricultural University, P.O. Box 338, 6700 AH Wageningen, The Netherlands.

Introduction

In all vertebrates investigated so far, white blood cells (WBC), particularly macrophages and lymphoid cells, are a prerequisite for a proper functioning of the immune system. In contrast to higher vertebrates, however, the WBC of fish are not well characterized. Although fish leucocytes can be differentiated in lymphocytes, plasma cells, monocytes, macrophages and several types of granulocytes, based on their ultrastructural resemblance to mammalian WBC, at present most of these cell types cannot be unambiguously identified, using light microscopic or flowcytometric techniques. Therefore, the characterization of WBC of fish would benefit very much from the availability of monoclonal antibodies (MAbs) recognizing cell-specific membrane antigens or products.

This contribution describes some attempts to produce MAbs against carp immunoglobulin (Ig), generally accepted as a B lymphocyte marker, and against T lymphocytes. In higher vertebrates several classes of Ig produced by different B cell types can be distinguished: for instance monomeric IgG, dimeric IgA and pentameric IgM. So far, only one Ig-class has been identified in teleost fish, i.e., a tetrameric IgM-like molecule, although there is some circumstancial evidence for the presence of a special mucosal Ig class in teleosts (Rombout *et al.* 1989). This paper also deals with the development of MAbs specifically reacting with mucus Ig.

Production of Ig-specific MAbs.

Mice were hyperimmunized intraperitoneally with a preparation of

partially purified serum Ig of carp, obtained after $(NH_4)_2SO_4$-fractionation followed by a size-exclusion chromatography step. After fusion of mouse splenocytes with myeloma cells, clones producing Ig-immunoreactive MAbs were selected using the ELISA technique, with serum Ig coated to the bottom of the microtiter plate wells. This procedure resulted in 18 MAbs (WCIs) showing a strong immunoreaction with serum Ig (Secombes *et al.*, 1983). However, three of these MAbs were also shown to exhibit a positive reaction with carp thymocytes (precursor T cells), although according to the current concepts of the vertebrate immune system these cells do not possess or produce Ig. Western blotting after SDS-PAGE of whole serum or its partially purified Ig component, showed that all WCIs react with the 75 kD heavy (H) chain of Ig (Fig. 1), but that the thymocyte-positive MAbs stain some additional protein bands. Thus, most WCIs seem to be a suitable reagent for the detection of serum Ig or Ig-bearing B cells (including Ig-secreting plasma cells). Because of its high affinity for Ig (Schots *et al.*, 1988) WCI 12 is at present used routinely in various assays, on frozen tissue or on cell suspensions. In this way, the relative numbers of B cells in blood and different lymphoid organs have been estimated by flow cytometry. In addition Ig-bearing cells are being characterized at the ultrastructural level by using an immunogold method on living cells followed by the preparation for electron-microscopy. In this analysis, Ig molecules appear to be present in clusters at the surface of B lymphocytes (Fig. 2), and most plasma cells.

Production of mucus Ig-specific MAbs

As already mentioned, some of our experimental data are indicative of a specific mucosal immune response in fish, suggesting implicitly the presence of a distinct Ig molecule.

However, mucus as well as serum Ig seem to incorporate H and L (light) chains of similar molecular weights (75 kD and 25 kD, respectively) and the forementioned WCIs also react with H chains of both Ig populations (Fig. 1). In order to obtain reagents which might discriminate between the two carp Ig preparations, we hyperimmunized mice with partially purified Ig from skin mucus. MAbs reacting in a dotblot assay with mucus Ig, but not with serum Ig, were selected for further investigation. This procedure resulted in 20 MAbs that were tested again on Western blots of serum and mucus Ig. From these results the following classification was

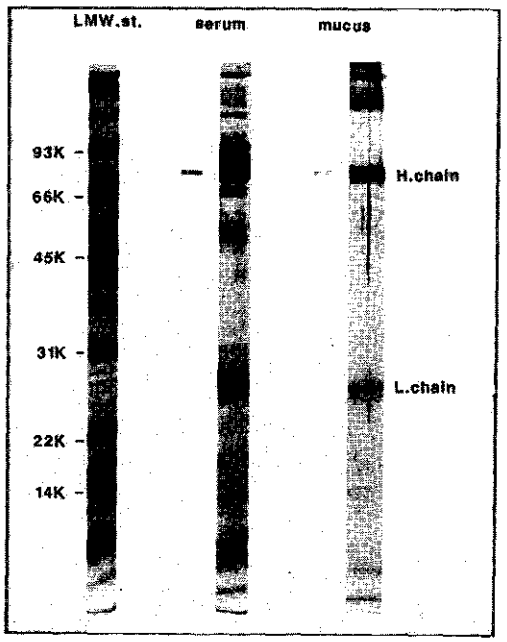

Figure 1. SDS-PAGE electrophoresis and subsequent Western blotting of purified samples of serum and mucus Ig. H and L chains of both Igs are shown to have similar molecular weights and WCI 12 react with H chains of both Igs.

derived:

1. MAbs still reactive with H chains of both Igs.
2. MAbs reactive with the H chain of mucus Ig only.
3. MAbs staining a broad diffuse 75 kD band and cross-reactive with the secretory component (75 kD!) of human IgA.
4. MAbs reactive with other as yet unidentified molecules of around 90 and 110 kD present in our mucus Ig-preparations.

Although these results are still preliminary it can be concluded that mucus Ig appears to differ at least in part from the Ig present in serum. In addition, mucus Ig might also contain a protective secretory component, like mammalian IgA.

Production of T lymphocyte-specific MAbs.

Because not all Ig$^-$ lymphocytes in fish are expected to be T-like lymphocytes, the production of MAbs specifically reacting with T cells and/or their subpopulations would be of great value. So far, two attempts have been undertaken, and will be described in chronological order.

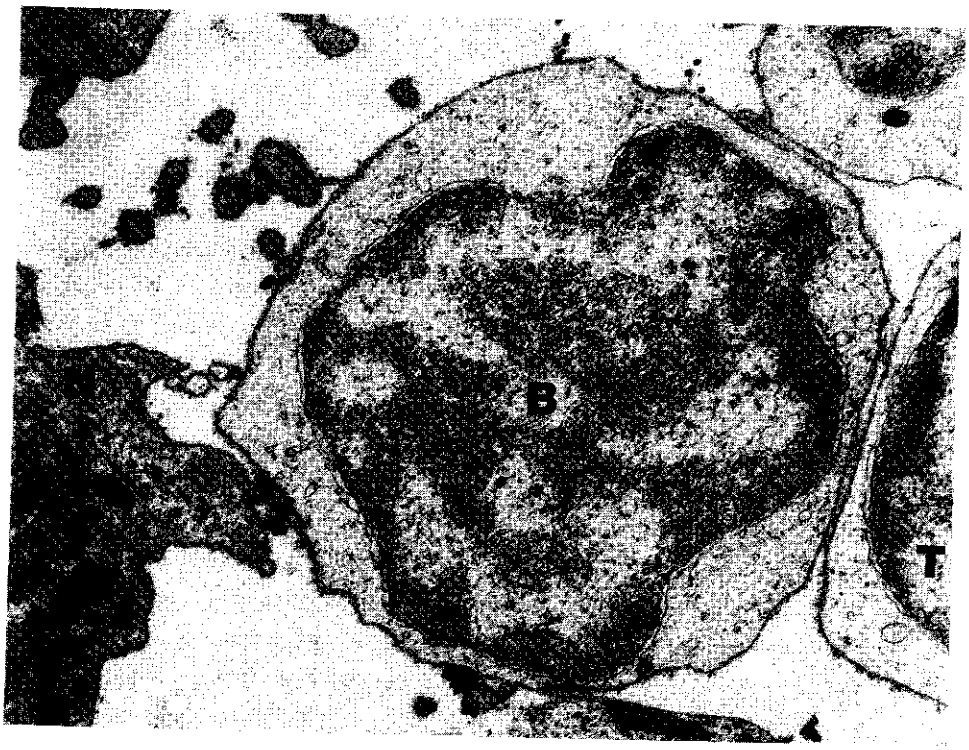

Figure 2. Electronmicrograph of WCI 12- and WCT 23-double- stained leucocytes. Gold particles of 40 nm represent WCI 12-labelled Ig molecules present in clusters at a B cell plasma membrane and gold particles of 10 nm represent WCT 23-labelled antigens at the surface of a B and a T-like cell and on a neutrophilic granulocyte (N). X 25,000.

A. Mice have been immunized with carp thymocytes, presumed to be a source of T-like cells and their precursors, and MAb-producing clones have been selected by means of an ELISA, in which thymocytes are coated to the wells. This attempt revealed 27 MAbs positively reacting with thymocytes, but most of them were reactive with serum Ig as well (Secombes *et al.*, 1983). Recently Western blot results have shown that these MAbs, designated WCT, are reactive with the H chain of Ig. However, most of the WCT MAbs also stain one or more additional bands in Western blots of serum proteins or total lymphocyte lysates. When these MAbs were applied for electron microscopy using an immunogold method, they appeared to react with all WBC (Fig. 2), but not with red blood cells. Apparently,

thymocytes seem to have antigens that share common determinants with antigens present on Ig molecules and on the surface of all WBC. It can therefore be concluded that this approach did not result in the isolation of T cell-specific MAbs.

B. In a second approach, mice have been immunized with Ig^- lymphoid cells isolated from head kidney and freed from Ig^+ lymphocytes by sorting on a FACS. After fusion, clones have been selected for their capacity to stain only part of the lymphoid cells, using a modified cytocentrifuge method that allowed us to distinguish different leucocyte cell types. Like is the case for WCTs, most of the clones isolated produce MAbs reacting with all WBC. However, four MAbs could be selected reacting with only a part of the lymphoid cells and in addition a part of the leucocytes (probably neutrophils) was stained. Similar attempts to produce T cell-specific MAbs for catfish have resulted in antibodies with a comparable immunoreactivity, i.e., positive for T cells and neutrophils (Miller *et al.* 1987; Ainsworth, 1990). However, recent FACS-results have shown that the MAbs obtained in this study were not reactive with membrane antigens of neutrophils and only react with a part of the Ig^- lymphoid cells. Whether all T-like cells or only a subpopulation of these cells are recognized by these MAbs remains to be investigated. Despite these results it can be concluded that the production of MAbs specifically reacting with fish T cells is much more difficult compared to the production of such MAbs for mammals. One explanation for these difficulties might be that the molecules responsible for distinct immune functions are less evolved in fish, and still have some common determinants which seem to be more immunogenic to higher vertebrate species than the more specific determinants. Different immunisation procedures might be needed to increase the chance for T cell-specific MAbs. One possible approach can be the induction of tolerance in mice for the common determinant(s) by injection of very high dose of carp Ig, before immunization with T cells. Another possibility may be to block these determinants by means of treatment of leucocyte cell suspensions with WCT MAbs prior to their use for immunization.

References

Ainsworth A.J. (1990) Characterization of monoclonal antibodies to channel catfish, *Ictalurus punctatus*, leucocytes. Vet Immunol. Immunopathol., in press.

Diepen J.C.E., Wagenaar G.T.M. & Rombout J.H.W.M. (1990) Immunocytochemical detection of membrane antigens at carp leucocytes using light and electron microscopy. J. Fish Biol., submitted.

Miller N.W., Bly J.E., Ginkel F. van, Ellsaesser C.F., & Clem L.W.(1987) Phylogeny of lymphocyte heterogeneity: identification and separation of functionally distinct subpopulations of channel catfish lymphocytes with monoclonal antibodies. Dev. Comp. Immunol. 11: 739-747.

Rombout J.H.W.M., Berg A.A. van den, Berg C.T.G.A. van den, Witte P., & Egberts E. (1989) Immunological importance of the second gut segment of carp. III. Systemic and/or mucosal immune responses after immunization with soluble and particulate antigen. J. Fish Biol. 35: 179-186.

Schots A., Leede B.J. van der, DeJongh E., & Egberts E. (1988) A method for the determination of antibody affinity using a direct ELISA. J. Immunol. Methods 109: 225-233.

Secombes C.J., Van Groningen J.J.M., & Egberts E. (1983) Separation of lymphocyte subpopulations in carp, *Cyprinus carpio* L., by monoclonal antibodies: immunohistochemical studies. Immunology 48: 165-175.

PROSPECTS FOR MONOCLONAL ANTIBODIES

H. Huttinga
Research Institute for Plant Protection (IPO), Binnenhaven 12, 6709 PD Wageningen

Introduction

When in 1975 Köhler and Milstein published their paper on the 'Continous cultures of fused cells secreting antibody of predefined specificity', a wide perspective was opened for the production of specific antibodies against all kind of 'difficult' antigens. Also in the field of plant production a lot of new research was started with the hope that it now would be possible to produce antibodies to plant viruses that till then could not sufficiently be purified to produce a specific polyclonal antiserum in e.g. a rabbit; to produce specific antibodies against pathovars of plant-pathogenic bacteria; to produce antibodies that can distinguish between viable pathogens and pathogens that have been killed, e.g. by heat treatment of plant material; etc.

Now, 15 years later, we have to admit that it has not been as easy as we thought it would be. Of course: many nice results have been obtained and you have been given many examples of that in the papers presented in this book. I just want to add one example from my own field: plant virology. The group of Ramon Jordan in Beltsville produced a monoclonal antibody that reacts specifically with all known potyviruses. They prepared it by injecting a mixture of potyviruses and subsequently selecting for the cell line that produces antibodies against an antigen that all these viruses have in common. The antibody is now commercially available in a test kit. The production of such an antibody would not have been possible using the polyclonal-antibody technique.

As said already, despite all the successes achieved, we have to admit that till now the monoclonal-antibody technique did not bring all the things we expected it to bring for the field of plant production. This is mainly caused by the fact that plants contain sugars, like arabinose, that are not found in animals and to mammals elicit a strong immune

response. This means that most of the B-cells that are triggered in an immunized animal, produce antibodies against these impurities in the antigen preparation. As a consequence the selection of the desired cell line is often laborious and many times it was impossible.
In this paper at first I like to discuss methods for the enrichment of desired B-cell populations before fusion, that will facilitate the selection of the desired cell line lateron. Furthermore I'll tell you something about new possibilities to use monoclonal antibodies.

Enrichment of desired B-cell populations

Many techniques have been described for the enrichment of B-cell populations (Cosenza & Köhler, 1972; Mage *et al.*, 1977; French *et al.*, 1986; Hockfield, 1987) but surprisingly it took a long time before they were applied to solve problems encountered with antigens obtained from plant material. George & Converse (1988) were the first to use them for anti-cauliflower mosaic virus hybridoma formation. They obtained an enriched B-cell population by immune-complex masking, B-cell reduction by solid-phase immunoadsorption, and complement-mediated B-cell lysis. In the first method they added mouse antibodies, which were prepared against purified healthy Chinese cabbage (HCC), to a cauliflower mosaic virus preparation purified from Chinese cabbage (CaMV-CC), before it was used to immunize mice. In the second method B-cells isolated from a mouse immunized with CaMV-CC were incubated in a polystyrene petri dish coated with HCC. In the third method an anti-CaMV-CC splenocyte suspension was incubated with anti-HCC-idiotype immunoglobulin, centrifuged and the pellet resuspended in a solution of complement components. The three methods led to 49, 72, and 54% increases, respectively, in the number of anti-CaMV clones found during screening. Hsu *et al.* (1990) successfully induced immune tolerance or suppression of immune response to plant pathogens by injecting neonatal BALB/c mice 4 times with healthy plants extracts on days 1, 3, 5, and 7 after birth. Subsequently, groups of mice each were immunized with partially purified tomato spotted wilt virus (TSWV) at age 5, 7, or 9 wk, respectively. Percentages of TSWV-specific hybridomas were 83%, 50%,and 40% for groups of mice immunized at 5, 7, and 9 wk of age, respectively. For control groups that obtained no neonatal injections of healthy plant extracts, 0%, 7%, and 7% of TSWV-specific hybridomas were found.

It is clear that many methods are still to be explored for improving the production of monoclonal antibodies for antigens purified from plant material. It is good to note that the 'Programmacommissie Landbouwbiotechnologie' supplied a grant to the Laboratory for Monoclonal Antibodies which will enable it to hire a reseach worker and a technical assistant for a period of 4 years to do research on these topics. The model systems that will be used in this study are: beet necrotic yellow vein virus, the plant-pathogenic bacterium *Erwinia chrysanthemi*, and the fungus *Verticillium dahliae*. The results of this research will certainly be of great value for everybody producing monoclonal antibodies against antigens associated with plants.

New possibilities to use monoclonal antibodies

Monoclonal antibodies and the production of mini-antibodies. If one has produced a cell line that excretes a monoclonal antibody it is relatively simple to isolate the particular immunoglobulin and its mRNA in sufficiently large quantities to determine the complete amino acid sequence and the base sequence of the mRNA. Based on those data Huston *et al.* (1988) were able to produce a single-chain antibody binding site for digoxin in *Escherichia coli* and to show that it closely mimics the antigen binding affinity and specificity of the parent antibody. In a similar way Welling *et al.* (1989), starting from the amino acid sequence of the H2-region of a monoclonal antibody against lysozyme, produced only the hyper-variable regions of that antibody. Those small protein molecules, when used in a column procedure bound to a carier by the N-termini, were able to specifically bind lysozyme from a mixture of proteins. The experiments showed that it is not necessary to make a large protein with a highly specific three-dimensional structure to provide the specific binding site: the mini-antibody peptide will adjust itself to the structure of the antigen while binding to it.

These results indicate that it will be easy to make socalled mini-antibodies and to use them in building biosensors: devices in which antibody and microelectronic technologies are combined to measure small molecules, like for instance pesticides, in environmental matrices (Stanbro *et al.*, 1988). Biosensors seek to exploit the rapidity and the specificity of biomolecular reactions, the consequences of which result in a change in the electrical or optical properties of the analyte that

can be transformed into a change in the measured voltage. (Mini-)antibodies are attractive for use in biosensors because of their specific reactivity and also because of their relative stability as compared with other proteins such as enzymes.

Monoclonal antibodies produced in plants. If the amino-acid sequence and the base sequence of the mRNA of a monoclonal antibody is known it is also rather simple to introduce the gene for it into other organisms. Horwitz *et al.* (1988) showed that by inserting cDNA copies of the chimeric light-chain and heavy-chain genes of an anti-tumor antibody into vectors containing an appropriate yeast promotor, it was possible to construct yeast strains that secrete mouse-human chimeric antibody and its Fab fragment into the culture medium. Hiatt *et al.* (1989) reported: "cDNAs derived from a mouse hybridoma mRNA were used to transform tobacco leaf segments followed by regeneration of mature plants. Plants expressing single gamma or kappa immunoglobulin chains were crossed to yield progeny in which both chains were expressed simultaneously. A functional antibody accumulated to 1.3% of total leaf protein in plants expressing full-lengths cDNAs containing leader sequences. Specific binding of the antigen recognized by these antibodies was similar to the hybridoma-derived antibody".

Introducing functional antibodies in plant cells in a way as described above, may open new possibilities to control diseases. E.g. it might be feasible that transgenic plants, producing appropriate antibodies in a constitutive way, are protected against infection by plant pathogens. Furthermore such transgenic plants, producing large macromolecules that do not pass through plant cell walls, may be used to specifically accumulate small organic molecules (toxins, pesticides) that are permeable to the cell wall. Thus providing, for instance, new options for the recovery of an array of environmental contaminants.

'Monoclonal' antibodies produced in bacteria. Huse *et al.* (1989) developed a completely novel system to produce (monoclonal) antibodies. They produced a 'combinatorial library' for the genes needed for the production of the antigen binding site, starting with mRNA taken from a mouse immunized against p-nitrophenylphosphonamidate (NPN). The library was constructed in two steps. At first separate Fab heavy and light chains libraries were constructed in bacteriophage lambda expression vectors. In the second step these two libraries were combined, resulting in a library of clones each of which potentially coexpresses a Fab heavy

and a Fab light chain. The actual combinations are random and do not necessarily reflect the combination present in the B-cell population of the parent animal.

The combinatorial library was screened at the level of single-phage plaques. In an experiment in which one million phage plaques were screened approximately 100 clones were identified on basis of their ability to produce assembled and functional Fab fragments that bind NPN. The authors claim that their method is much faster then the conventional production of monoclonal antibodies. The production and screening of thousands of hybridoma-derived monoclonal antibodies may take years; with the new method millions of new antibodies from bacteria can be made and screened in less than a week.

Conclusion

Immunological methods are a major tool in many areas where a lot of testing have to be performed. Just to give you an example: in the Netherlands about 9 million enzyme-linked immunosorbant assays were done in 1989, to assess the health status of agricultural and horticultural propagating material. The quality of the tests is highly depended on the quality of the antibodies used. If monoclonal antibodies can be used this means that highly reproducible tests can be designed and used all over the world, now and in the future. We have seen that there are many possibilities to improve the speed and the efficacy of the production of monoclonal antibodies, and that hybridomas can be used as starting points for the isolation of genes that can produce antibodies in other organisms. We have also seen that a broad range of monovalent Fab antigen binders can be produced starting with the mRNAs of immunized mice.

However, in my opinion the last option needs a lot more of developmental research as compared to the hybrodoma technique and therefore it is more then justified to encourage the Laboratory for Monoclonal Antibodies to continue their research on the development of monoclonal antibodies for the benefit of agricultural research.

References

Cosenza, H. & Köhler, H., (1972). Specific suppression of the antibody response by antibodies to receptors. *Proc. Natl. Acad. Sci. USA* **69**: 2701-2705.

French, D., Fischberg, E., Buhl, S. & Scharff, M.D., (1986). The production of more useful monoclonal antibodies. 1. Modifications of the basic technology. *Immunol. Today.* **7**: 344-346.

George, R.A. & Converse, R.H., (1988). Methods for the enrichment of desired B-cell populations before anti-cauliflower mosaic virus hybridoma formation. *Phytopathology* **78**: 1631-1636.

Hiatt, A., Cafferkey, R. & Bowdish, K., (1989). Production of antibodies in transgenic plants. *Nature* **342**: 76-78.

Hockfield, S., (1987). A Mab to a unique cerebellar neuron generated by immunosuppression and rapid immunization. *Science* **237**: 67-70.

Horwitz, A.H., Chang, C.P., Better, M., Hellstrom, K.E. & Robinson, R.R., (1988). Secretion of functional antibody and Fab fragment from yeast cells. *Proc. Natl. Acad. Sci. USA* **85**: 8678-8682.

Hsu, H.T., Wang, Y.C., Lawson, R.H., Wang, M. & Gonsalves, D., (1990). Splenocytes of mice with induced immunological tolerance to plant antigens for construction of hybridomas secreting tomato spotted wilt virus-specific antibodies. *Phytopathology* **80**: ???-???.

Huse, W.D., Sastry, L., Iverson, S.A., Kang, A.S., Alting-Mees, M., Burton, D.R., Benkovic, S.J. & Lerner, R.A., (1989). Generation of a large combinatorial library of the immunoglobulin repertoire in phage lambda. *Science* **246**: 1275-1281.

Huston, J.S. Levinson, D., Mudgett-Hunter, M., Tai, M.-S., Novotny, J., Margolies, M.N., Ridge, R.J., Bruccoleri, R.E., Haber, E., Crea, R. & Oppermann, H., (1988). Protein engineering of antibody binding sites: recovery of specific activity in an anti-digoxin single-chain Fv analogue produced in *Escherichia coli*. *Proc. Natl. Acad. Sci. USA* **85**: 5879-5883.

Köhler, G. & Milstein, C., (1975). Continuous cultures of fused cells secreting antibody of predefined specificity. *Nature* **256**: 495-497.

Mage, M.G., McHugh, L.L. & Rothstein, T.L., (1977). Mouse lymphocytes with and without surface immunoglobulins. Preparative scale separation in polystyrene tissue culture dishes coated with specifically purified anti-immunoglobulin. *J. Immunol. Methods* **15**: 47-56.

Stanbro, W.D., Newman, A.L. & Hunter, K.W., (1988). Applying biotechnology and microelectronics for environmental analysis. *In: Hedin, P.A., Menn, J.J. & Hollingworth, R.M. (eds.) Biotechnology for Crop Protection. ACS Symposium Series* **379**: 331-337.

Welling, G.W., Geurts, T., Gorkum, J. van, Damhof, R.A., Bloemhoff, W., Drijfhout, J.W. & Welling-Wester, S., (1989). Synthetische antilichamen voor antigeen-binding in biosensoren. *Abstracts 3T Biosensor Symposium, Utrecht, 14 December 1989.*