A serological approach to the identification of potato cyst nematodes

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A serological approach to the identification of potato cyst nematodes

Proefschrift

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STELLINGEN

1. Het is onjuist op basis van de positie in een SDS-PAGE gel te concluderen dat eiwitten afkomstig zijn uit de nematode zelf dan wel uit de door deze in de plant geinduceerde reuzecel.

   Jones, M.G.K., Physiol. Plant Pathol. 16, 359-367, 1980.

2. Het door Bojesen gehanteerde model voor de schatting van de bindingsparameters van antilichamen is in strijd met de wet van de massawerking.

   Bojesen, E., Scand. J. Immunol. 25, 69-82, 1987

3. Het is niet waarschijnlijk dat 8-methoxypsoraleen in "photophoresis" vervangen kan worden door het fotoactieve 2,2'-5,2''-tertiofeen.

   Yemul S. *et al.*, Proc. Natl. Acad. Sci. USA 84, 246-250, 1987.
   Edelson, R. *et al.*, N. Engl. J. Med. 316, 297-303, 1987.

4. De door Schwarz *et al.* gepresenteerde drie-dimensionale weergave van het antigene oppervlak van het eiwit-hormoon humaan chorion gonadotropine lijkt eerder ingegeven door de hun omgevende Oostenrijkse bergen en de belangstelling van de auteurs voor "computer graphics", dan dat dit werkelijk bijdraagt aan een beter inzicht in de distributie van de epitopen op dit eiwit.

   Schwarz, S., *et al.*, Endocrinology 118, 189-197, 1986.

5. De veronderstelling dat kleur het paringsgedrag van guppy vrouwtjes bepaalt is een te eenvoudige voorstelling van zaken.

   Breden, F., & Stoner, G., Nature 329, 831-833, 1987.

6. Het gezegde dat veel monoclonale antilichamen een "predefined" specificiteit hebben wekt valse verwachtingen voor wat betreft de reaktiviteit van deze antilichamen, zeker in het licht van de onverwachte kruisreakties die veel monoclonale antilichamen vertonen.

   Dit Proefschrift.

7. Het gebruik van het lichtgevoelige delta-aminolevulinezuur als milieuvriendelijk insecticide wordt tot nu toe ernstig beperkt door de benodigde modulator 2,2'-dipyridyl en door de herbicide werking van deze combinatie.

Rebeiz, C.A. *et al.*, In: Light activated pesticides, ACS symposium series no. 339 (James, R.H. & Kelsey, R.D. eds.), p. 296-328, 1987.

8. De door Patton en Avise ontwikkelde methode voor het opsporen van synapomorfe allozymen aan de hand van "out-group" vergelijkingen wordt niet ondersteund door de "neutral theory of molecular evolution".

Patton, J.C., & Avise, J.C., J. Molec. Evol. 19, 244-254, 1983
Kimura, M., The neutral theory of molecular evolution. Cambridge University Press, 1983.

9. Dat delen en vermenigvuldigen bewerkingen zijn die leiden tot een tegenovergesteld resultaat is slechts beperkt tot de rekenkunde. In de biologie zijn deze bewerkingen altijd complementair.

10. De wisselende prestaties van veel amateur topsporters zijn eerder te wijten aan een amateuristische werkwijze van veel sportbonden dan aan de amateurs zelf.

Proefschrift A. Schots

A serological approach to the
identification of potato cyst nematodes

Wageningen, 28 oktober 1988

Voor Marina

en

mijn ouders

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Arjen Schots
augustus 1988

CONTENTS

CHAPTER I

INTRODUCTION

Potato cyst nematodes are a major threat to potato crop. They originated in the Andean regions of South America where they co-evolved with their principal host, the potato, and were introduced in Europe with potatoes imported for breeding purposes, in the latter half of the 19th century. Today, the parasites are present in nearly all temperate zones where potato is cultivated. Overcropping, before and during the two world wars increased soil infestation such that, in the forties and fifties, severe losses became manifest in the principal potato growing areas. At present, potato cyst nematodes embody a considerable loss to the farmer, either because of harvest reduction or because of denial of certification for seed potatoes and other crops with adhering soil. On world scale, losses caused by potato cyst nematodes are estimated to constitute around 10% of the annual potato yield (Brodie, 1984). This figure does not include indirect losses such as those due to crop rotation schemes (obligatory in several countries), where alternate crops are of less value, soil disinfection, legislative control, and resistance breeding programs.

Untill 1923, potato cyst nematodes were considered to be the potato strain of the sugar beet cyst nematode Heterodera schachtii (Schmidt). In that year, Wollenweber (1923) classified this potato strain as Heterodera rostochiensis (Woll.). In the seventies, it became apparent that in Europe two species of potato cyst nematodes are present, which do not freely interbreed: Globodera rostochiensis (Skarbilow (Woll.)) and G. pallida (Stone). Although these species are supposed to have diverged several millions of years ago (Bakker, 1987), a striking similarity in morphology has been retained.

Resistant potato varieties are, together with crop rotation and soil disinfection with various nematicides, essential for the control of potato cyst nematodes. Both Globodera species have their own virulence spectra with respect to genes for

resistance transferred to potato. The success of these resistant cultivars is limited by the occurrence of virulent cyst nematode populations. At present eight pathotypes are recognized, five within *G. rostochiensis* (Ro_1 - Ro_5) and three within *G. pallida* (Pa_1 - Pa_3; Kort *et al.*, 1978), and large areas are infested with both species. New cultivars of potato are required to be resistant against most or all pathotypes of *G. rostochiensis* and *G. pallida*. Resistance is mainly derived from *Solanum tuberosum ssp. andigena* (Juz. and Buk.) (Toxopeus & Huijsman, 1953) and *S. vernei* (Bitt. and Wittm.) (Huijsman, 1974), and is mediated by pathotype specific genes. Since the number of genes transferring resistance to potato cyst nematodes is restricted, the development of new resistant potato varieties has become increasingly difficult.

A simple and quick screening test to characterize and quantitate field infestations of potato cyst nematodes according to species, would create the possibility to develop potato varieties with a limited but defined spectrum of resistance. Thus crop rotation schemes can be made more flexible by carefully selecting suitable cultivars, soil disinfection can probably be reduced, and the use of the available resistant potato cultivars can be largely expanded.

Present methods for distinguishing potato cyst nematode species and their pathotypes are laborious and time consuming, and therefore expensive. Only specialists can distinguish between the two species, but not pathotypes, on morphological characteristics. Usually the presence of species and their pathotypes in soil samples is determined in a greenhouse or in closed containers, on a series of test plants with different genes for resistance (differentials). A differentation based on biochemical entities offers better perpectives. Polyacrylamide gel electrophoresis is an appropriate method for the identification of closely related nematode species. Differences found in protein patterns of potato cyst nematode pathotypes actually inspired the view that in Europe two species are present (Trudgill & Carpenter, 1971; Trudgill & Parrott 1972). Since then many reports have been published which demonstrate differences in protein composition between *G. rostochiensis* and

G. pallida with various techniques, such as isoenzyme electrophoresis (Fleming & Marks, 1983; Wharton et al., 1983, Fox & Atkinson, 1984; Fox et al., 1986), disc-gel electrophoresis (Trudgill & Carpenter, 1971; Trudgill & Parrott 1972; Greet & Firth, 1977; Franco, 1979), isoelectric focussing (Fleming & Marks, 1982, 1983; Ohms & Heinicke, 1983; Fox & Atkinson, 1984), and two-dimensional gel electrophoresis on macro- and microscale (Bakker & Gommers, 1982; Stegemann et al., 1982; Ohms & Heinicke, 1985; Bakker & Bouwman-Smits, 1988; Bakker et al., 1988). Likewise, other soil inhabiting nematodes like Meloidogyne, Heterodera, Radopholus, and Labronema have been characterized with electrophoretic techniques (Dalmasso & Bergé, 1978; Bergé et al., 1981; Huettel et al., 1983; Ferris et al., 1985, 1986, 1987). Bakker (1987) has shown that it is even possible to go beyond the level of species identification by using two-dimensional gel electrophoresis to differentiate population types of both potato cyst nematode species. It is, however, anticipated that these techniques currently are too laborious and expensive for routine purposes.

At present, serological techniques may offer the best opportunities for large scale identification of the two species. Immunoassays, for example, are applied in agriculture for the detection and quantification of crop diseases, pesticides, and naturally occurring compounds. The advantages of immunoassays, over other assays, are their specificity, rapidity, simplicity, and relative inexpensiveness, which render them very suitable for routine applications. It can therefore be expected that the development and application of immunoassays will still increase in the near future (Lankow et al., 1987). Before an immunoassay can be performed, antisera have to be developed. However, antisera produced by the classical immunisation protocols using protein extracts of either one of the potato cyst nematode species, have been found to cross-react too strongly (Webster & Hooper, 1968; Wharton et al., 1983) to be of value as immunological differentiating reagents, although improved sensitivity of conventional antisera was obtained by the use of a much more refined crossed immunoelectrophoresis technique (Wharton et al., 1983).

Recently, a method for obtaining antibodies has been described which offers the potential to overcome problems of cross-reactivity and standardization encountered with conventional antisera. With this technique, developed by Köhler & Milstein (1975), a single antibody-producing cell is isolated from immunized mice, and fused *in vitro* with a mouse lymphoid tumour cell. In the resulting hybrid cell (hybridoma) the characteristics of both parental cell types are combined, *i.e.* specific (monoclonal) antibody production, and the capacity for *in vitro* multiplication. Thus, the humoral immune response of a mouse against a complex immunogen containing many structures, each of which gives rise to an individual antibody, can be dissected into its separate component antibodies. Concomitantly, the production of antibody is immortalized and standardized. Moreover, rare antibody specificities whose potential reactivities never become manifest *in vivo*, might be generated *in vitro* by cell fusion with single members of the antigen reactive B cell pool (Metzger *et al.*, 1984). As a result, hybridomas have been described which produce monoclonal antibodies able to dicriminate between isoenzymes and other variants of protein antigens (Berzofsky *et al.*, 1980; Tzartos *et al.*, 1980; East *et al.*, 1982; Smith-Gill *et al.*, 1982; Schroer *et al.*, 1983; Metzger *et al.*, 1984; Hollander *et al.*, 1986). Most monoclonal antibodies presently used in agriculture are directed against viruses, and a few against bacteria and fungi (Halk & De Boer, 1985). Thus they can also be expected to facilitate the differentiation of nematode species.

To identify the two potato cyst nematode species *G. rostochiensis* and *G. pallida* with monoclonal antibodies, species-specific proteins should be available as antigens. When they have to be applied in an immunoassay, such proteins should be stable, ubiquitous, and available in amounts appropriate for immunization. Their occurrence should be independent from pathotype and physiological conditions when the ultimate goal is the development of a quantitative immunoassay to determine the proportion of both nematode species in a soil sample. Several species-specific proteins from different stages of *G. rostochiensis* or *G. pallida* have been identified (Bakker *et al.*,

1988; Bakker & Bouwman-Smits, 1988), and some of these proteins have been shown to resist heat denaturation. In chapter II, we describe the isolation and partial characterization of such thermostable species-specific proteins from eggs of G. rostochiensis and G. pallida. These proteins have subsequently been used as antigens for the development of monoclonal antibodies (chapter IV).

Despite the many advantages of monoclonal antibodies over conventional antibodies, their application may also encounter difficulties (Goding, 1983). First, cross-reactions due to the structural relationship of antigens, or as a consequence of multiple specificities of an individual monoclonal antibody, might still be present (Sperling et al., 1983; Fox & Siraganian, 1986). Second, a slight alteration of the antigenic determinant may result in a loss of recognition by the antibody due to its high degree of specificity. Third, cross-linking of antigen and monoclonal antibody will only develop if the antigen possesses two or more identical antibody-binding sites, which might constitute a major disadvantage for a precipitin assay. Fourth, monoclonal antibodies have highly individual physical and chemical properties not related to antigen binding, which might interfere with their use in an assay, because of phenomena like euglobulin precipitation, or cryoprecipitation. These disadvantages imply that, once monoclonal antibodies are raised against an antigen, both antigen and antibody have to be characterized thoroughly, before they can be used to develop a routine test. In chapters IV and V, such a characterization is described for the monoclonal antibodies and antigens used to design an ELISA for the discrimination of G. rostochiensis and G. pallida.

In chapter III a method is described to determine binding constants of antibodies using Scatchard plots (Scatchard, 1949), and a non-linear regression algorithm (Munson & Rodbard , 1980). The binding constant, a measure of the strength of the bond between antibody and antigen, is a suitable parameter for the specificity of an antibody and is of particular relevance when (monoclonal) antibodies are intended for use as differentiating reagents, since antibody specificity is often related to

affinity (Berzofsky et al., 1980; Tzartos et al., 1980; East et al., 1982; Smith-Gill et al., 1982; Schroer et al., 1983; Metzger et al., 1984). In chapter VI, the reactivities of some selected monoclonal antibodies with protein antigens from G. rostochiensis and G. pallida in a direct ELISA have been predicted, based on the previous determined antibody binding constants and the concepts for the interactions between antigen and antibody as described by Rodbard & Bertino (1973).

In the final chapter, the results of this study are integrally discussed, with special attention to their prospects for improvement of potato cultivation, by means of a quick and reliable qualitative and quantitative determination of potato cyst nematode field infestations.

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CHAPTER II

SEROLOGICAL DIFFERENTIATION OF THE POTATO CYST NEMATODES GLOBODERA PALLIDA AND G. ROSTOCHIENSIS: PARTIAL PURIFICATION OF SPECIES SPECIFIC PROTEINS.

SUMMARY

Two major groups of heat stable proteins have been purified by heat denaturation from homogenates of eggs of the potato cyst nematodes Globodera rostochiensis and G. pallida. SDS-polyacrylamide gel electrophoresis of protein homogenates from six G. rostochienis populations and seven G. pallida populations revealed two bands specific for G. rostochiensis and three bands specific for G. pallida. Two-dimensional electrophoresis showed that the two bands specific for G. rostochiensis consisted of two polypeptides differing slightly in isoelectric point, as did one of the bands specific for G. pallida. Conventional antisera made against protein homogenates of either Globodera species showed a complete cross-reaction with the species-specific proteins. The perspectives of the differences in protein composition between G. rostochiensis and G. pallida, established in this study, for a quantitative differentiation of mixed field populations of the two Globodera species, involving monoclonal antibodies, is discussed.

INTRODUCTION

The potato-cyst nematode species Globodera pallida (Stone) Behrens and G. rostochiensis (Woll.) Behrens, are so closely related that until 1973 (Stone, 1973), no distinction was made between them, and both were combined within the species Heterodera rostochiensis. Within each species, a number of pathotypes have been characterized according to their ability to overcome certain resistance genes that have been introduced into the potato plant (Kort, Ross, Rumpenhorst & Stone, 1978). Since morphological differentiation between the two species is laborious there is a growing need for a simple and quick screening test to characterize and monitor field infestations of potato-cyst nematodes based, for example, on electrophoretic or serological characteristics of both species.

In support of this concept, differences have been reported in polyacrylamide disc-gel electrophoretic protein patterns between homogenates from 2nd-stage larvae, or adult females, of G. pallida and G. rostochiensis (Greet & Firth, 1977; Trudgill & Carpenter, 1971) as well as between those from South American and British pathotypes of G. pallida (Franco, 1979). Using isoelectric focussing, the isoenzyme patterns for a number of enzymes were shown to be pathotype specific (Wharton, Storey & Fox, 1983; Fox & Atkinson, 1984), and proteins with characteristically different isoelectric points were described for G. pallida and G. rostochiensis (Fleming & Marks, 1982, 1983; Fox & Atkinson, 1984). With two-dimensional gel electrophoresis according to the method of O'Farrell (1975), comparable results were obtained by Bakker & Gommers (1982). They found distinctive variations in protein patterns from homogenates of young females from the two Globodera species.

Despite the differences in protein composition of potato-cyst nematodes mentioned above, conventional antisera prepared from crude cyst nematode homogenates (Webster & Hooper, 1968; Wharton et al. 1983), failed to differentiate the two species because of an extensive cross-reaction.

In this report, we describe the properties of some species-specific proteins and their partial purification from eggs of

G. pallida and G. rostochiensis. The use of these proteins for large scale characterization of cyst nematode infestations with monoclonal antibodies is discussed.

MATERIAL AND METHODS

Preparation of protein extracts

Potato plants variety 'Eigenheimer', susceptible to all pathotypes, were inoculated with cysts of the G. rostochiensis and G. pallida populations listed in Table 1, and grown in pots in a greenhouse at 18 °C and 16 h daylight. Fresh cysts were recovered from the soil and squeezed, and the eggs were collected on a 20 μm sieve. Eggs were homogenized with a tissue grinder at 4 °C in 10 mM Tris-HCl buffer, pH 7.4, with or without 5% (v/v) β-mercaptoethanol. Homogenates were centrifuged in an Airfuge (Beckman, Palo Alto, Ca., U.S.A.) for 10 min at 105.000 x g, and the supernatant fraction collected. Protein concentrations were determined as described by Bradford (1976) with Coomassie Brilliant Blue G250, by measuring the shift in extinction at 595 nm in a spectrophotometer.

Electrophoresis

One-dimensional polyacrylamide slab-gel electrophoresis was carried out with 2-3 μg of protein/lane essentially as described by Laemmli (1970), using a 12 or a 17.5% (w/v) acrylamide separation gel, and a 6% (w/v) acrylamide stacking gel. Two-dimensional polyacrylamide slab gel electrophoresis was performed according to the method of O'Farrell (1975), using Biolyte 4/6 (Bio-Rad, Richmond, Ca., U.S.A.) to prepare the first dimension isoelectric focussing gel. Proteins in the gels were visualized with Coomassie Brilliant Blue R250 or with a silver stain according to the method of Oakley, Kirsch & Morris (1980).

Preparation of antisera

In order to obtain rabbit antisera suitable for detection of cyst nematode proteins on nitrocellulose blots, a volume of

the supernatant from homogenized eggs containing 1 mg of protein was mixed with an equal volume of electrophoretic sample buffer consisting of 10% (w/v) glycerol, 5% (v/v) β-mercaptoethanol, 2.3% (w/v) sodium dodecylsulphate, and 0.01% (w/v) bromophenol blue, in 62.5 mM Tris-HCl, pH 7.4. The sample was subjected to an electrophoretic run into a 6% (w/v) acrylamide stacking gel which had been layered on top of a 140 x 1.5 mm 25% (w/v) acrylamide separation gel slab, 100 mm in length. Electrophoresis was stopped when the bromophenol blue had reached the separating gel, and the stacking gel was washed with an aqueous solution of 25% (v/v) isopropanol and 10% (v/v) ethanol. Thereafter, the gel was minced and freeze dried, and the powdered pieces were ground into a suspension with 1 ml of phosphate-buffered saline (PBS), pH 7.2. Half of this suspension, mixed with an equal volume of Freund's Complete Adjuvant (Difco, Detroit, U.S.A.), was injected subcutaneously in the dorsal region of a rabbit at several sites. A similar injection was given 6 weeks after the first, but this time with Freund's Incomplete Adjuvant (Difco), and 14 days later the animals were bled for antisera.

Western blotting of proteins

Transfer of proteins from acrylamide gels onto nitrocellulose was carried out essentially as described by Towbin, Staehelin & Gordon (1979), with a slight modification of the visualization procedure. To detect specific binding between electrophoretically separated cyst nematode proteins, and rabbit anti-*G. pallida* or *G. rostochiensis* antisera, nitrocellulose blots were incubated for 2 h at room temperature with a 1:3000 dilution of goat anti-rabbit antiserum conjugated to horseradish peroxidase (Bio-Rad). The blots were then treated for colour development with a solution of 0.005% (w/v) 4-chloro-1-naphthol in 20 mM Tris-HCl, pH 7.2, containing 500 mM NaCl, 20% (v/v) methanol, and 0.015% (w/v) H_2O_2. After washing in distilled water to stop the reaction, the blots were dried, and immediately photographed.

RESULTS

At least three major bands, differing consistently in electrophoretic mobility were observed with G. rostochiensis and G. pallida in one-dimensional polyacrylamide slab-gel electrophoresis (Fig. 1). The proteins corresponding to these bands were relatively abundant, and were present in all Globodera populations studied. Moreover, in all thirteen populations screened by us the differences in electrophoretic pattern correlated with the taxonomic classification of G. rostochiensis and G. pallida, respectively (see Table 1).

An easy and simple purification method for some of these species-specific proteins was found. When homogenates of both species were heated 'au bain marie' for 10 min at 100 °C followed by centrifugation for 10 min at 105,000 x g, the gel electrophoretic profile, obtained after separation on a 17.5 % polyacrylamide gel, showed some proteins differing slightly in molecular weight (M_r) between G. rostochiensis and G. pallida (Fig. 1 C,D). All proteins were present in the heated supernatant fraction in considerable quantities, and were not subject to any noticeable degree of denaturation, following to an incubation at 100 °C for 30 min.

The thermostable proteins have been further characterized with two-dimensional gel electrophoresis using iso-electric focussing in the first dimension. The results are shown in Figure 2. These patterns showed that the protein of G. rostochiensis with a M_r of 20.7 kD consisted, as with the corresponding proteins in G. pallida, of two proteins with a M_r of 20.6 kD and 20.8 kD, and an iso-electric point (pI) of 5.2 and 5.3 respectively. The protein bands of G. rostochiensis and G. pallida with M_r of 18.0 kD and 17.0 kD, respectively, also consisted of two proteins differing in pI.

Conventional rabbit antisera prepared with egg homogenates from G. pallida, or G. rostochiensis, contained antibodies which bound to the heat-stable proteins from the species from which the immunizing material was derived. This can be seen by their reaction on a protein-blot prepared from a one-dimensional polyacrylamide slab gel (Fig. 3). Despite the electropho-

retically distinctive characteristics of the heat-stable proteins of either Globodera species both antisera were however, unable to distinguish them, due to extensive cross-reactivity.

Table 1. Populations of Globodera rostochiensis and G. pallida screened for the presence of species-specific proteins as indicated in the text.

Pop. no.	Original pathotype and designation	collection location
1.	Ro_1	Wageningen, The Netherlands, MIER
2.	Ro_1	Bergh, The Netherlands, A19
3.	Ro_1	Hardenbergh, The Netherlands, C-133
4.	Ro_3	Hoogeveen, The Netherlands C-129
5.	Ro_4	Emmen, The Netherlands, F-515
6.	Ro_5	Harmerz, Federal Republic of Germany, H
7.	Pa_1	Glarryford, Northern Ireland, 1337
8.	Pa_2	New Leake, Great Britain, ST
9.	Pa_2	Veendam, The Netherlands, HPL-1
10.	Pa_3	Cadishead, Great Britain E-1202
11.	Pa_3	Far øer, Denmark, E-1215
12.	Pa_3	Frenswegen, Federal Republic of Germany, FR
13.	Pa_2	?, The Netherlands, D-1

DISCUSSION

Considerable attention has been paid to identification of plant-parasitic nematodes by methods other than those based on morphological characteristics. Differences in isoenzyme patterns, for instance, have been used to distinguish Meloidogyne species (Dalmasso & Bergé, 1983), Heterodera avenae strains (Bergé, Dalmasso, Person, Rivoal & Thomas, 1981), and the potato

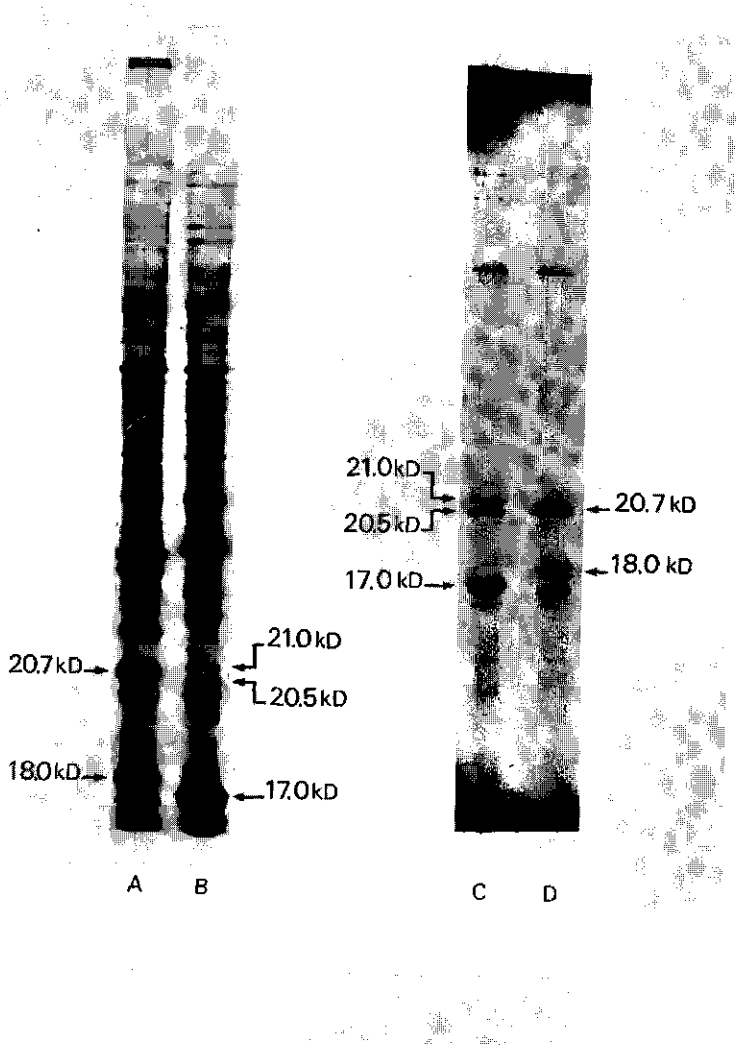

Figure 1. *SDS*-protein patterns made with homogenized eggs of Globodera pallida and G. rostochiensis. Lanes A and B show a total protein pattern of eggs from G. rostochiensis (lane A) and from G. pallida (lane B) (12% gel). Major species-specific proteins are indicated. Lanes C and D show the results of a partial purification of the species-specific proteins by heating a protein homogenate of eggs from G. pallida (lane C) or G. rostochiensis (lane D) at 100 °C for a period of 10 minutes (17.5 % gel). Molecular weights are indicated in kilodaltons.

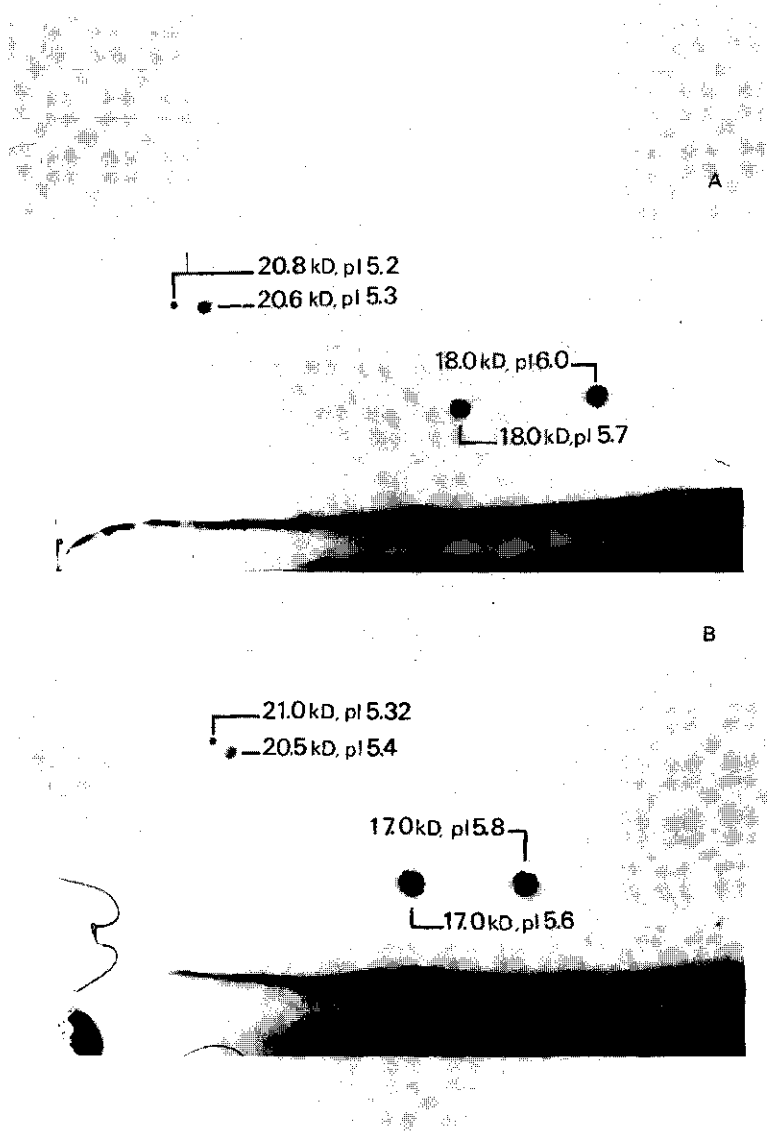

Figure 2. Enlarged section of a 2-dimensional protein pattern (12% gel) showing the molecular weights (in kilodaltons, kD) and the iso-electric points (pI) of the thermostable proteins of Globodera rostochienis (Fig. 2A) and G. pallida (Fig. 2B).

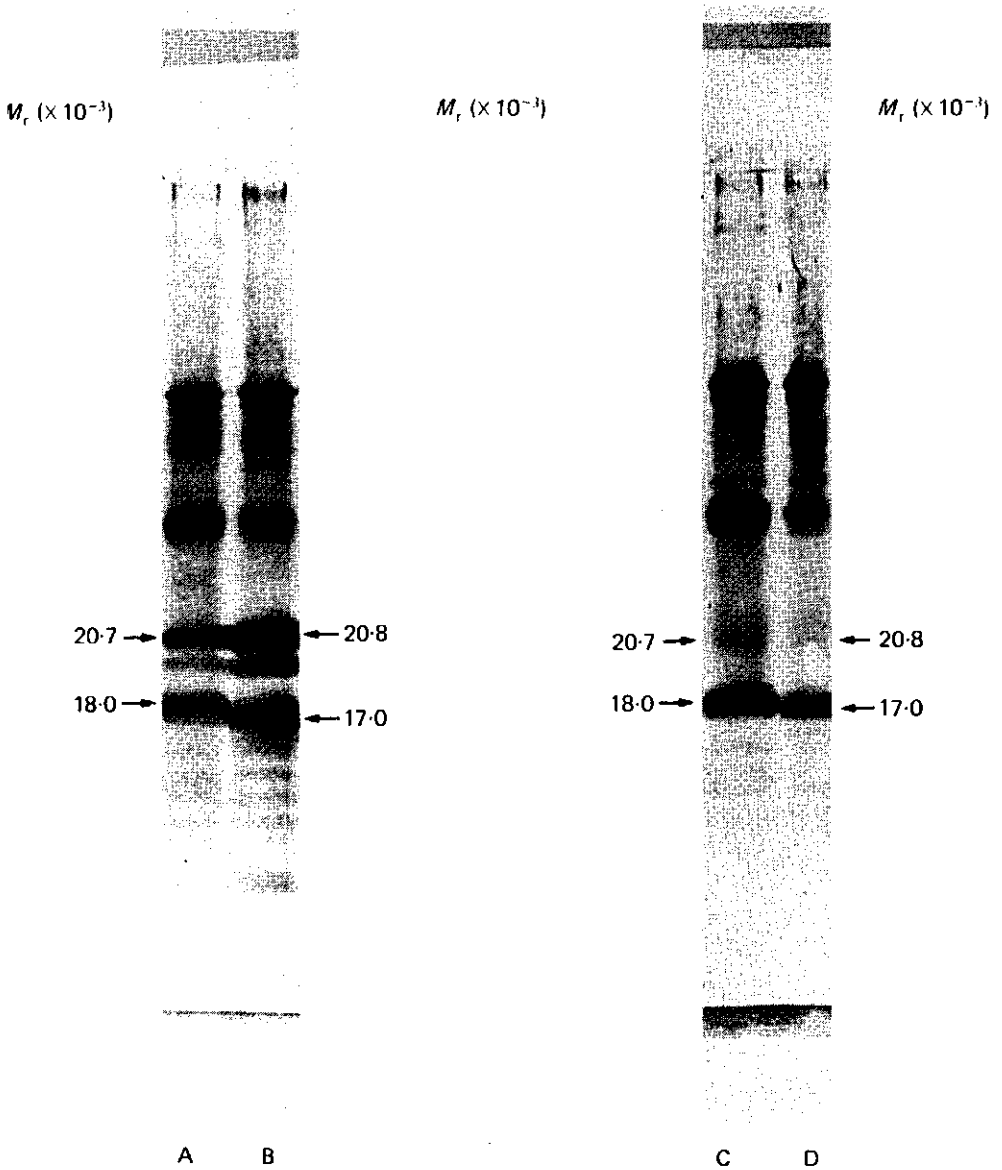

Figure 3. Immunoblots showing the reaction of antiserum raised against a protein homogenate from G. rostochienis (lanes A and B) or G. pallida (lanes C and D) reacting with thermostable proteins of Globodera rostochiensis (lanes A and C) and G. pallida (lanes B and D). Proteins were separated on a 17.5% polyacrylamide gel and transferred to nitrocellulose. Although some differences in reaction pattern between both antisera is seen, a strong cross-reaction is also observed.

cyst nematode complex (Wharton *et al.* 1983). Although Fleming & Marks (1983) have reported a potential discriminatory method by differentiating *G. pallida* and *G. rostochiensis* populations on the basis of their phosphoglucomutase isoenzyme distribution, the procedures involved using this type of assay systems are at present too laborious to render them suitable for large-scale routine tests. The same holds true for the use of other electrophoretic systems having the potential to differentiate between populations of potato cyst nematodes, such as polyacrylamide disc-gel electrophoresis (Franco, 1979; Greet & Firth, 1977; Trudgill & Parrott, 1972), isoelectric focussing (Fleming & Marks, 1982, 1983; Fox & Atkinson, 1984), and two-dimensional gel electrophoresis (Bakker & Gommers, 1982; Stegemann, Francksen & Rumpenhorst, 1982). Moreover, the reproducibility of the protein extraction procedures and the stability of the proteins *per se* are of crucial significance when the assay systems are based upon a quantitative determination of their presence. This might explain the discrepancies found between morphological data and the results from a densitometric evaluation of two species-specific proteins separated by isoelectric focussing, in a trial of several *Globodera* populations made up of differing proportions of *G. pallida* and *G. rostochiensis* (Fleming & Marks, 1982).

Conventional serological techniques for the identification of plant-parasitic nematodes have also been used. Despite strong cross-reactions obtained with the antisera used, some species of Meloidogyne (Misaghi & McClure, 1974) and Heterodera (Webster & Hooper, 1968) could, however, be identified. An improvement of the sensitivity of the assay could be obtained by the use of a much more refined crossed-immunoelectrophoretic system (Wharton *et al.* 1983).

None of the techniques mentioned, however, are suitable for routinely differentiating *Globodera* field populations, either because of their complexity or their low degree of sensitivity. In this study, we presented a simple and reproducible method to isolate some water-soluble, species-specific proteins from *Globodera* egg homogenates. The stability and relative abundance of these proteins renders them suitable for the development of a

rapid and sensitive quantitative identification assay which could be used to monitor mixed field populations of Globodera. A high degree of similarity between the species-specific proteins of the two Globodera species is suggested by the slight differences in molecular weight and isoelectric point, and the cross-reactivity with conventionally prepared rabbit antisera. It is, however, still reasonable to assume that the proteins possess differences in antigenic determinants. The hybridoma technique offers the opportunity, when these proteins are used as antigens, of isolating cell lines producing monoclonal antibodies reacting exclusively with either G. pallida or G. rostochiensis. An approach to isolate these hybridomas is now under investigation.

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CHAPTER III

A METHOD FOR THE DETERMINATION OF ANTIBODY AFFINITY USING A DIRECT ELISA

SUMMARY

Antibody affinity is of major significance for immunoassays. Since affinity may be influenced by the immunoassay methodology it is important to determine this parameter under the conditions of the assay used. Here a method is described for the determination of binding constants (K) in a direct ELISA with the use of the computer programme LIGAND. Five of the antibodies studied bound to their antigen with two classes of antigen binding sites, while all the other antibodies studied reacted with only a single class of antigen binding site. The accuracy of the method and the implications for antigen-antibody reactions are discussed.

INTRODUCTION

Enzyme immunoassays are, today, of major importance as an analytical tool in human and veterinary medicine, the food industry and agriculture. In such assays, conventional antisera are increasingly replaced by monoclonal antibodies (McAbs) with predefined specificity. With McAbs, a precise estimation of physical parameters is possible since these reagents possess uniform characteristics. To define the limitations of an immunoassay and predict the behaviour of a McAb in an immunoassay, knowledge of these physical parameters is of major importance.

One of the crucial physical parameters in every immunoassay is the affinity. As reported by Péterfy et al. (1983), commonly used immunoassays like SP-RIA, ELISA, Farr assay, complement-mediated hemolysis, and precipitation reactions are insensitive to antibodies of very low affinity. Also, in an extensive review, Steward & Lew (1985) noted the importance of antibody affinity in the performance of immunoassays.

Affinity of antibodies, expressed as a binding constant, is determined at an equilibrium state. Feldman (1972) developed a mathematical theory to characterize the equilibrium composition of a multicomponent cross-reactive ligand-binding system by solving the mass action equations. The theory is a generalization of that of Scatchard (1949). Several statistical and graphical methods based on the theory of Feldman (1972) have been described for the analysis of ligand-binding assays in general. However, most are applied to radioligand assays (Rodbard & Tacey, 1978; Munson & Rodbard, 1980; Thakur et al., 1980).

In contrast to RIA, little research has been done on the determination of antigen-antibody affinity in ELISA-systems. Friguet et al. (1985) developed a proper method for the determination of antibody affinity, without the need for labelling antibodies. However, a disadvantage of their method is the requirement for relative large amounts of antigen. In the present report, we describe a method for the determination of antibody affinity in an ELISA system that can be used with small

amounts of antibody and antigen. The binding constants were calculated using the computer program LIGAND described by Munson & Rodbard (1980).

MATERIALS AND METHODS

Isolation and purification of McAbs

Monoclonal antibodies reacting with carp immunoglobulin (cIg) were produced from mice immunized with cIg (WCI-series) or carp thymocytes (WCT-series) as recently described (Egberts et al., 1982; Secombes et al., 1983). The production of monoclonal antibodies specific for thermostable proteins isolated from the potato cyst nematode Globodera pallida (WGP-series) will be reported elsewhere (Schots et al., 1988). These antibodies are listed in Table I.

For the large scale preparation of antibodies, ascites was produced in BALB/c mice. Antibodies were purified from ascites on a hydroxylapatite column (Bio-Rad, Richmond, CA.), connected to an HPLC (Waters model 510), using a gradient from 100-400 mM Na-phosphate buffer pH 6.8 with 0.1 mM $CaCl_2$ and 0.02% NaN_3. Fractions of 1 ml were collected and tested for the presence of antibody in an ELISA. The ELISA was performed as described below, using as a second antibody an affinity purified goat-anti-mouse IgG (H + L) preparation coupled to horse radish peroxydase (GAM-HRP, Bio-Rad).

Conjugation to HRP

Purified antibodies were conjugated to horse radish peroxydase (HRP) using the two step glutaraldehyde method of Avrameas & Ternynck (1971). After conjugation, the remaining unconjugated peroxydase molecules were removed with a 50% ammonium sulphate precipitation. The ratio antibody:HRP was determined by reading the absorbance of the conjugates at 280 and 403 nm and calculating the total protein concentration from a $E^{1\%}_{1cm}$ of 14.0 at 280 nm and the concentration of HRP from a $E^{1\%}_{1cm}$ of 22.8 at 403 nm.

Table I. The monoclonal antibodies used for the affinity binding studies.

Antibody	Isotype	Immunizing antigen	Reactivity with
WCI 4	IgG_1	cIg	cIg
WCI 7	IgG_1	cIg	cIg
WCI 12	IgG_1	cIg	cIg
WCI 14	IgG_1	cIg	cIg, carp thymocytes
WCT 5	IgG_1	carp thymocytes	cIg, carp thymocytes
WCT 9	IgG_1	carp thymocytes	cIg, carp thymocytes
WCT 11	IgG_1	carp thymocytes	cIg, carp thymocytes
WCT 16	IgA	carp thymocytes	cIg, carp thymocytes
WCT 26	IgG_1	carp thymocytes	cIg, carp thymocytes
WGP 2	IgG_1	TSPaP	TSPaP
WGP 3	IgG1	TSPaP	TSPaP

Determination of the specific activity of antibodies conjugated to HRP

The specific activity of all conjugated antibodies (McAb-HRP) was determined as follows: 50 µl of a twofold dilution series of a conjugate in PBS/0.05% Tween 20/0.1% BSA were introduced into eight wells of a microtitreplate (Omnilabo, Breda, The Netherlands) followed by 50 µl substrate solution, comprising 0.2 M citric acid/0.4 M Na-phosphate/0.08% o-phenylenediamine/ 0.08% H_2O_2, pH 4.3. Microtitreplates were placed in the dark for 30 min. After this incubation, the reaction was stopped by the addition of 50 µl 2.5 M H_2SO_4. The extinction was then read at 492 nm in a microtitreplatereader (SLT-labinstruments, Gröding, Austria). The specific activities of the McAb-HRP conjugates were calculated from these results as O.D. units produced per mole antibody under the conditions of the ELISA used.

Direct "affinity-ELISA"

The ELISA was performed according to Klasen et al. (1982), with a modification in the post-coatingbuffer where a concentration of 0.5% BSA was used, and with omission of the second antibody step. The antigen coating concentrations used were 6.5 µg/ml for cIg and 1.0 µg/ml for potato cyst nematode proteins. At least 8 dilutions were made for each McAb-HRP conjugate and experiments were performed in duplicate (WCT/WCI antibodies) or triplicate (WGP antibodies). After the addition of the substrate solution, the amount of McAb bound to the antigen was calculated from the extinction readings using the specific activity of the McAb-HRP conjugate, determined as described above.

Computational methods

Calculation of the binding constants of the McAbs on the basis of the theory of Feldman (1972) was performed with the computer program system LIGAND as developed by Munson & Rodbard (1980). LIGAND version 2.3.12 was used which included the possibility of fitting a cooperativity parameter β (Munson & Rodbard, 1984). Input files were made using LOTUS 1-2-3. The general model $Var(Y) = 0.0001Y^2$ (Rodbard et al., 1976) was used as a weighting model (constant percent error). LIGAND was run on an Olivetti M24 personal computer. With each datafile a fit for a one-site and a two-site model with or without cooperativity was made. When more then one fit is succesful, the program automatically compares the results using an F-test to determine the best fit.

RESULTS

The binding constant (K) belonging to an antibody-antigen complex can be calculated as a measure of the strength of the bond between antigen and antibody. Together with the binding constant, the binding capacity (R) can be calculated to express the number of binding sites (in mol/liter) available for all antibody molecules. From the results of the ELISA, binding constants and binding capacities were calculated using the

computer program system LIGAND, and are listed in Table II.

LIGAND is a computer program system useful for the evaluation of data obtained with all kinds of ligand-binding systems. In the ELISA performed as described, the antibody is used as a ligand and the antigen, absorbed to the microtitreplate, as a binding group. The program LIGAND describes a heterologous system, that will give statistical evidence whether the antigen has one or two (or even more) apparent binding sites for the antibody and, wether the binding characteristics are the result of a cooperative effect. The results shown are the average of several independent experiments. For all data sets obtained from the ELISA, a fit was tried with the one-site as well as with the two-site model, with and without the assumption of cooperativity. When succesful fits were obtained with more than one model, the program compares the fits using an F-test.

Six of the eleven antibodies tested showed a succesful fit with the one-site model only without involving cooperativity. The outcome of the F-test, comparing the one-site model with and without cooperativity and a two-site model without cooperativity, showed that for four of the antibodies the two-site model without cooperativity was favoured in all cases. For one antibody, WCI 12, LIGAND gave statistical evidence for a two-site model with cooperativity for K_1.

As an example of the antibodies reacting with one binding site a Scatchard-plot was made for WCT 26 of the ratio of bound to free antibody against the concentration of bound antibody (Figure 1). However, four of the eleven antibodies tested (WCI 7, WCT 5, WGP 2 and WGP 3) showed a reaction with two apparent classes of binding sites. In Figure 2, a Scatchard-plot for WCT 5 is presented. Compared with the other antibodies, the second binding site of WCT 5 reacts with a remarkably high affinity (ratio $K_1:K_2$ = 1:10). Although the coefficients of variation for both binding constants of WCT 5 tend to be higher for the two-site model (compared to the one-site model), the two-site model was significantly better as was shown by the F-test (the F-value was 28.61 with a critical level (P) of 0.000).

Figure 3 and Table III show the effect of six different fits for antibody WCI 12 on the height and accuracy of the

estimated parameters. The fits obtained with a one-site model without the assumption of cooperativity (Fig. 3A) and with the assumption of positive cooperativity (cooperativity parameter β_1 > 1, Fig. 3B) were the least accurate ones. The assumption of a one-site model with negative cooperativity (cooperativity parameter β_1 < 1, Fig. 3C) or a two-site model without cooperativity (Fig. 3D) resulted in two fits of nearly the same accuracy. However the two-site model showed extremely high coefficients of variation for K_2 and R_2. Significantly better fits were obtained using a two-site model with the assumption of cooperativity for either K_1 (Fig. 3E) or both binding sites (Fig. 3F). The latter assumption resulted in a fit with very high coefficients of variation for K_2, β_2 and R_2. Moreover, the fit was not significantly better than the fit which uses the two-site model with positive cooperativity for only K_1. We therefore conclude that the binding characteristics of monoclonal antibody WCI 12 to carp immunoglobulin are best described by a model assuming two binding sites with positive cooperativity for K_1 (Fig. 3E).

DISCUSSION

The antibodies studied can be divided in two major groups: those reacting with one apparent binding site and those reacting with two apparent binding sites.

Binding of protein antigens to a solid support might change the structure of the antigen and thus influence antibody affinity. Depending on the nature of the alteration, the binding constant for a given combination of antibody and antigen can either increase or decrease. If one binding site is found, it is expected that the antigenic structure of an epitope is not changed too drastically upon absorption of the antigen to the plastic of the microtitreplate. However, if such structural changes occur, it is possible that an epitope can be presented to the antibody in more than one form, if not already present on the native antigen molecule. The antibody will then bind to the

TABLE II

BINDING CONSTANTS (K) AND BINDING CAPACITIES (R) OF THE MONOCLONAL ANTIBODIES STUDIED WITH THEIR COEFFICIENTS OF VARIATION (%CV)

The results were obtained with the computer program system LIGAND (version 2.3.12). If convergence was reached with both the one-site model and the two-site model, the two results were compared using an F-test. In all these instances the two-site model proved to be the most favourable

McAb	K_1 $\times 10^{-7}$ (l/M)	%CV	K_2 $\times 10^{-7}$ (l/M)	%CV	R_1 $\times 10^{11}$ (M)	%CV	R_2 $\times 10^{11}$ (M)	%CV	DF [c]	RSS [d]	F [e]	P [f]
WCI 4	2.04	38	–	–	4.68	16	–	–	8	3350.03		
WCI 7 [a]	37.46	14	–	–	4.79	7	–	–	28	12477.91	152.11	0.000
WCI 7 [b]	74.19	8	0.18	41	3.43	4	6.33	22	26	982.48		
WCI 12 [a]	31.95	4	–	–	9.51	3	–	–	52	4357.78	21.17	0.000
WCI 12 [b]	36.78	4	0.01	730	8.78	3	34.42	682	50	2359.96		
WCI 14	0.82	71	–	–	41.19	64	–	–	15	17312.03		
WCT 5 [a]	5.37	7	–	–	13.16	5	–	–	28	3146.76	28.61	0.000
WCT 5 [b]	14.47	31	1.36	39	4.57	35	12.59	9	26	983.03		
WCT 9	4.18	16	–	–	37.20	12	–	–	17	5621.88		
WCT 11	0.63	20	–	–	11.38	14	–	–	26	14584.44		
WCT 16	9.09	6	–	–	40.00	4	–	–	28	3480.49		
WCT 26	21.30	5	–	–	31.45	4	–	–	26	1979.47		
WGP 2 [a]	368.71	9	–	–	6.31	9	–	–	78	12520.04	39.72	0.000
WGP 2 [b]	4980.90	41	151.17	24	0.33	44	8.69	13	76	6121.89		
WGP 3 [a]	952.10	13	–	–	4.54	11	–	–	39	4442.43	13.85	0.000
WGP 3 [b]	7387.71	120	467.22	31	0.05	71	7.00	22	37	2540.73		

[a] Fit using a one-site model.
[b] Fit using a two-site model.
[c] DF, degrees of freedom.
[d] RSS, residual sum of squares.
[e] F, F-value, resulting from the calculations for an F-test.
[f] P, critical level.

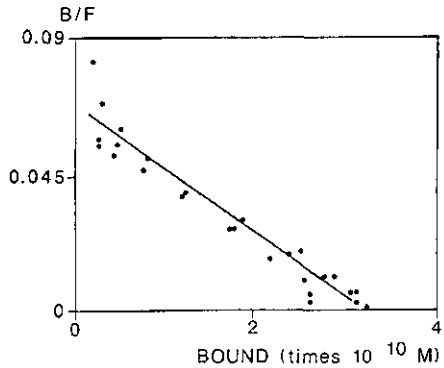

Figure 1: Scatchard plot for antibody WCT 26, a monoclonal antibody reacting with carp immunoglobulin. The antigen shows one binding site to which the antibody can bind. On the X-axis the concentration of bound antibody is given in mol/l, on the Y-axis the bound to free ratio for the antibody is given.

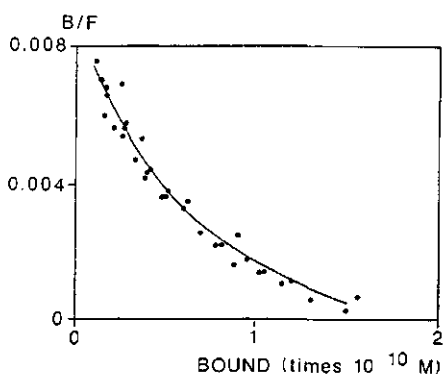

Figure 2: Scatchard plot for antibody WCT 5, a monoclonal antibody binding to carp immunoglobulin apparently at two sites. Axes as in Figure 1.

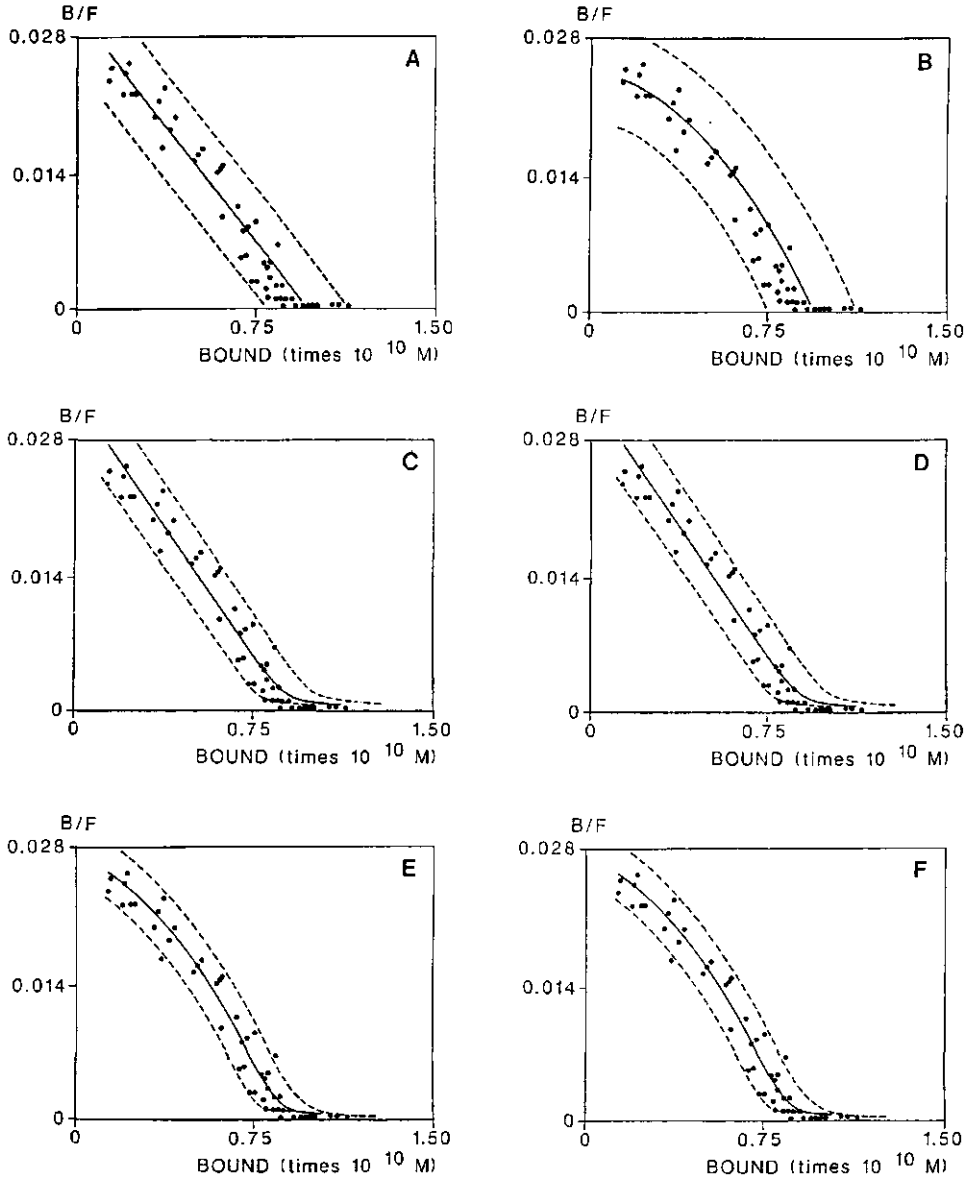

Figure 3: Scatchard plots with different fits for antibody WCI 12 with confidence limits. **A.** one-site model, **B.** one-site model with cooperativity parameter $B_1 > 1$, **C.** one-site model with $B_1 < 1$, **D.** two-site model, **E.** two-site model with positive cooperativity for K_1, **F.** two-site model with cooperativity for both apparent binding sites. Axes as in Figure 1.

TABLE III

COMPARISON OF DIFFERENT FITS FOR ANTIBODY WCI 12

The results were obtained by fitting different models using the computer program system LIGAND (version 2.3.12). With all indicated models convergence was reached. See Table II for explanation of symbols.

Model	K_1 $\times 10^{-7}$ (l/M)	%CV	K_2 $\times 10^{-7}$ (l/M)	%CV	β_1	%CV	β_2	%CV	R_1 $\times 10^{11}$ (M)	%CV	R_2 $\times 10^{11}$ (M)	%CV	DF	RSS	F	P
One-site no cooperativity	31.93	4	–	–	–	–	–	–	9.51	3	–	–	52	4357.25	25.58	0.000
One-site cooperativity ($\beta_1 > 1$)	25.76	17	–	–	2.21	34	–	–	9.26	34	–	–	51	5264.72	44.54	0.000
One-site cooperativity ($\beta_1 < 1$)	18.56	4	–	–	0.006	8	–	–	17.47	3	–	–	51	2383.49	11.41	0.000
Two-site no cooperativity	36.78	4	0.01	720	–	–	–	–	8.79	3	32.24	672	50	2361.23	16.73	0.000
Two-site cooperativity for K_1	31.68	12	0.08	99	1.97	23	–	–	8.28	23	8.71	69	49	1414.09	0.79	0.379
Two-site cooperativity K_1 and K_2	31.37	12	0.08	>1000	2.05	23	0.94	902	8.26	23	8.96	902	48	1391.30		

different epitopes with different affinity constants. In this way, we can explain why for the antibodies WCI 7, WCI 12, WCT 5, WGP 2 and WGP 3 the two-site model, resulting in two apparent binding sites, was favourable. Such a case, i.e., a divalent antigen reacting with a univalent antibody, has already been considered by Berson & Yalow (1959).

Antibody affinity can also be influenced by the binding of more antibody molecules to the same antigen molecule. In the case of a multivalent antigen (e.g., cIg), binding of a second antibody molecule could be enhanced by a previously bound molecule. Here, we have demonstrated statistical evidence for positive cooperativity for one of the binding sites (K_1) of cIg for antibody WCI 12 (Fig. 3E and Table III). This phenomenon of binding enhancement has been described for mixtures of two individual McAbs (Tosi et al., 1981; Ehrlich et al., 1982; Lefrancois & Lyles, 1982; Lemke & Hämmerling, 1982; Lubeck & Gerhard, 1982). In radioimmunoassays, using conventional antisera, a similar feature has been reported. A rise in the Bound/Free ratio for antigen with increasing mass of labeled antigen was found by Matsukura et al. (1971). Rodbard & Bertino (1973) developed a theory to describe this phenomenon of cooperativity for radioimmunoassays.

In most literature on antibody affinity or avidity determinations, only one binding site has been reported. In the case of anti-hapten antibodies the binding constant has often been determined by equilibrium dialysis (Lehtonen & Eerola, 1982; Péterfy et al., 1983; Bose et al., 1986). The occurrence of one binding constant for these antibodies might be a consequence of the fact that, in binding to a first site, small organic molecules do not alter the binding parameters for a second site. Application of data obtained from equilibrium dialysis to a solid phase immunoassay, where haptens are coupled to a protein carrier, involves the risk of either under- or overestimation of the binding constants as discussed above. Nevertheless, in cases where the affinities of antibodies for protein antigens have been determined, one binding site has often been suggested. This is possible when a model describing a homologous system is used. However, our data demonstrate that for several monoclonal

antibodies the binding characteristics between antigen and antibody can not be explained by such a model. A model describing a heterologous system is needed to describe the binding characteristics between antigen and antibodies WCI 7, WCI 12, WCT 5, WGP 2 and WGP 3. LIGAND (Munson & Rodbard, 1980; 1984) does provide such a system, and it can be of great help for the estimation of binding contants of monoclonal antibodies reacting with multivalent antigens. As stated by its creators, and confirmed in this study, LIGAND is certainly applicable to a wide variety of ligand-binding studies.

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CHAPTER IV

SEROLOGICAL DIFFERENTIATION OF THE POTATO-CYST NEMATODES GLOBODERA PALLIDA AND G. ROSTOCHIENSIS. II. PREPARATION AND CHARACTERIZATION OF SPECIES-SPECIFIC MONOCLONAL ANTIBODIES.

SUMMARY

Hybridomas producing antibodies which react with thermostable protein antigens isolated from the potato cyst nematode species Globodera rostochiensis (TSRoP) and G. pallida (TSPaP) were isolated. Three of the isolated hybridomas (WGP 1, WGP 2 and WGP 3) produce antibodies that react with preferent affinity with protein antigens isolated from G. pallida, and two (WGR 11 and WGR 12) produce antibodies which bind preferentially to G. rostochiensis. Binding constants were determined to quantitate the differences in affinity of WGP 1, WGP 2, WGP 3, WGR 11 and WGR 12 for the protein antigens from both nematode species, and to assess the similarities in affinity for either protein antigen with respect to the other non-specific antibodies. In immunoblotting experiments, a binding could be demonstrated for most antibodies, to two thermostable proteins with apparent molecular weights of 20.7 kD for G. rostochiensis and 20.5/21.0 kD for G. pallida. The reactivities of the monoclonal antibodies with thermostable protein antigens from other common cyst nematodes was also investigated. All monoclonal antibodies which are not specific for TSRoP or TSPaP, bind to thermostable proteins of these cyst nematode species. The use of some of the isolated monoclonal antibodies for the improvement of the diagnosis of potato cyst nematodes in soil samples is discussed.

INTRODUCTION

The nematode species Globodera rostochiensis (Skarbilow (Woll.)) and G. pallida (Stone) are a major threat to potato tillage. Their presence in the soil embodies a potential financial loss to the farmer either because of harvest reduction, or because of rejection of seed potatoes (like other crops with adhering soil) for certification. One method to control potato cyst nematodes is the use of resistant potato varieties. These varieties are required to be resistant against most or all pathotypes of both nematode species. However, the two Globodera species have their own partially overlapping virulence spectra with respect to the genes for resistance transferred from other Solanaceae to potato. It would, therefore, be desirable to know whether a species is present singly, or in a mixed population. A quick and reliable routine test to identify the sibling species would thus be of great importance. Morphological species differentiation is arduous and unreliable because of intra- and interspecific variation in shape and size (Bakker et al., 1988; Stone 1977). Differentiation based on biochemical or serological entities should offer better perspectives. Many biochemical approaches, including immuno-electrophoresis, pyrolysis gas-liquid chromatography, and analysis of DNA restriction fragment length polymorphism, to distinguish potato cyst nematodes and other plant parasitic nematodes have been described (see for a review Fox & Atkinson, 1986). These methods, however, are not suited for routine applications.

Although several animal species have been identified with conventional antisera to protein antigens (Lowenstein, 1985), such reagents, when made with protein homogenates of either nematode species, show extensive cross-reactions in immunoblotting (Schots et al., 1987) and ELISA experiments (data not published). It might still be possible to discriminate both nematode species with conventional antisera using more refined immunochemical techniques. It is, however, anticipated that the higher degree of sophistication and/or labour required for such a design will counteract the profits from an increase at the discriminatory level.

Table 1. Apparent molecular weights (in kD) and isoelectric points (pI) of thermostable proteins isolated from Globodera rostochiensis (TSRoP) and G. pallida (TSPaP).

TSRoP molecular weight	pI	TSPaP molecular weight	pI
18.0	6.0	17.0	5.8
18.0	5.7	17.0	5.6
20.6	5.3	20.5	5.4
20.8	5.2	21.0	5.3

Monoclonal antibodies (Köhler & Milstein, 1975) hold the promise of better differentiating reagents. They usually recognize a single epitope, with a concomitant reduction of cross-reactivity. Moreover, rare antibody specificities whose potential reactivities never become manifest in vivo, might be generated in vitro by cell fusion with single members from the antigen-reactive B cell pool (Metzger et al., 1984). Thus, the development of hybridomas producing antibodies able to discriminate between isoenzymes, and other variants of protein antigens, differing in only one or a few amino acids, has been described by a number of authors (Berzofsky et al., 1980; Tzartos et al., 1981; East et al., 1982; Smith-Gill et al., 1982; Schroer et al., 1983; Metzger et al., 1984; Hollander et al., 1986).

Recently, we have isolated some species-specific thermostable proteins from eggs of G. rostochiensis (TSRoP) and G. pallida (TSPaP) (Schots et al., 1987). Both TSRoP and TSPaP have been characterized to consist of four proteins differing somewhat in apparent molecular weight (M.W.) and iso-electric point (pI) (Table 1). These results offer the possibility to use these proteins as antigens for the production of differentiating antisera. In this report we describe the development of hybridomas producing monoclonal antibodies binding preferentially to TSPaP or TSRoP. The application of these, and other cross-reactive, monoclonal antibodies to the development of a routine test to differentiate G. rostochiensis and G. pallida after

recovery from soil samples, will be discussed.

MATERIALS AND METHODS

Isolation of TSRoP and TSPaP antigens

Potato plants variety 'Eigenheimer' were inoculated with cysts of a G. rostochiensis population designated Ro_1 mier and cysts of a G. pallida population designated $PaHPL_1$ and grown in pots in a greenhouse at 18 °C and 16 h daylight. Newly formed cysts were recovered from the soil using a flotation method (Southey, 1970). Most of the remaining organic material was separated from the cysts using acetone. Cysts were air dried and stored at 4 °C until use. To prepare egg homogenates, about 10^5 dry cysts (equivalent to approximately 20 mg thermostable proteins) were soaked in distilled water overnight, and squeezed (Seinhorst & Den Ouden, 1966). Eggs were separated from the cyst shells on a 150 µm sieve and collected by centrifugation (5 min, 2000 x g). After washing three times with distilled water and twice with 10 mM Tris.HCl, pH 7.4, the eggs were finally resuspended in 40 ml of 10 mM Tris.HCl, pH 7.4. Egg homogenates were prepared by passing a frozen suspension three times through a french press. Watersoluble thermostable proteins of G. rostochiensis (TSRoP) and G. pallida (TSPaP) were obtained by heating the homogenate 'au bain marie' for 10 min. at 100 °C, followed by centrifugation for 10 min. at 15,000 x g. Protein concentrations were determined as described by Bradford (1976).

Immunization of mice

Several immunization-protocols have been used to isolate hybridomas producing antibodies which react with either TSRoP or TSPaP.

1) TSRoP and TSPaP were conjugated to keyhole limpet hemocyanin (KLH; Sigma, St. Louis, MO., USA) using the heterobifunctional reagent N-succinimidyl 3-(2-pyridyldithio)propionate (SPDP; Pharmacia, Uppsala, Sweden). The procedure was essentially as described by the manufacturer, with a modification in the reduction of the 2-pyridyl disulphide groups of

KLH, which was performed in a 0.1 M sodium phosphate buffer (pH 7.5) containing 0.1 M NaCl, by the addition of 50 mM dithiothreitol. The reduction was stopped after 5 minutes by removing dithiothreitol by gel filtration on a prepacked Sephadex G-25 (Pharmacia) PD-10 column.

For immunization the protein conjugate was precipitated on aluminumhydroxide. Five milligrams of conjugate in 10 ml phosphate buffered saline (PBS) were mixed with 4.6 ml 1 M $NaHCO_3$, followed by the addition of 10 ml of a solution of 10 % (w/v) aluminum potassium sulphate in PBS under continuous stirring. The resulting suspension was left for 16 hours at 4 °C, centrifuged (15 min., 900 x g) and the pellet resuspended in PBS. Balb/c mice were immunised twice intraperitoneally (i.p.) with 200 µl of this suspension corresponding to 50 - 100 µg of protein.

2) 100 µg TSRoP or TSPaP were spotted on nitrocellulose (BA 85, Schleicher & Schüll, Dassel, FRG), which was then left 16 hours at room temperature to dry. The nitrocellulose was mixed with 100-200 µl of PBS, frozen in liquid nitrogen, and ground to a suspension of fines in order to pass it through a 0.5 x 16 mm injection needle. With this suspension, Balb/c mice were first immunised intrasplenically, according to Spitz et al. (1984), and, where appropriate, along the intraperitoneal route in a second immunization.

3) 200 µg of TSRoP or TSPaP were subjected to preparative SDS-PAGE in a mini-protean II cell (Bio-Rad, Richmond, Ca., USA) essentially according to Laemmli (1970), using a 12 % (w/v) separation, and a 6 % (w/v) stacking gel. After termination of electrophoresis the proteins in the gel were blotted to nitrocellulose according to Towbin et al. (1979). Following transfer of the proteins, part of the nitrocellulose was cut off and stained with a solution of 0.2 % (w/v) amido black in 50 % (v/v) methanol and 10 % (v/v) acetic acid, to visualize the proteins. Strips corresponding to the protein bands of 18 kilodalton (kD) of TSRoP, or of 17 kD of TSPaP were then cut out. Grinding of the nitrocellulose and immunization of the Balb/c mice was carried out as described under 2.

The mice were bled 10 days after each immunization to

monitor the immune response. Mice having a titer of at least 1:20000, being the dilution of the immune serum giving an optical density reading of 0.1 in an enzyme linked immunosorbent assay (ELISA), were given a booster, 4-6 weeks after the last immunization, and used for cell fusion experiments. The ELISA procedure is described under screening of cell culture supernatants.

Cell fusion

Cell fusions were carried out with SP 2/0-Ag 14 myeloma cells as described by Lane et al. (1985), using polyethyleneglycol 4000 GA (Merck, Darmstadt, FRG) as fusion agent. After fusion, the cells were resuspended in a hybridoma selective medium consisting of RPMI 1640 with 2 mM L-glutamine, 1 mM pyruvic acid, 50 IE/l penicillin, 50 IE/l streptomycin, 0.1 mM hypoxanthin, 6 μM azaserine, 5 % (v/v) fetal calf serum and 5 % (v/v) newborn calf serum. Occasionally, human endothelial cell supernatant (Astaldi et al., 1980) was included to 10 % (v/v), to improve the growth of the fusion products. Usually, one third of the cells from a fusion were divided in 100 μl lots over 600 microtiterplate wells each containing 10^4 peritoneal mouse cells. The remaining cells were cultured in two 75 cm^2 bottles containing 10^6 peritoneal mouse cells (20 ml/bottle) for 4 days, and then frozen in a medium consisting of RPMI 1640 with 40 % (v/v) fetal calf serum and 10 % (v/v) dimethylsulfoxide. Peritoneal cells were obtained by flushing the peritoneal cavity of Balb/c or NMRI mice with a solution of 11.6 % (w/v) saccharose in PBS.

Screening of cell culture supernatants

Cell culture supernatants were screened for the presence of specific antibodies using an ELISA. Briefly, TSRoP or TSPaP were coated to polystyrene microtiterplates at 0.1 μg/well. Postcoating was carried out with 0.5 % (w/v) bovine serum albumin (BSA) and 0.05 % (w/v) Tween 20 in PBS. The microtiterplates were filled with 100 μl/well of cell culture supernatant diluted 1:1 with 0.1 % Tween 20 in PBS. Interaction of antibodies with TSRoP or TSPaP was determined with a goat-anti-mouse immuno-

globulin/horse radish peroxydase conjugate (Bio-Rad) diluted 1:3000 in PBS with 0.1 % (w/v) BSA and 0.05 % Tween 20, followed by an incubation with 0.04 % (w/v) o-phenylenediamine and 0.04 % (w/v) H_2O_2 in a 0.2 M sodiumphosphate/0.1 M citric acid buffer (pH 5.0). Absorbance at 492 nm was read in an EAR 400 Easy Reader (SLT Labinstruments, Gröding, Austria).

Cloning of hybridomas

Hybrid cell lines were cloned by limiting dilution. The resulting clones were tested for the secretion of antibody like the parental hybridoma cell line. Usually 3-4 of the fastest growing clones showing positive reactions were chosen for preservation. Hybridomas were cloned for a second and a third time in case a promising cell line had not stabilized after cloning once.

Preparation of ascites fluid

Adult Balb/c mice were primed by an i.p. injection of 0.2 ml pristane (2,6,10,14-tetramethylpentadecane; Sigma), followed not less than 7 days later by an i.p. injection of around 5.10^6 hybridoma cells. After the development of an ascitic tumour, samples of the peritoneal fluid were withdrawn at regular intervals and freed from cells by centrifugation (5 min, 400 x g). To remove fibrin, the ascitic fluid was incubated with 1 mM $CaCl_2$ for 30 min. at room temperature, and centrifuged (15 min., 15.000 x g). The samples were stored at -20 °C.

Heavy chain analysis

Antigen specific hybridoma supernatants were concentrated 25 times using a Minicon B 125 concentrator (Amicon, Danvers, Ma., U.S.A.) and analyzed for the presence of mouse immunoglobulin heavy chains in a double diffusion Ouchterlony assay, using 0.9 % agarose in Gelman buffer (Gelman, Ann Arbor, Mi., U.S.A.). Purified goat immunoglobulins (Meloy, Springfield, Va., U.S.A.) specific for IgA, IgG_1, IgG_{2a}, IgG_{2b}, and IgG_3 mouse immunoglobulins, and rabbit immunoglobulins (Central Laboratory of the Dutch Red Cross, Amsterdam, The Netherlands) specific for mouse IgM immunoglobulin, were used.

Calculation of Binding Constants

The calculation of antibody binding constants from the results of a direct ELISA has been previously described (Schots et al., 1988). Briefly, antibodies were purified using Na_2SO_4 precipitation and anion exchange chromatography, and then conjugated to horse radish peroxydase (HRP; Boehringer, Mannheim, FRG) according to the method of Nakane & Kawaoi (1974). The immunoglobulin content, and specific activity, of the conjugates were then determined. Finally, binding constants and binding capacities were calculated using the computer program LIGAND (Munson & Rodbard, 1980).

Electrophoresis and blotting

Electrophoresis was carried out on a Mini Protean™ II Dual Slab Cell apparatus (Bio Rad) using gels of 0.75 mm thickness. One dimensional gel-electrophoresis was performed with a protein equivalent of 300 eggs per lane essentially according to Laemmli (1970). For native gels, a separating gel of 7 % (w/v) acrylamide in 0.375 M Tris.HCl pH 8.8 and a stacking gel of 3 % (w/v) acrylamide in 0.125 M Tris.HCl pH 6.8 were used. For denaturing SDS gels, the acrylamide percentages were 13 % (w/v) for the separation and 4 % (w/v) for the stacking gel, respectively.

Two dimensional electrophoresis (2-DGE) was carried out with a protein equivalent of 600-800 eggs per gel. Iso-electric focussing in the pH region 5-5.5 was essentially according to O'Farrell (1975) as modified by Bakker et al. (1988), using for the first dimension 6.6 cm long gels in 7.6 cm long 50 µl-capillaries mounted in a micro-gel electrophoresis chamber (Ernst Schütt, Göttingen, FRG). SDS-PAGE in the second dimension was carried out using a 13 % (w/v) acrylamide separation gel.

Apparent molecular weights were estimated using a mixture of phosphorylase B, bovine serum albumin, ovalbumin, carbonic anhydrase, soybean trypsin inhibitor, and lysozyme as reference proteins (Bio Rad low molecular weight standards). The gels were either used for immunoblotting experiments, or stained according to a modification of the silver staining procedure described by Oakley et al. (1980).

Blotting of gels was performed by electrotransfer of the

proteins from the gel to an immobilon membrane (Millipore, Bedford, Ma.) according to Kyshe-Andersen (1984). Immunodetection of the transferred proteins followed a modification of the procedure described by Ey & Ashman (1986). First, residual binding sites on the immobilon membrane were blocked by incubating the immobilon sheets in 10 mM Tris.HCl pH 7.5, 150 mM NaCl and 1 % (w/v) BSA for 1 hr. Then, the immobilon sheets were incubated overnight at room temperature with the relevant monoclonal antibody appropriately diluted in 10 mM Tris.HCl pH 7.5, 150 mM NaCl, 0.5% (w/v) BSA, 1 % (v/v) Triton X-100, 0.5 % (w/v) sodiumdesoxycholate and 0.1 % (w/v) sodiumdodecylsulphate, followed by three washes in PBS. Thereafter, the membranes were incubated for 2 hrs at room temperature with a goat-anti-mouse antibody conjugated to alkaline-phosphatase (Bio-Rad), diluted 1:500 in the same buffer as the monoclonal antibody. The immobilon sheets were then rinsed thoroughly 3-4 times in PBS, and finally immersed in 100 mM Tris.HCl pH 9.5, 100 mM NaCl, 5 mM $MgCl_2$, 0.017 % (w/v) 5-bromo-4-chloro-indolylphosphate (Boehringer), and 0.034 % (w/v) nitro blue tetrazolium (Sigma), until colour had sufficiently developed.

RESULTS

Three different immunization protocols and routes have been used to generate the hybridomas listed in Table 2. One fusion experiment, carried out after a secondary immunization with a TSPaP-KLH conjugate, resulted in the isolation of three hybridomas producing antibodies binding preferentially with TSPaP. Similar experiments carried out with TSRoP-KLH conjugates, however, did not succeed in hybridomas producing antibodies specific for TSRoP.

We therefore tried a different approach, in which the mice were immunised 'single shot intraspleen' with the antigen spotted on nitrocellulose, accepting the disadvantage that the majority of the resulting hybridomas might be of the IgM isotype. Among the many hybridomas screened, none showed the production of antibodies binding preferentially to TSRoP;

neither were any other hybridomas isolated producing antibodies with apparent specificity for TSPaP.

From preliminary immunoblotting experiments (data not shown) we concluded that most hybridomas produced antibodies reacting with the 20.6/20.8 and/or 20.5/21 kilodalton band from TSRoP and TSPaP, respectively. A third approach (immunization schedule 3) was therefore chosen which made use of SDS-PAGE separated proteins blotted on nitrocellulose as the antigen. This approach resulted in two hybridomas, WGR 11 and 12, producing antibodies binding preferentially with TSRoP.

To establish the specificities of hybridomas WGP 1, 2, 3, and WGR 11 and 12, and to investigate whether the antibodies produced by the other hybridomas isolated showed identical affinities for TSPaP and TSRoP, binding constants (K's) and binding capacities (R's) for these antibodies were calculated according to Schots et al. (1988). The binding constant of an antibody-antigen complex is an estimate of the strength of the bond between antigen and antibody. The binding capacity expresses the number of antigen binding sites (in moles/liter) available to the antibody molecules. Binding constants and capacities were calculated from the results of a quantitative direct ELISA using the computer program LIGAND (Munson & Rodbard, 1980), and are listed in Table 3. Antibodies WGP 2 and 3 clearly favour binding to TSPaP. Antibody WGP 1 binds with "intermediate" preference to protein antigens isolated from Globodera pallida. Antibodies WGR 11 and 12 favour binding to TSRoP. The binding parameters of all other antibodies studied do not differ significantly between the two antigen preparations. Statistical evidence was obtained from LIGAND that both TSRoP and TSPaP have two apparent binding sites for the antibodies WGP 2, 3, 8, and WGR 1, 8, 11 and 12, as exemplified by the two binding constants (K_1 and K_2) in Table 3.

Scatchard-plots (Scatchard, 1949) of bound antibody (B) against the ratio of bound to free antibody (B/F) can be made from the data resulting from the ELISA-experiments. In Figure 1, a Scatchard-plot is given for the binding of antibody WGR 2 to TSPaP or TSRoP. WGR 2 has similar binding constants for TSPaP and TSRoP (Table 3). The binding constant can be inferred from

the slope of the line. The binding capacity, which is also identical for both antigens (Table 3), corresponds to the intercept of the line with the X-axis. Since the Scatchard-plot is a straight line, it can be concluded that WGR 2 apparently has a single affinity for both TSPaP and TSRoP. In contrast, for the binding of antibody WGP 3 to TSRoP or TSPaP, curved Scatchard-plots are obtained as shown in Figure 2A and B. This curved shape can be explained by two apparent binding sites being recognized on both antigens by this antibody. In the case of a curved Scatchard-plot, the binding constants are given by the slope of the two asymptotes of the curve, while the binding capacities are defined as the intercepts of these asymptotes with the X-axis. From Figure 2 can also be derived that at the same concentration of bound antibody, the B/F ratio for the binding of WGP 3 to TSPaP is always a factor 300 larger compared with that for binding to TSRoP (Figure 2C). Consequently, to obtain a similar quantity of WGP 3-TSRoP complex at an antigen concentration equal to that needed for the formation of the WGP 3-TSPaP complex, the concentration of the antibody has to be raised several hundred times. Scatchard-plots obtained with WGP 1 can be interpreted in a similar way (Figure 3), although compared to WGP 3 the difference in affinity of WGP 1 for TSPaP relative to TSRoP is reduced. The straight lines of these plots indicate that WGP 1 recognizes only one apparent binding site on its antigen.

From previous work (Schots et al., 1987) we concluded that both TSRoP and TSPaP consist of four proteins (for apparent M.W.'s and pI's see Table 1), which can only partly be resolved in 1-dimensional electrophoresis. Similar results were obtained here with SDS-PAGE using a micro-electrophoresis system (Figures 4, 5A and 5B). Immunoblotting experiments were carried out to determine which of these proteins were recognized by the monoclonal antibodies from the hybridomas WGP 1, 2, 3, 7 and 8, and WGR 1, 2, 8, 11 and 12. The results, part of which are displayed in Figure 6, show that the monoclonal antibodies with a similar affinity for TSPaP and TSRoP, i.e., WGP 7 and 8 and WGR 1, 2 and 8, together with WGP 1, which has a slightly higher affinity for TSPaP, all bind to the same protein band of 20.7 kD

of G. rostochiensis (Figure 6). With 2-DGE, this protein band resolves into two protein spots, both of which are recognized by the respective monoclonal antibodies (Figure 5B and 5D). The same antibodies, and those from WGP 2 and 3, having a preponderant specificity for TSPaP, all recognize the two protein bands of 20.5 and 21.0 kD from G. pallida (Figures 5A, 5C and 6). We were, however, not able to detect any proteins on the immunoblots with antibodies WGR 11 and 12.

Under native conditions of gelelectrophoresis, species-specific proteins from second stage larvae of G. rostochiensis or G. pallida exhibit Rf values of 0.35, 0.60, 0.65 and 0.75, or 0.24, 0.55, 0.59 and 0.80, respectively (Bakker et al., 1988). Most of these proteins are also represented in thermostable protein preparations from egg homogenates. Blotting experiments showed that the proteins with Rf values of 0.60 and 0.65 from TSRoP and 0.55 and 0.59 from TSPaP correspond with the proteins having apparent molecular weights in an SDS-PAGE of 20.7 kD, and 20.5/21.0 kD respectively.

To verify the specificity of the panel of monoclonal antibodies for protein antigens from the two potato cyst nematode species we have also tested their reactivities with thermostable protein antigens isolated from eggs of 6 other common cyst nematodes: the white and yellow beet cyst nematodes Heterodera schachtii (Schmidt) and H. trifolii (Goffart), the pea cyst nematode H. goettingiana (Liebscher), the cabbage cyst nematode H. cruciferae (Franklin), and the grass cyst nematodes H. bifenestra (Cooper) and H. mani (Mathews). The results of these tests are displayed in Table 4. All monoclonal antibodies which exhibit no preference for either TSRoP or TSPaP, also bind to the proteins of the six other nematode species. WGP 1 is the only antibody which binds specifically with thermostable proteins of the two potato cyst nematode species, although G. pallida proteins are recognized with preference. WGP 2 and 3 only bind to TSPaP. WGR 11 and 12 prefer binding to TSRoP, although some other cyst nematode species are recognized as well.

Table 2. A selected panel of hybridomas producing antibodies against TSRoP and/or TSPaP. The method of immunization is indicated with a numerical as described in materials and methods. WGP and WGR hybridomas are derived from fusion experiments carried out with splenocytes from mice immunized with TSPaP or TSRoP, respectively.

Hybridoma	Isotype	Immunization Schedule	Reactivity with TSPaP	Reactivity with TSRoP
WGP 1	IgG_1	1	+	±
WGP 2	IgG_1	1	+	-
WGP 3	IgG_1	1	+	-
WGP 6	IgG_1	1	+	+
WGP 7	IgM	1	+	+
WGP 8	IgM	1	+	+
WGP 10	N.D.	3	+	+
WGR 1	IgM	2	+	+
WGR 2	IgM	1	+	+
WGR 8	IgM	2	+	+
WGR 10	IgM	2	+	+
WGR 11	IgM	3	±	+
WGR 12	IgM	3	±	+

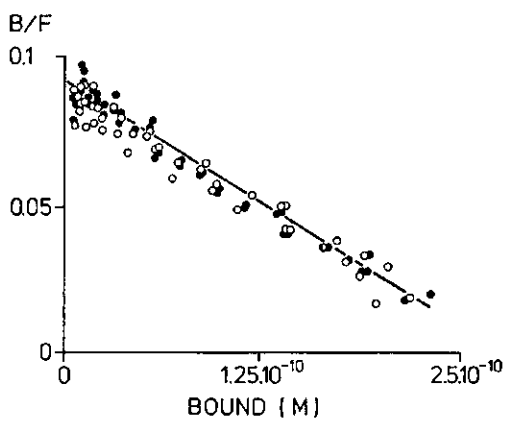


Figure 1. Scatchard plot of bound antibody (B) against the ratio of bound to free antibody (B/F) of antibody WGR 2. Open circles reactivity with G. pallida, Closed circles reactivity with G. rostochiensis.

Table 3. Binding constants (K) and binding capacities (R), as calculated with the computer program system LIGAND, for some of the isolated monoclonal antibodies conjugated to horse radish peroxydase (for methodology see ref. 22).

Hybridoma	Parameters for binding							
	TSRoP				TSPaP			
	K_1[a]	K_2[a]	R_1[b]	R_2[b]	K_1[a]	K_2[a]	R_1[b]	R_2[b]
WGP 1	0.09		7.46		0.53	3.35		
WGP 2	6.09	0.07	0.08	0.04	498.09	15.12	0.33	0.87
WGP 3	1.51	0.03	1.91	1.29	596.78	20.60	0.06	0.34
WGP 7	0.29		5.71		0.37		5.36	
WGP 8	2.72	0.05	0.28	0.76	2.02	0.04	0.48	0.14
WGR 1	4.97	0.05	0.34	0.89	3.74	0.03	0.52	0.20
WGR 2	3.11		28.09		3.35		27.27	
WGR 8	3.71	0.02	0.87	3.81	3.66	0.09	0.48	0.12
WGR 11	1.04	0.004	936.46	6000	0.08	0.0003	5810.8	20000
WGR 12	6.96	0.05	490.14	2000	0.35	0.003	2844.0	6000

[a] Binding constants (K) times 10^8 l/M; [b] binding capacities times 10^{-12} M

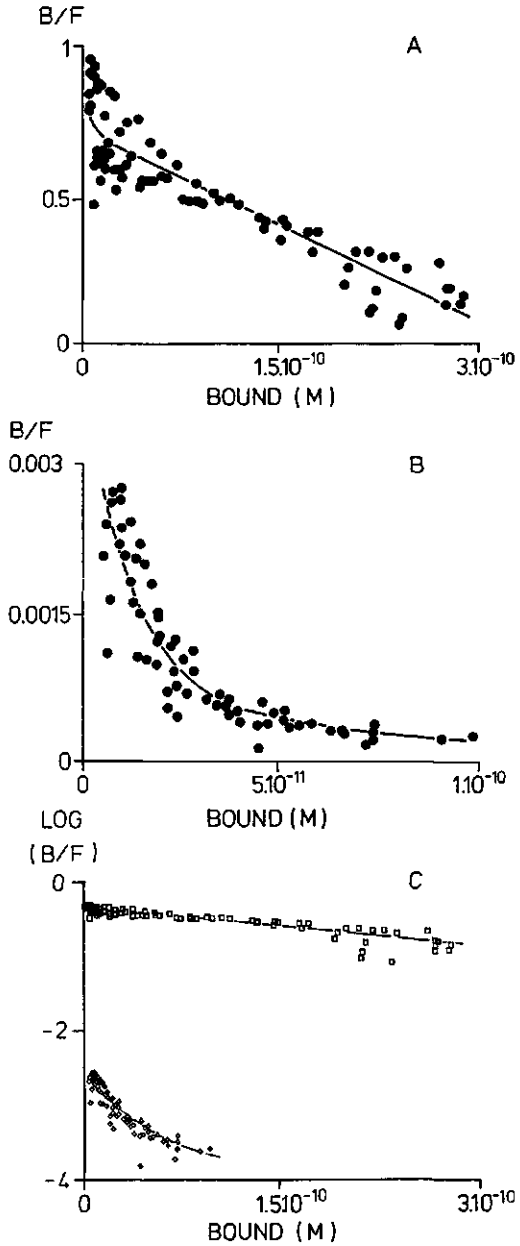


Figure 2. Scatchard plots for monoclonal antibody WGP 3 by displaying bound (B) antibody against log bound/free (B/F). **A.** Reactivity with thermostable protein antigens isolated from G. pallida, **B.** Reactivity with thermostable protein antigens from G. rostochiensis, **C.** Comparison of reactivity of WGP 3 with antigens from G. pallida (squares) or G. rostochiensis (diamonds).

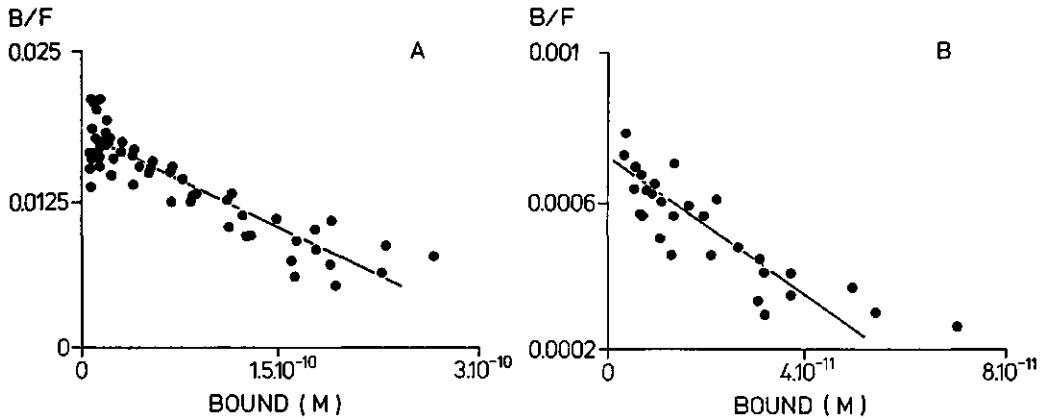


Figure 3. Scatchard plots for monoclonal antibody WGP 1. **A.** Reactivity with thermostable protein antigens from G. pallida, **B.** Reactivity with thermostable protein antigens from G. rostochiensis.

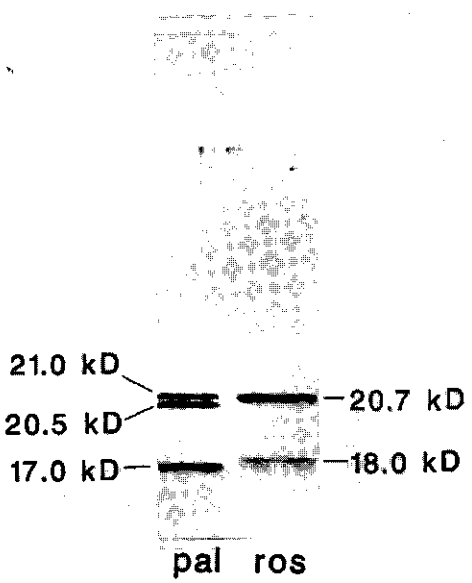


FIGURE 4. One-dimensional electrophoretic (PAGE) protein patterns of thermostable proteins isolated from G. pallida (designated pal) and G. rostochiensis (designated ros).

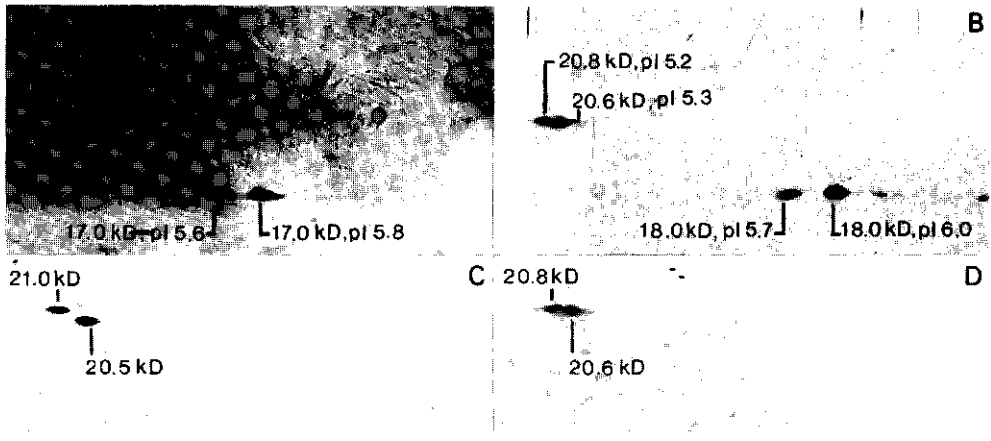


Figure 5. Two-dimensional SDS-PAGE of thermostable proteins from G. pallida **(A,C)** and G. rostochiensis **(B,D)**, before **(A,B)** and after **(C,D)** immunoblotting with monoclonal antibody WGR 8.

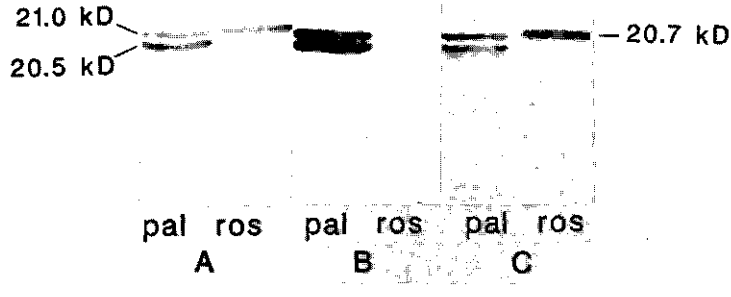
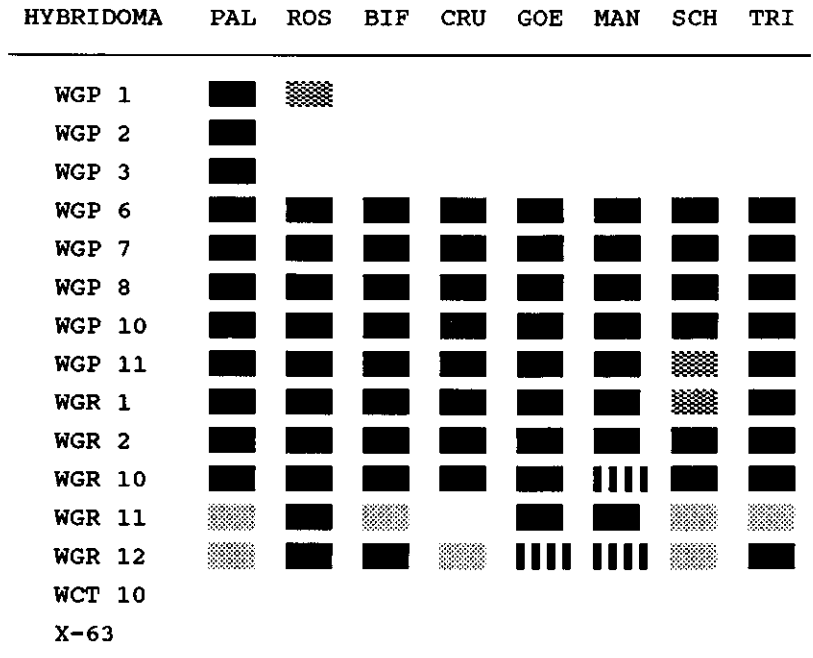


Figure 6. SDS-PAGE of thermostable protein antigens of G. rostochiensis and G. pallida followed by immunoblotting. Antigens are indicated as pal for G. pallida and ros for G. rostochiensis. Lanes **A:** Reaction of monoclonal antibody WGP 1; Lanes **B:** Reaction of monoclonal antibody WGP 2; Lanes **C:** Reaction of monoclonal antibody WGR 1.

Table 4. Reactivity of a selected panel of McAb raised against thermostable proteins from G. pallida (PAL) or G. rostochiensis (ROS) and two control antibodies, X-63-IgG$_1$ and WCT 10-IgM (raised against carp immunoglobulin) with thermostable proteins isolated from the two potato cyst nematode species, and six other common cyst nematodes: Heterodera bifenestra (BIF), H. cruciferae (CRU), H. goettingiana (GOE), H. mani (MAN), H. schachtii (SCH) and H. trifoli (TRI). The antibodies were tested for their ability to bind to thermostable proteins isolated from 50 eggs. No symbol: 0-10 % binding, ▒: >10-50 % binding, ▓: >50-80 % binding, ■: >80-120 % binding, ▌▌▌▌: >120 % binding.



DISCUSSION

There are several examples of the successful identification of species using conventional antisera (Lowenstein, 1985). However, in the case of sibling species this methodology might lack the discriminatory potential because of too large a number of identical epitopes. Theoretically, monoclonal antibodies can be used to overcome this problem. We have indeed been able to isolate hybridomas producing antibodies binding preferentially to thermostable proteins from G. rostochiensis or G. pallida. However, these monoclonal antibodies still show different degrees of cross-reactivity with the other antigen preparation.

In this study it is once more shown that both thermostable protein preparations consist of four proteins differing in pI and M.W.. Based on the results obtained with the immunoblotting experiments in this study, it is presumed that the 20.6/20.8 kD proteins from TSRoP and the 20.5/21.0 kD proteins from TSPaP are homologous, since they are recognized by a single monoclonal antibody. This idea is supported by the fact that the discrepancies in pI between the antigenic TSRoP and TSPaP proteins is small (about 0.1 pH unit for each protein) and differences in apparent molecular weights are negligible. It became further apparent that the 20.6/20.8 kD proteins of TSRoP or the 20.5/21.0 kD proteins of TSPaP are always recognized as a pair. These results, and the facts that (a) the apparent molecular weights of these proteins are nearly equal, and (b) the pI's hardly differ (0.1 pH unit in both cases), suggest that the two antigenic proteins in each species are modifications of the same protein (intra-species variation). This may be the result of differences in posttranslational processes.

Antibodies WGR 11 and 12, having a preponderant affinity for TSRoP, show no reactivity with either TSRoP or TSPaP in immunoblotting experiments. This might be explained by their relative low binding constants. Furthermore, monoclonal antibodies showing a good reactivity in an ELISA do not necessarily produce a good result in another immunoassay (Haaijman et al., 1984). However, WGR 11 and 12 were obtained in a fusion experiment where splenocytes were used obtained from

mice immunised with the 18 kD band of TSRoP (Table 2). It is therefore not unlikely that both antibodies do bind to this protein.

Because we might be dealing with homologous protein antigens from the two sibling Globodera species, the question may be raised why we have obtained monoclonal antibodies with such a strong preference for TSPaP (WGP 2 and 3), and not for TSRoP, although WGR 11 and 12 bind better to TSRoP. Among the essential properties of an antigenic site are its accessibility to an antibody molecule (Novotny et al., 1986, 1987), its flexibility (Tainer et al., 1984; Geysen et al., 1987; Getzoff et al., 1987) or a combination of both (Berzofsky, 1984). It can not be excluded that equivalent structures which are accessible and/or flexible on TSPaP, are in a different conformation or absent on the corresponding protein antigens from G. rostochiensis. This suggestion might conflict, however, with the thermostable properties of TSRoP and TSPaP. Thermostability often is the result of salt bridges on the protein surface, electrostatic interactions, and specific amino acid modifications, that stabilize secondary structures and their interactions (Perutz, 1978; Grütter et al., 1979). It is therefore not surprising that for some proteins, amino acid substitutions have been shown to exert a drastic effect on their thermostability (Yutani et al., 1977; Grütter et al., 1979; Perry & Wetzel, 1984). However, if TSRoP and TSPaP differ in amino acid composition, this apparently has no effect on thermostability. Therefore, such amino acid substitutions are expected to be conservative (e.g., Asp for Glu), hardly affecting ionic bonds (i.e., thermostability), but modifying the protein's antigenic properties (i.e., accessibility to antibody molecules, flexibility, or both). Such a phenomenon has been described for myoglobin (Berzofsky, 1984).

As already has been discussed by others (Berzofsky et al., 1980; Tzartos et al., 1981; East et al., 1982; Smith-Gill et al., 1982; Schroer et al., 1983; Metzger et al., 1984; Hollander et al., 1986), antibody specificity often is a matter of affinity. We have therefore determined binding constants for the antibodies produced by 10 of our hybridomas. The nonspecific antibodies WGP 7 and 8, and WGR 1, 2 and 8 have binding

constants for TSRoP and TSPaP that do not significantly differ. Antibodies WGP 1, 2 and 3 show a preferent binding to TSPaP, and WGR 11 and 12 react better with TSRoP, as exemplified by the respective binding constants. Although binding to TSRoP is detectable, WGP 2 and 3 can be used in concentrations where binding to TSRoP is negligible, while binding to TSPaP in the ELISA system used is maximal. We therefore consider WGP 2 and WGP 3 as species-specific, while WGP 1, WGR 11 and 12 can be designated as of intermediate specificity. Similar results were obtained by Metzger et al. (1984) with a panel of hybridomas producing antibodies to hen egg white lysozyme. Most of these monoclonal antibodies showed a considerable cross-reactivity with lysozymes from other avian species, except for one which only bound to lysozyme from the Bobwhite quail. With respect to our intention to develop a routine potato cyst nematode screening assay, these results, and the observations that monoclonal antibodies sometimes exhibit a specificity for seemingly unrelated antigens (Sperling et al., 1983; Fox & Siraganian, 1986), urged us to check the reactivity of our antibodies with proteins isolated from other common cyst nematodes. It was thus shown that all antibodies which have a similar affinity for TSPaP and TSRoP, also bind to thermostable protein antigens isolated from six other common cyst nematodes. As for the reactivities of WGR 11 and 12 with the thermostable proteins from these Heterodera species, varying results were obtained. The broad reactivity pattern of most monoclonal antibodies correspond with the observation that many, if not all, cyst nematodes of the species Globodera and Heterodera possess thermostable proteins, some having an apparent molecular weight of approximately 17 or 21 kD (Schouten et al., unpublished results).

The calculation of binding constants is of major advantage to the modelling of an "ELISA-field-test". With the method outlined by Rodbard & Bertino (1973), binding constants can be employed to describe the reaction of an antibody with its corresponding antigen simply by solving two equations with two unknowns. Such calculations are useful to predict the effect of different concentrations of antigen and antibody on the scope of

the reaction, and to define the limitations of an assay. Besides, in the case of mixed field populations, they are effective in defining the effect of various concentrations of G. rostochiensis protein on the intensity of the reaction with anti-TSPaP-antibody.

With the reagents and information obtained in this study, it seems possible to develop an ELISA that can be routinely used to screen soil samples for the presence of potato cyst nematodes. The species present in the sample may be identified, and, in the case of mixed field populations, their proportions determined. To achieve this goal, maximally three assays seem necessary. First, WGP 1 has to be used, to confirm whether potato cyst nematodes are present in the soil sample. In a second assay, the total number of cyst nematodes is estimated using an antibody which binds equally well to TSRoP and TSPaP, while in a third assay the number of G. pallida individuals is determined with one of the antibodies WGP 2 or 3. The number of G. rostochiensis individuals can than be calculated by subtraction, taking the result of all three assays into account. The development of such a test is presently under investigation.

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CHAPTER V

SEROLOGICAL DIFFERENTIATION OF THE POTATO CYST NEMATODES GLOBODERA PALLIDA AND G. ROSTOCHIENSIS. III. SELECTION OF REAGENT ANTIBODIES FOR IMMUNOASSAYS.

SUMMARY

The topological relationships between the antigenic sites defined by six monoclonal antibodies (McAbs) directed against thermostable protein antigens from the two potato cyst nematode species Globodera rostochiensis and G. pallida were investigated with a competition ELISA. The rationale of this approach was to select antibodies for a routine assay which discriminates both species in soil samples. Five antigenic sites were delineated on the protein antigens from either species. For two of these sites it could be shown that they are located close to each other. The possibility to select reagent antibodies with this information is demonstrated.

INTRODUCTION

Monoclonal antibodies (McAbs) have become a powerful tool in the analysis of cell populations, (protein) antigens and physiological systems. For a proper and directed application of McAbs, for instance in immunoassays, a structural analysis of the antigen with respect to its recognition by antibody molecules is desired. The last decade, many such data have become available for protein antigens, not the least because of the contribution of McAbs (see Benjamin et al., 1984 for a review). For the majority of the proteins which have thus been immunologically characterized, the amino acid sequence and the three dimensional structure are known, and a structural analysis has also been facilitated by virtue of well documented, naturally occurring molecular variants. However, for many protein antigens such data and variants are not (yet) available. To obtain nonetheless some insight in the antigenic structure of a protein, especially the number and the topological relationship of the antibody-reactive sites or epitopes, competition immunoassays can be performed (Kohno et al., 1982, Miller et al., 1983; Cook et al., 1985; Schwarz et al., 1986). The results obtained from such experiments might be of help to select, for reagent purposes, antibodies of high specificity, and to evaluate cross-reactivity phenomena.

The two sibling potato cyst nematode species Globodera rostochiensis and G. pallida are the causative agents of major losses to potato crop. Among the methods to control their multiplication are the use of potato varieties with resistance to several pathotypes of these Globodera species, and soil disinfection. To expand and direct the use of resistant potato varieties, and to reduce soil disinfection, a quick and reliable routine test for the identification of potato cyst nematodes would be most welcome. We are presently developing an ELISA to distinguish the two sibling species in soil samples. This assay makes use of McAbs directed against proteins isolated from either species (Schots et al., 1988). Most of these McAbs recognize two thermostable proteins in a mixture of four isolated from either species (see Table 1A for some charac-

teristics). In the same study it has been argued that these two proteins are variants, i.e., their origins go back to the same genetic entity, differences in apparent molecular weight and iso-electric point being due to different posttranslational processing.

This study was undertaken to properly select reagent antibodies for a routine laboratory test which can identify the two potato cyst nematode species. For this test, McAbs are available with (a) preponderant specificity for thermostable protein antigens from G. pallida (TSPaP), i.e., WGP 2 and 3, (b) a slight preference for TSPaP, i.e., WGP 1, (c) a slight preference for the thermostable protein antigens from G. rostochiensis (TSRoP), i.e., WGR 11 and 12, and (d) no preference for either of the two protein antigens, i.e., all remaining McAbs (Schots et al., 1988). Furthermore, the McAbs WGP 1, 2 and 3, only recognize thermostable proteins isolated from G. rostochiensis and/or G. pallida, while all other McAbs also exhibit reactivities with thermostable proteins from other commonly occurring cyst nematodes. The criteria whereupon selection is based are the specificity, i.e., the antigenic site recognized by the antibody on the protein antigens from both Globodera species, and the (topological) relationship of this site with those recognized by other antibodies.

MATERIAL AND METHODS

Preparation of protein antigens

Protein antigens were isolated from the potato cyst nematodes G. rostochiensis population MIER (TSRoP), and G. pallida population HPL-1 (TSPaP), as described before (Schots et al., 1987; 1988).

Production and conjugation of monoclonal antibodies

The production of monoclonal antibodies (McAbs) WGP 1, 2, 3, 7 and 8, and WGR 1, 2 and 8, and their initial characterization, have been previously described (Schots et al., 1988; see also Table 1B). The McAbs were purified from ascites fluid by Na_2SO_4-

Table 1. A. Characteristics of the antigenic TSPaP and TSRoP proteins

TSRoP molecular weight	pI	TSPaP molecular weight	pI
20.6	5.3	20.5	5.4
20.8	5.2	21.0	5.3

B. Binding constants (K) and binding capacities (R), as calculated with the computer program system LIGAND, for some of the isolated monoclonal antibodies conjugated to horse radish peroxydase.

	Parameters for binding							
Hybridoma	TSRoP				TSPaP			
	K_1[a]	K_2[a]	R_1[b]	R_2[b]	K_1[a]	K_2[a]	R_1[b]	R_2[b]
WGP 1	0.09		7.46		0.53		3.35	
WGP 2	6.09	0.07	0.08	0.39	498.09	15.12	0.33	8.69
WGP 3	1.51	0.03	1.91	12.92	596.78	20.60	0.06	33.53
WGP 7	0.29		5.71		0.37		5.36	
WGP 8	2.72	0.05	0.28	7.60	2.02	0.04	0.48	13.71
WGR 2	3.11		28.09		3.35		27.27	

[a] Binding constants (K) times 10^8 l/M;

[b] binding capacities times 10^{-12} M

precipitation (Klein, 1985), and conjugated to horse radish peroxydase (HRP) according to Nakane & Kawaoi (1974).

Performance and analysis of the competition ELISA

The ELISA was carried out essentially as previously described (Schots et al., 1988). Briefly, microtitre plates were coated with 0.1 μg of protein and postcoated with BSA. Thereafter, the plates were incubated with an McAb, together with a defined quantity of another antibody coupled to horse radish peroxydase (HRP). The amount of antigen-bound McAb-HRP was then detected with o-phenylenediamine as a substrate, by registration of the optical density at 492 nm in a microtitre plate reader. A starting concentration of the unconjugated McAb was chosen such that it made allowance for the binding constants of the HRP labeled antibodies for TSPaP or TSRoP (Schots et al., 1988).

RESULTS

The reactivities of a panel of six McAbs with the 20.6/20.8 kD proteins of TSRoP, and the 20.5/21.0 kD proteins of TSPaP were investigated by competition ELISAs to establish a topology of the antigenic sites on these antigens. The rationale of this approach was to select antibodies for a routine laboratory test. Each McAb was labelled with HRP, and examined for its ability to bind to either TSRoP, or TSPaP, in the presence of serial dilutions of nonlabeled homologous, as a control, or heterologous antibody. The assay conditions were based upon previously determined parameters of the McAbs as reported in Table 1B, and cross-validation for reciprocal binding effects was experimentally built in by employing all HRP-labeled McAbs also as unlabeled competitor. Antibodies whose binding was reduced by 20 % or more with respect to the result in the absence of the competing antibody, were considered to be inhibited, whereas binding of antibodies in the presence of the unlabelled one to more than 120 % of the control level was supposed to be enhanced.

For the interpretation of the results the following premises

were made: (a) the antibodies were considered to interact with sovereign antigenic sites when in a reciprocal combination each antibody does not affect the binding of its labelled counterpart (i.e., ELISA signal levels between 80 and 120 % of control); (b) binding of an unlabelled antibody alters the conformation of a separate antigenic site for a labelled one when unidirectional, or bidirectional, enhancement of binding is observed; (c) in case a particular antibody combination shows unilateral inhibition of binding, both antibodies either occupy closely spaced binding sites on the antigen, or antibody binding to one site induces a conformational change in another remote site of the antigen, expressed in its decreased interaction with the second antibody; and (d) reciprocal inhibition of antibody binding suggests full or partial identity of antigenic sites, but can also be the result of interactions dealt with in proposition c.

The efficacy of an immunoassay depends on the affinity of the antibody for the antigen. In a competition immunoassay two antibodies are compared, each with a different affinity for the antigen. It is, therefore, necessary to correct for these differences by adjusting the mutual concentrations of the antibodies. This might be difficult when the discrepancies in affinity are large. Since such a situation may exist for some antibody combinations on binding to TSPaP, we have first analyzed the experiments carried out with TSRoP as an antigen, where the binding constants of the six antibodies do not differ very much. The data of the competition ELISAs on TSRoP are listed in Table 2, and allow the following conclusions: i) reciprocal inhibition was seen in the combinations of WGP 2 with WGP 3 and WGP 8, respectively. This might be explained by partial identity, or very close proximity of the antigenic binding sites for these antibodies, or be the result of mutual conformational effects between the separate binding sites of either WGP 3 or 8, and that for WGP 2; ii) all other combinations of antibodies of the panel are characterized by the absence of effects on antibody binding, by unilateral inhibition, or by reciprocal or non-reciprocal enhancement, indicative of independent binding sites for the remaining McAbs

WGP 1, 7 and WGR 2.

The results from the competition ELISA carried out with the six McAbs and TSPaP are given in Table 3. From these data it can be concluded that: i) WGP 2 and 3 clearly inhibit each others binding without interference from WGP 8; ii) in only few of the other combinations unilateral inhibitory or enhancing effects were observed, suggesting independent binding of WGP 1, 7, 8 and WGR 2 to TSPaP.

In a previous study (Schots et al., 1988), it has been argued that the 20.6/20.8 kD proteins of TSRoP and the 20.5/21.0 kD proteins of TSPaP are homologous. This impresssion is based on the observations that these proteins from the two nematode species: (a) are thermostable; (b) are recognized by the same McAbs, but not always with similar affinities; (c) show small discrepancies in iso-electric points; and (d) differ only slightly in apparent molecular weights. It might, therefore, be assumed that the topology of antigenic sites on both antigens is similar. This concept is substantiated by the interpretation of the competition data from our panel of McAbs into a topology of the antigenic sites on both antigens, with the exception of the interrelations of the epitopes established for McAbs WGP 2, 3, and 8. These discrepancies, however, might be explained by the large degree of differences between the binding constants of WGP 2 as well as WGP 3 for the two antigens.

Table 2. Data resulting from competition ELISA's carried out with six McAbs directed against thermostable protein antigens isolated from the potato cyst nematode species G. rostochiensis (TSRoP). The data are presented as a percentage of the control reaction, i.e., the reaction in absence of the competing nonconjugated antibody.

	WGP 1	WGP 2	WGP 3	WGP 7	WGP 8	WGR 2
WGP 1-HRP	44	65	62	100	123	91
WGP 2-HRP	146	26	48	119	74	89
WGP 3-HRP	101	56	51	61	57	103
WGP 7-HRP	140	124	126	49	61	95
WGP 8-HRP	134	67	192	115	41	101
WGR 2-HRP	106	99	97	99	44	1

Table 3. Data resulting from competition ELISA's carried out with six McAbs directed against thermostable protein antigens isolated from the potato cyst nematode species G. pallida (TSPaP). The data are presented as a percentage of the control reaction, i.e., the reaction in absence of the competing nonconjugated antibody.

	WGP 1	WGP 2	WGP 3	WGP 7	WGP 8	WGR 2
WGP 1-HRP	1	103	104	113	109	96
WGP 2-HRP	116	20	19	115	103	107
WGP 3-HRP	113	21	21	101	105	108
WGP 7-HRP	135	118	95	37	85	106
WGP 8-HRP	138	101	106	119	43	97
WGR 2-HRP	93	107	96	102	14	1

DISCUSSION

In the present study at least four antigenic sites were delineated by a panel of six McAbs on both TSRoP and TSPaP antigens. WGP 1 and 7, and WGR 2 each recognize an independent epitope, while the interrelationship of the epitopes recognized by WGP 2, 3 and 8 remains to be clarified. In a number of studies where the topology of epitopes on several species-specific variants of a protein was analyzed with a panel of cross-reactive McAbs, their distribution has been shown to be very similar (East et al., 1982; Smith-Gill et al., 1982; Berzofsky et al., 1982; Berzofsky, 1984; Schroer et al., 1983; Metzger et al., 1984; Benjamin et al., 1984). Since, as already discussed, we have good reasons to believe that TSRoP and TSPaP are homologous, the McAb-delineated antigenic sites also can be supposed to show a comparable distribution over both proteins.

From the results of the competition ELISA, it is possible to obtain information on the reciprocal interactions of two antibodies binding to the same antigen. With such information, predictions can be made for the topological relationship of the respective antigenic sites. However, one should take care not to arrive at conclusions too fast. For example, if inhibition is observed in both, reciprocal, antibody combinations, it is usually accepted to be the consequence of sterical inhibition, but in this study this inference could not be made with regard to the epitopes recognized by WGP 2, 3 and 8.

In several combinations, only one of which was reciprocal (WGP 1/WGP 8 on TSRoP), enhancement of antibody binding was observed. This phenomenon has been described for many protein and viral antigens, in direct competition as well as sandwich competition assays (Tosi et al., 1981, Ehrlich et al., 1982, Lefrancois & Lyles, 1982, Lemke & Hämmerling, 1982, Lubeck & Gerhard, 1982, Nemazee & Sato, 1982, Clegg et al., 1983, Heinz et al., 1984, Cook et al., 1985, Jackson et al., 1985). One explanation might be that the antibody binds with higher affinity to the complexed antigen than to the antigen alone, because of a conformationally changed antigenic determinant. Nemazee & Sato (1982) and Heinz et al. (1984) have argued, that

this feature is a normal component of the humoral immune response, because it would increase the immunogenicity of an antigen when complexed with an antibody. Enhancement can also be explained by an increase through conformational effects on the number of binding sites, i.e., the binding capacity, for an antibody, without their alteration of the affinity (Lemke & Hämmerling, 1982). It should be possible to differentiate between these two mechanisms of enhancement by determination of the binding constants of an antibody with and without the second ligand present.

The information obtained in this, and a previous study (Schots et al., 1988), can be used to choose reagent antibodies for a routine ELISA to qualitatively and quantitatively assay the potato cyst nematode species G. rostochiensis and G. pallida in soil samples. To keep the greatest possible flexibility for the application of the McAbs in an immunoassay, one should avoid the simultaneous use of antibodies which demonstrate interference in binding to the antigen. Furthermore, high binding constants and binding capacities are preferred, as is a properly restricted specificity. In addition, the physico-chemical properties of the antibodies should be considered, e.g., euglobulin precipitation properties of IgM antibodies. Thus, WGP 7 and 8 can be eliminated because they are of the IgM isotype, exhibit relatively low binding constants and binding capacities (Table 1B), are very sensitive to binding interference by other antibodies (Tables 2 and 3), and also react with a number of common cyst nematodes other than G. rostochiensis and G. pallida. Part of these qualifications also fit the McAb WGR 2, but it demonstrates a high binding capacity for the antigens, without interference by other antibodies. Concerning the remaining antibodies, WGP 2 and 3 both show a unique specificity for TSPaP, while the affinity of WGP 1 for TSPaP is six times higher than that for TSRoP.

Thus, WGP 2 or 3 can be used to estimate the number of G. pallida individuals in a soil sample. The number of G. rostochiensis individuals, however, can not be directly determined from an ELISA with one of the available McAbs. It can be calculated by subtraction from the results of immunoassays

carried out with WGP 2 or 3, and WGP 1, respectively. However, WGP 1 exhibits different affinities for TSPaP and TSRoP, which might result in difficulties in the assesment of the number of G. rostochiensis individuals. Therefore, one of the other aspecific McAbs, e.g. WGR 2, would be preferred to determine the total number of potato cyst nematodes, were it not that they also exhibit reactivities with proteins from other common cyst nematodes.

Thus a combined use of WGP 1 and WGR 2 seems necessary to estimate the number of G. rostochiensis individuals. A detailed description of an assay system based on these considerations will be the subject of a future study.

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CHAPTER VI

PREDICTION OF THE REACTIVITY OF MONOCLONAL ANTIBODIES IN IMMUNOASSAYS: CHARACTERIZATION OF AN ELISA TO DETECT POTATO CYST NEMATODES IN SOIL SAMPLES.

SUMMARY

Monoclonal antibodies (McAbs) are typified by uniform physical characteristics. The behaviour of McAbs in an immunoassay can therefore be predicted once the major binding parameters have been determined. Here, a method is described to characterize an indirect ELISA for the qualitative and quantitative determination of the potato cyst nematodes Globodera rostochiensis and G. pallida. This assay makes use of McAbs with properly determined binding constants (K's). The practical implications of such a characterization are demonstrated, with special reference to the phenomenon of "cross-reactivity". Experimental validation was performed using a routine to fit sigmoidal curves. The conditions to be fullfilled for the correct application of the method are discussed.

INTRODUCTION

Since the development of the hybridoma technique by Köhler & Milstein (1975) it is possible to generate large quantities of an antibody with uniform characteristics. Once such characteristics have been determined, they can be used to predict the behaviour of that antibody in an immunoassay. In a previous paper (Schots et al., 1988[a]), a method for the determination of one of the important binding parameters, the binding constant (K), has been described. The binding constant, a measure of the strength of the bond between antigen and antibody, defines the detection limit of an immunoassay, i.e., the ability to distinguish a low analyte concentration as being statistically different from zero (Malan, 1986). The binding constant can be used to calculate the amount of antibody which binds to the corresponding antigen at various concentrations of antigen and antibody (Rodbard & Bertino, 1973; Munson & Rodbard, 1984). By performing such calculations it is possible to obtain information on the ranges of antigen and/or antibody concentrations in which a detectable reaction can be expected.

In our laboratory, an ELISA is under development for the identification in soil samples of the sibling potato cyst nematode species Globodera rostochiensis and G. pallida. Hitherto three suitable monoclonal antibodies (McAbs) have been selected: (1) McAb WGP 2, which has a preponderant specificity for thermostable protein antigens from G. pallida (TSPaP); (2) McAb WGR 2 which binds equally well to thermostable protein antigens from G. rostochiensis (TSRoP) and G. pallida, but also to thermostable protein antigens from other prevailing cyst nematodes and (3) McAb WGP 1, which binds preferentially to TSPaP and also shows a considerable binding for TSRoP, but does not bind to thermostable proteins from other common cyst nematodes. The proportion of G. pallida in a test sample can be properly detected with an ELISA using McAb WGP 2. The proportion of G. rostochiensis has to be calculated from the reaction of the test sample with McAb WGR 2, since we have not yet been able to isolate an McAb with preferent specificity for TSRoP. McAb WGP 1 is used solely to exclude the possibility that thermo-

stable proteins from other cyst nematode species are interfering. These three McAbs were selected because they all recognize a different epitope, present on both thermostable protein antigens isolated from G. rostochiensis and G. pallida (Schots et al., unpublished results).

In this report, a theoretical validation of the proposed assay is described. With the theory of Rodbard & Bertino (1973) and the previous determined binding constants of the monoclonal antibodies (Table 1; Schots et al., 1988[b]), the antibody quantities are calculated that bind at varying concentrations of antigen, or vice versa. From these data one is then able to determine the concentration of antibody which results in sufficient binding with antigens isolated from G. pallida and G. rostochiensis, or, in the case of WGP 2, which lacks detectable binding with antigens isolated from G. rostochiensis. These theoretical results were confirmed in an ELISA carried out with antibodies and antigens in the calculated concentration ranges.

MATERIAL AND METHODS

Reagents

TSRoP and TSPaP were isolated from potato cyst nematode eggs as described by Schots et al. (1987).

The preparation and partial characterization of mouse IgG_1 anti-TSPaP McAbs WGP 1 and 2, and mouse IgM anti-TSPaP/TSRoP McAb WGR 2 have been described by Schots et al. (1988[b]). The binding constants of these antibodies are presented in Table 1. Monoclonal antibodies were purified from ascites fluid with a Na_2SO_4 precipitation (Klein, 1985).

ELISA

The ELISA was carried out as follows: 96-well microtitre plates (Labstar High, Costar Europe Ltd, Badhoevedorp, The Netherlands) were coated overnight at room temperature with 100 µl of a two-fold serially diluted thermostable protein preparation from egg-homogenates of G. rostochiensis or G. pallida in 0.1 M Na-carbonate pH 9.5, the starting concentration being

equivalent to the contents of 200 eggs. The next day the microtitre plates were washed, and filled with 200 µl/well of incubation buffer consisting of PBS with 0.5 % BSA (Organon Teknika, Boxmeer, The Netherlands) and 0.05 % Tween 20 (Merck, Darmstadt, FRG). After 1 hr, the plates were washed, and 100 µl of McAb WGP 2 at a concentration of $3.41*10^{-5}$ mg/ml (equivalent to $2.24*10^{-10}$ M), 100 µl of McAb WGR 2, at a concentration of $1.88*10^{-3}$ mg/ml (equivalent to $2.09*10^{-9}$ M), or 100 µl of McAb WGP 1, at a concentration of $1.17*10^{-3}$ mg/ml (equivalent to $7.70*10^{-9}$ M), all diluted in incubation buffer, were added to each well. After incubation for 2 hours at 37 °C the plates were washed, and 100 µl affinity purified goat-anti-mouse IgG(H+L)-HRP (Bio-Rad, Richmond, Ca.), diluted 1:2000 in incubation buffer, were added to each well. After 2 hours at 37 °C, the plates were thoroughly washed, and the binding of the antibodies to the antigens was then visualized by adding 100 µl substrate solution: 0.1 M citric acid, 0.2 M Na-phosphate, 0.04 % o-phenylenediamine, and 0.04 % H_2O_2, pH 5.0. The plates were placed in the dark for 30 minutes and, the reaction was stopped by adding 50 µl 2.5 M H_2SO_4. The extinction was then read in a microtitre plate-reader (SLT-labinstruments, Gröding, Austria) at 492 nm.

In this ELISA set-up we have tried to establish conditions where the relative proportions of protein antigen that binds to the plastic of the microtitre plate are fairly constant. Antigens were applied at a concentration of less then 200 ng/well, since beyond that level the percentage of proteins binding to the plastic might decline (Salonen & Vaheri, 1979; Cantarero et al., 1980; Larsson et al., 1987).

Mathematical and Computational methods

Curve fitting. The results of an ELISA carried out as described above, usually show, when graphically displayed, a typically smooth, symmetrical and sigmoidal curve with the varying concentration of antigen portrayed on a logarithmic scale. Such a curve can be described by the four parameter logistic function (De Lean et al., 1978; Rodbard et al., 1978):

$$Y = \frac{a - d}{1 + (X/c)^{b}} + d \qquad (1)$$

where "X" and "Y" are the dose (amount of antigen) and response (O.D. 492 nm), respectively, and "a", "b", "c" and "d" denote the following parameters: response at zero dose ("a"), slope factor ("b"), 50 % maximally efficient dose or ED_{50} ("c"), and response at "infinite" dose ("d"). Calculation of these four parameters was performed from the data obtained in the ELISA experiments, using the computer program ALLFIT as developed by De Lean et al. (1978). ALLFIT employs a general nonlinear, least-squares curve fitting routine based on the Marquardt-Levenberg modification of the Gauss-Newton algorithm. Input files were made with LOTUS 1-2-3. ALLFIT was run on an Olivetti M24 personal computer.

Prediction of antibody reactivity. The binding constant (K) belonging to an antigen-antibody complex is a measure of the strength of the bond between antigen and antibody. It is an intrinsic property of an antibody, and can be used to predict the quantity of antibody that will bind to the antigen at equilibrium. The quantity of bound antibody can be calculated from (Feldman, 1972):

$$B = \frac{KRF}{1 + KF} \qquad (2)$$

where "B" denotes the bound antibody concentration; "F" the free antibody concentration; "K" the binding constant of the antibody and "R" the (initial) antigen concentration.

Sometimes, e.g. in the case of WGP 2, an antibody has two apparent affinities for an antigen. The quantity of bound antibody is then calculated from (Feldman, 1972; Rodbard & Bertino, 1973; Munson & Rodbard, 1984):

$$B = \frac{K_1 RF(1 + 2K_2 F)/2}{1 + K_1 F + K_1 K_2 F^2} \qquad (3)$$

where "K_1" denotes the binding constant for the 1st apparent binding site on the antigen, and "K_2" the binding constant for the 2nd apparent binding site on the antigen. The other symbols are the same as in formula (2).

However, in formula (2) and (3) both "B" and "F" are unknowns, and to solve these equations for a given concentration of Ag or Ab the following formula can be applied:

$$B = T - F \qquad (4)$$

with T as the total antibody concentration and the other symbols as in formula (2).

With equations (2) and (4), or equations (3) and (4), we have two equations and two unknowns. A Newton-Raphson algorithm in the computer program KINETICS was employed to solve these equations for varying concentrations of antigen. KINETICS is written in GW-BASIC, and a compiled version is available on request.

RESULTS

Based on the values of the binding constants of WGP 1, WGP 2 and WGR 2 determined before (Schots et al., 1988), equations (2) and (4), or (3) and (4), have been solved for different concentrations of these monoclonal antibodies with varying concentrations of antigens. The goal of these calculations was to find antibody concentrations of WGP 2 where binding to TSRoP is negligible, but to TSPaP is significant. It was found that for WGP 2 antibodies such concentrations are between 1 and $5*10^{-10}$ M. From Figure 1 it can be derived that with a solution of $2.24*10^{-10}$ M WGP 2 monoclonal antibody, $5*10^{-11}$ to 10^{-9} M of solid phase coated antigen will be required to obtain a detectable level of antibodies binding to TSPaP, but not TSRoP. For one well of a microtitre plate, such an amount of antigen can be obtained from 0.5 to 10 eggs. The calculations also show that 10 to 100 times more TSRoP are required to observe significant binding of WGP 2. These antigen concentrations are thus in the range of 10^{-9} to 10^{-7} M, or 10 to 1000 egg equivalents of protein. The foregoing considerations were subsequently verified in an experiment where, after coating a microtitre plate with varying concentrations of TSPaP or TSRoP,

the quantity of bound WGP 2 monoclonal antibody was measured using a second antibody labeled with HRP as a tracer. The results are graphically displayed in Figure 2. The curve was fitted through the datapoints according to the four parameter logistic model using the computer program ALLFIT, and corroborates the theoretical calculations.

Next, it was essential to find an antibody concentration for WGR 2 where a reasonable signal could be obtained with concentrations of TSPaP in a similar range as those established for WGP 2. A concentration of WGR 2 antibodies between 1.5 and $5*10^{-9}$ M was calculated to meet this condition. At $2.09*10^{-9}$ M WGR 2 a theoretical dose response curve was obtained as presented in Figure 3. Under these circumstances, the concentration of antigen should be within a range of 10^{-10} to 10^{-8} M. Consequently, each well should be coated with the thermostable proteins from 1 to 100 egg equivalents of protein of either G. rostochiensis or G. pallida, or a mixture of both species. These considerations were confirmed in a validation experiment (Figure 4).

Monoclonal antibody WGR 2 has been shown to exhibit equal affinity constants for protein antigens from both potato cyst nematode species (Table 1). Therefore, preceding the determination of the proportion of G. rostochiensis in a soil sample, the number of G. pallida eggs has to be calculated from an assay with WGP 2. The proportion of G. rostochiensis can then be determined by subtracting the contribution of G. pallida from the results obtained in an ELISA carried out with WGR 2. Unfortunately, the reactivity of WGR 2 extends to other common cyst nematode species (Schots et al., 1988[b]). From Table 1 can be deduced that monoclonal antibody WGP 1 could be used to confirm the presence in a soil sample of other cyst nematode species than G. rostochiensis and G. pallida. WGP 1 binds with a slight preference to TSPaP, but concurrently displays a considerable reactivity with TSRoP. In an assay, WGP 1 should be applied in a concentration where a reasonable signal is obtained with TSPaP and TSRoP quantities, in the same range as those being used for WGP 2 and WGR 2. Calculations show that this condition is achieved with a concentration of $0.5\text{-}1*10^{8}$ M WGP 1

Table 1. Binding constants of the three McAbs used in this study for TSRoP and TSPaP, as calculated with the computer program system LIGAND (Schots et al., 1988[b]).

	TSRoP		TSPaP	
	K_1	K_2	K_1	K_2
	(*10^{-8} l/M)		(*10^{-8} l/M)	
WGP 1	0.09		0.53	
WGP 2	6.09	0.03	498.09	15.12
WGR 2	3.11		3.35	

Table 2. Estimation of the slope factor (parameter "b", formula (1)), of antibodies WGP 2 and WGR 2, belonging to some of the theoretical (A) and experimental curves (B), with the computer program ALLFIT. For details on the calculations, see Materials and Methods. For the experimental curves the coefficient of variation is given in brackets, as a percentage.

	A (theoretical)	B (practical)
WGP 2 on TSPaP	1.54	1.61 (4.0)
WGR 2 on TSRoP and TSPaP	1.12	1.12 (2.6)

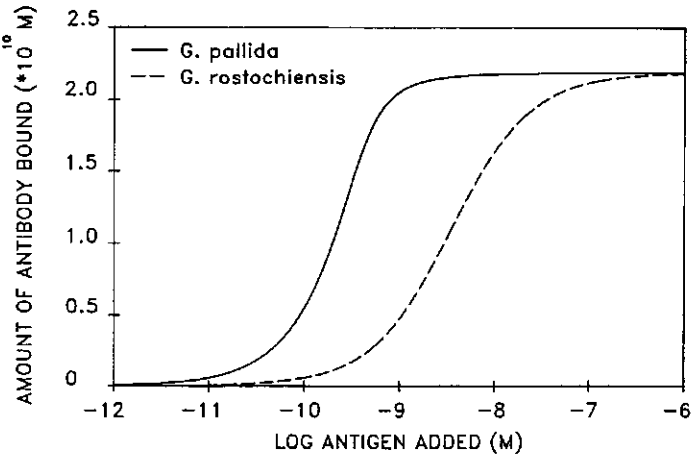

Figure 1. Theoretical curves for the binding of antibody WGP 2 at $2.24*10^{-10}$ M to varying concentrations of TSPaP or TSRoP. The data for these curves were calculated using the computer program KINETICS. On the X-axis the quantities of TSPaP or TSRoP applied to the microtitre plate are given, on the Y-axis the quantity of antibody bound.

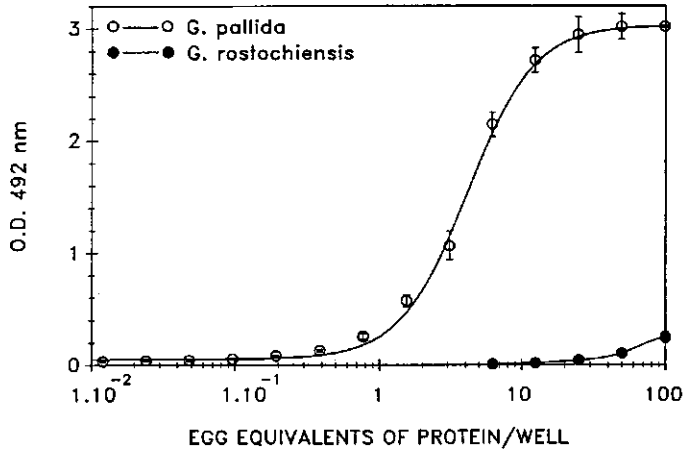

Figure 2. Reactivities of antibody WGP 2 at $2.24*10^{-10}$ M with varying quantities protein antigens isolated from either G. rostochiensis or G. pallida. The curves were fitted using the computer program ALLFIT. On the X-axis the antigen quantity corresponding to the number of eggs from which it was isolated is given, on the Y-axis the optical density readings at 492 nm.

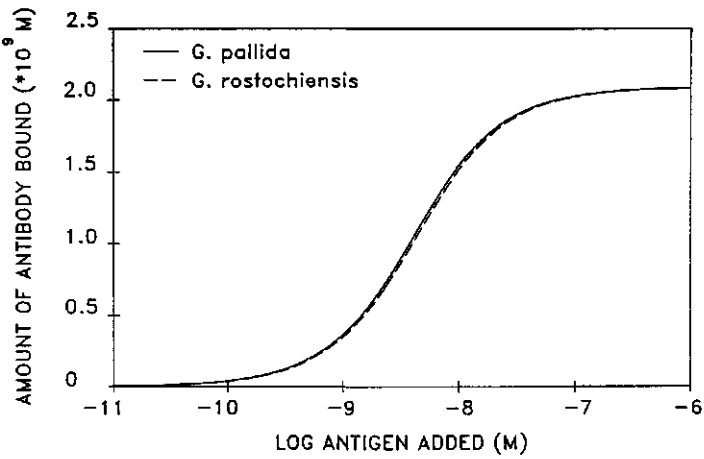

Figure 3. Theoretical curves for the binding of antibody WGR 2 at $2.09*10^{-9}$ M with either TSPaP or TSRoP. Axes and data calculation as in figure 1.

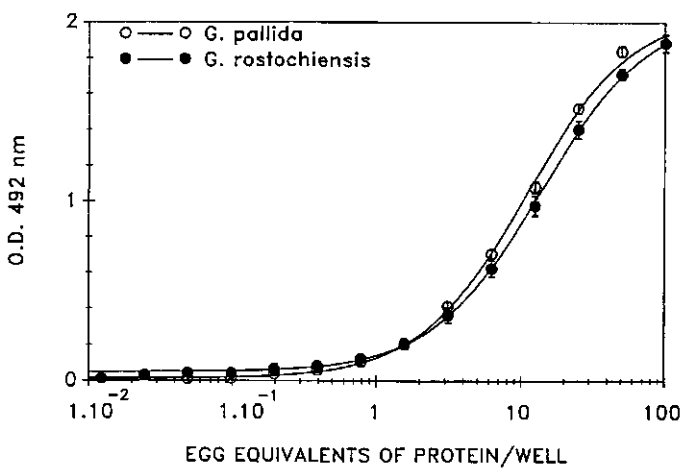

Figure 4. Reactivities of antibody WGR 2 at $2.09*10^{-9}$ M with antigens isolated from G. rostochiensis or G. pallida. The curves were fitted using the computer program ALLFIT. Axes as in figure 2.

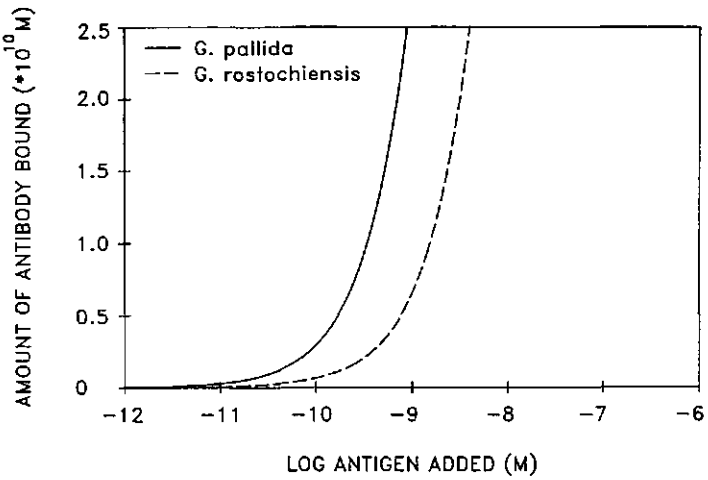

Figure 5. Theoretical curves for the binding of antibody WGP 1 at $7.70*10^{-9}$ M with either TSPaP or TSRoP. Axes and data calculation as in figure 1.

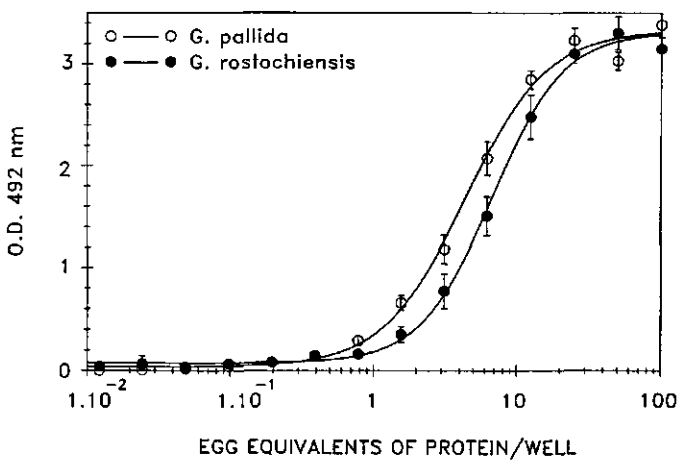

Figure 6. Reactivities of antibody WGP 1 at $7.70*10^{-9}$ M with antigens isolated from G. rostochiensis or G. pallida. The curves were fitted using the computer program ALLFIT. Axes as in figure 2.

antibodies. Computations of the amount of antibody bound to varying concentrations of antigen with $7.7*10^{-9}$ M WGP 1 result in a graph as represented in Figure 5. Because of the low binding constants of WGP 1 for the two antigen preparations relative to the other McAbs, maximal binding of WGP 1 to TSPaP or TSRoP will be obtained at the rather high antigen concentrations of more than 10^{-6} M and $5*10^{-6}$ M, respectively. Since for our purposes we are interested in the binding of WGP 1 within the antigen concentration range of 10^{-10} to 10^{-8} M, only this part of the curve is displayed. However, when the assay is executed, maximal binding of WGP 1 is already observed at an antigen concentration of $5*10^{-9}$ M, or 50 egg equivalents of G. rostochiensis or G. pallida thermostable proteins per well (Figure 6). This discrepancy between theory and praxis originates from the detection limit of the goat-anti-mouse IgG(H+L)-HRP conjugate used. With this second antibody as a tracer, no more than $2*10^{-10}$ M of antigen-bound mouse IgG can be quantified. The same conditions pertain for the assays with McAbs WGP 2 and WGR 2. However, at the concentrations established for these antibodies, the maximum amount of monoclonal antibody bound to the antigen is close to the detection limit of the conjugate which explains the similarity between the anticipated and observed curves as shown in Figures 1 to 4. Since McAb WGR 2 is an IgM, here only the component of the conjugate, with specificity for mouse Ig light chains is expected to bind, resulting in a similar phenomenon of signal limitation at a concentration of $2.09*10^{-9}$ M WGR 2 applied to the assay.

DISCUSSION

The reactivity pattern of a McAb with an antigen in an immunoassay can be predicted with the computer program KINETICS on the basis of the binding constants of the antibody, and the concepts for its interaction with the antigen as developed by Feldman, (1972) and Rodbard and Bertino (1973). From the curves describing the antibody-antigen interactions, parameters can be derived which are of practical use for the development of an

immunoassay. Thus, it is possible to calculate the antibody quantities required in an ELISA with respect to affinity, and to determine the detection limit of the assay under various conditions. Therefore, although validation is still desired, comprehensive empirical studies seem no longer necessary.

The efficacy of predicting the reactivity of our anti-Globodera McAbs was demonstrated in an ELISA for the detection in soil samples of the sibling species of potato cyst nematodes. From the calculations and the validation experiments it was concluded that a quantity of 1 to 50 eggs of both nematode species suffices to provide the thermostable proteins for antigen coating. At these antigen concentrations the number of G. pallida eggs present in a soil sample can be estimated, even in mixed Globodera populations, because TSRoP does not interfere in the reaction of WGP 2 with TSPaP, if this antibody is used in concentrations below $5*10^{-10}$ M. In similar assays, the concentrations of WGR 2 and WGP 1, used to determine the total amount of potato cyst nematodes present, and to investigate whether other cyst nematodes are interfering, were based on the quantity TSPaP needed to obtain a significant reaction with WGP 2.

The model for the interaction between antibody and antigen used in this study is based on the mass action law, i.e., the reactions in an immunoassay are considered to be equilibrium reactions. However, the binding of antibodies to an antigen in a solid phase immunoassay is diffusion-rate limited, and mass transport limitations are likely to occur in an ELISA at high antigen densities and/or binding constants (Nygren & Stenberg, 1985). In this study a fair correlation was found between the predicted and the experimental reactivities of the antibodies. It is, therefore, unlikely that in our experimental conditions mass transport limitations constitute a major factor, thus justifying the application of the mass action law.

Another important issue with respect to the model, is the method used to determine the binding constant, the major parameter on which the prediction is based. The binding constant of an antibody should be determined in an assay similar to the one in which its value is applied to predict the antibody's reactivity (Nygren & Stenberg, 1985; Schots et al., 1988[a]). In

this study an indirect ELISA was used to validate the predicted curves. A direct ELISA reaction could have been performed, but this would have required a concomitant increase of the quantity of the monoclonal antibody, now conjugated to HRP, with a factor 5 to 10, in order to obtain a signal of sufficient strength. Such an approach would have resulted in problems concerning binding of WGP 2 to TSRoP, as can be calculated with KINETICS (Figures 1, 3 and 5). Binding constants of the McAbs were, however, determined in a direct ELISA, and might differ from those of unconjugated McAbs in an indirect ELISA. To test for possible changes in affinity due to differing "K's" of conjugated and unconjugated McAbs, the slope factors (parameter "b", formula (1)) of the theoretical curves were calculated for McAbs WGP 2 and WGR 2, and compared with those from the experimental curves (Table 2). Significant discrepancies in slope factors were not observed, thus confirming that the affinities of unconjugated and conjugated McAbs do not differ drastically. For WGP 1, however, such comparisons were not possible, due to the low binding constant of this antibody and the concentration range of antigen used, which makes only the lower part of the dose-response curve of relevance (Figure 5).

The program ALLFIT was applied to fit a curve through the data points from the ELISA-experiments, in order to verify the predicted dose-response curves. This was possible since the curves exhibited a smooth, symmetrical and sigmoidal shape. Dose-response curves having a "bumpy-shape" due to the reaction of an antibody with two or more apparent binding sites, or a downward curvature at "infinite dose", as a consequence of a cooperativity phenomenon (Munson & Rodbard, 1984), can be fit with a recently described algorithm which uses parallel constrained splines (Guardabasso et al., 1987).

In conclusion, prediction of dose response curves as outlined in this study can be of great help in designing immunoassays with McAbs. Here, we have shown that a prediction can be performed with a concept, based on the mass action law, of the antibody-antigen interaction, which is described by Rodbard & Bertino (1973). The following conditions have to be met: (1) the affinity of the McAb should be determined properly; (2) The

antibody and antigen quantities used should be within limits defined by the mass action law, and (3) the antigen should be applied at a concentration not higher than 200 ng/well since beyond that level the percentage of proteins binding to the microtitreplate might decline. The method, as outlined, can be validated using an appropriate immunoassay and a suitable curve fitting routine, e.g., ALLFIT.

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CHAPTER VII

GENERAL DISCUSSION

Immunoassay for the differentiation of potato cyst nematodes

Distinction between Globodera rostochiensis and G. pallida, based on morphological, biochemical or serological characteristics, has been a topic of many investigations (see chapter I). However, none of the techniques described so far accomodates routine identification purposes. The development of the hybridoma technique (Köhler & Milstein, 1975) has offered new perspectives, because this way monoclonal antibodies with (pre)defined specificity can be obtained. In the work described in this thesis we have tried to establish a basis for the serological identification of the potato cyst nematodes species G. rostochiensis and G. pallida by means of monoclonal antibodies.

Thermostable species-specific proteins were isolated from both G. rostochiensis (TSRoP) and G. pallida (TSPaP), partially characterized (chapter II), and then used as an antigen for the production of species-differentiating monoclonal antibodies (chapter IV). Two antigenic polypeptides specific for G. rostochiensis were identified with apparent molecular weights and iso-electric points (pI) of 20.6 kD, pI 5.3 and 20.8 kD, pI 5.2 respectively. These values slightly differ from those of two antigenic polypeptides specific for G. pallida, which are 21.0 kD, pI 5.3 and 20.5 kD, pI 5.4. These species-specific proteins are probably homologues because of their similar physicochemical properties such as thermostability, their coloration in a silver stained SDS-PAGE gel, and the slight differences in iso-electric points and apparent molecular weights. Additional evidence for homology was obtained from immunoblotting experiments carried out with five non-specific monoclonal antibodies (WGP 7 and 8, and WGR 1, 2 and 8), and one monoclonal antibody (WGP 1) which binds with preference to TSPaP. Because these proteins from either species are always recognized as a pair by

the six monoclonal antibodies as well as by the monoclonal antibodies WGP 2 and 3 which only bind to thermostable proteins from G. pallida, we assume that their origins go back to the same genetic entity; their differences in apparent molecular weight and iso-electric point being the result of differences in posttranslational processing.

The abundance of these species-specific proteins in the soluble protein fraction, and their simple purification, are desirable traits for the development of an immunoassay. Homology of the proteins is another profitable character for an immunoassay to identify of G. rostochiensis and G. pallida, because similar entities are compared.

Before an immunoassay can be developed, monoclonal antibodies and antigens have to be characterized. This is necessary for the selection of reagent antibodies and the evaluation of cross-reactivity phenomena. It has recently become increasingly apparent that specificity of monoclonal antibodies is related to affinity. The latter depends on two binding parameters, the binding constant (K) and binding capacity (R). The binding or affinity constant is a measure for the strength of the bond between antibody and antigen. The binding capacity expresses the number of antigen binding sites available to all antibody molecules. In chapter III, a method is described for the determination of K and R for monoclonal antibodies. A direct ELISA was used and the binding parameters were estimated with the computer program LIGAND (Munson & Rodbard, 1980). LIGAND is a general non-linear curve-fitting program based on the mass action law, with appropriate statistical methodology. The model used describes a heterologous system, and binding parameters can also be estimated in cases where the antigen shows more than one binding site, or where cooperativity, either positive or negative (Munson & Rodbard, 1984), is involved. The method has been tested extensively using monoclonal antibodies against carp immunoglobulin.

In chapter IV, the binding constants from the monoclonal antibodies directed to TSRoP and TSPaP have been determined, and the reactivities with thermostable proteins from other commonly occurring nematodes has also been assessed. Four categories of

antibodies could be distinguished: i) WGP 2 and 3 only bind to TSPaP although a slight reaction with TSRoP was observed in case the concentration of antibody was increased several hundred times. Both antibodies do not recognize thermostable protein antigens from other cyst nematode species. These antibodies are considered to be specific for G. pallida; ii) WGP 1 reacts with the antigens from both potato cyst nematode species, but with a 6-fold higher binding constant for TSPaP compared to that for TSRoP. This antibody also does not exhibit any reactivity with thermostable proteins from other cyst nematodes. Therefore, WGP 1 is considered specific for potato cyst nematodes; iii) WGR 11 and 12 prefer binding to TSRoP, but a considerable extent of cross-reaction is observed with thermostable proteins from G. pallida and other cyst nematodes; iv) All other monoclonal antibodies do not differentiate between G. rostochiensis, G. pallida, or other cyst nematodes.

The question may be raised why we have obtained monoclonal antibodies with such a strong preference for TSPaP and not for TSRoP. Among the essential properties of an antigenic site are its accessibility, its flexibility, or a combination of both (chapter IV). It is possible that equivalent structures which are accessible and/or flexible on TSPaP, are in a different conformation or absent on the corresponding protein antigens from G. rostochiensis. This suggestion might conflict, however, with the thermostable properties of TSRoP and TSPaP. Thermostability is a fragile character easily lost by, for instance, amino acid substitutions. Therefore, differences in protein composition between the TSRoP and TSPaP proteins probably are the result of conservative amino acid substitutions that hardly affect the tertiary structure. The tertiary structure must therefore be very similar. This may result in a limited number of different epitopes. Epitopes giving rise to differentiating antibodies are apparently only present on TSPaP.

The relationship of the antigenic sites recognized by the monoclonal antibodies was studied in competition ELISA experiments as described in chapter V. McAbs were used which bind to the 20.6/20.8 kD proteins from TSRoP and/or the 20.5/21.0 kD proteins from TSPaP. The competition ELISAs delineate at least

four antigenic sites recognized by six antibodies. The antibodies WGP 1, 7 and WGR 2 recognize independent epitopes on TSRoP and TSPaP, which are probably located in all three cases at similar positions on both proteins. The topological relationship of the antigenic sites for WGP 2, 3 and 8 could not be evaluated, probably because of differences in affinity of these antibodies for TSPaP.

The information obtained in this study on the reactivities of monoclonal antibodies with antigens from G. rostochiensis, G. pallida, and other cyst nematodes, their physical parameters, and the epitopes they recognize, offers possibilities to select reagent antibodies for the development of a routine immunoassay based on the following considerations: i) the presence of potato cyst nematodes in a soil sample can be assessed with WGP 1 because it does not bind to thermostable protein antigens of other generally occurring cyst nematodes; ii) the number of G. pallida individuals can be determined with WGP 2 or WGP 3. Numbers of G. rostochiensis can than be calculated from the results of this assay, and that carried out with WGP 1; iii) when necessary, WGR 2 can be used to determine the total number of cyst nematode eggs in a soil sample.

In chapter VI, the theoretical ELISA reactivity patterns of WGP 1, and 2, and WGR 2 have been predicted on the basis of their binding constants, and the concepts on the interaction between antibody and antigen as developed by Rodbard & Bertino (1973). The reactivity patterns were experimentally validated. It is shown that an appropriate signal will be obtained with protein quantities derived from 1 to 50 eggs of either potato cyst nematode species. Based on these experiments an ELISA can be designed which may be used as a routine test for the identification in soil samples of the potato cyst nematode species G. rostochiensis and G. pallida.

Prospects

With the available specific and non-specific monoclonal antibodies, the development of a serological identification method, such as an ELISA, offers excellent opportunities for

large scale identification of the two species. Immunoassays are increasingly applied in agriculture for the detection and quantification of crop diseases, pesticides and naturally occurring compounds. Information obtained from such immunoassays is useful in determining crop rotation patterns, cultivar selection, pesticide selection, pesticide application timing, harvest dates, post harvest handling and many other management aspects. The advantages of immunoassays, over other assays, are found in their speed, simplicity, and relatively low cost. It has to be expected that the development and application of immunoassays will increase in the near future (Lankow et al., 1987). Compared to medical immunoassays, some major differences are found in the matrices to be analyzed in agriculture. In medicine the matrix is usually blood serum or urine. Agricultural chemists and biologists deal with a wide variety of matrices ranging from relatively simple ground water, to complex soils and macerated crop samples. These different conditions add layers of complexity that are not routinely encountered in the clinical laboratory. Furthermore, the complex structure of fungi, bacteria and nematodes make it difficult to develop specific antibodies that could be used in diagnostic tests.

Before we have the disposal of a routine laboratory test for the identification of the potato cyts nematodes G. rostochiensis and G. pallida, based on serological entities as outlined in this thesis, some research is still required. Homogenisation of nematodes is difficult, as a consequence of the structure of the cuticle. A reproducible and simple method based on physical and/or (bio)chemical entities must be developed. Furthermore, the effect on the ELISA and the homogenisation procedure, of organic material remaining in the cyst isolates, in dependence on different types of soil, has to be investigated. Also, the effect of soil fumigants on the protein antigens should be known, i.e., how long TSRoP and TSPaP remain stable in a dead animal and which effect soil fumigants exert on the antigenic properties of these proteins. Finally, the whole assay procedure must be standardized.

Once these problems are solved, an immunoassay is available for the identification of G. rostochiensis and G. pallida. Such

an assay has many advantages compared to other methods for the identification of these parasites as stated in the introduction.
i) An immunoassay is very sensitive. In our hands protein equivalents of less than 1 egg can be detected.
ii) The proportion of G. rostochiensis and G. pallida can be accurately assessed, in case both species are present in a soil sample. Moreover, information on the absolute number of eggs can be obtained without extra effort.
iii) The results of an immunoassay are not influenced by the physiological status of the nematodes, such as the diapause of the eggs.
iv) An immunoassay can be performed rapidly within a period of 24 hours.
v) Immunoassays are cheap and computerizable. Large numbers of samples can be processed in a relatively short period of time.

One can envisage some implications of the introduction of the proposed immunoassay for the cultivation of potatoes, and the control of potato cyst nematodes. Crop rotation and the use of nematicides, together with the cultivation of resistant potato varieties, are essential to control G. rostochiensis and G. pallida. Several disadvantages of this strategy can be mentioned. First, crop rotation limits the possibility of growing potatoes on plots with a low exploitability for other crops. Second, nematicides compose 60% of all pesticides used in the Netherlands, and therefore increasingly encounter objections concerning environmental health. Furthermore, several nematicides, i.e., aldicarb, oxamyl, and ethoprophos show decreasing effectiveness after successive applications for some years because of accelerated degradation as the result of microbial adaptation (Smelt et al., 1987). Recently, accelerated degradation, after repeated application, was also found for the fumigants 1,3-dichloropropene and methamsodium (Smelt, personal communication). Third, the success of resistant cultivars is restricted by the occurrence of virulent cyst nematode populations, the pathotypes.

Because many fields are infested with potato cyst nematodes, current breeding programs are focussed on resistance against most or all pathotypes of both species. This is a major

constraint for the introduction of new cultivars. A reliable screening test to characterize field populations according to species will facilitate the incorporation of genes for resistance effective against either G. rostochiensis or G. pallida. The low reproduction rates (potato cyst nematodes have only one generation cycle in a growing season), and the low dispersal abilities are in favour for such a control strategy. As far as is known, selection towards alleles for virulence is also relatively slow. Resistant potato cultivars can therefore be used for several years.

Furthermore, the resistant cultivars currently available in the Netherlands can be used more efficiently. For example, in case G. pallida is found in a plot, it is useless to grow cultivars with resistance derived from Solanum tuberosum ssp. andigena and S. vernei 58.1642/4, because it has clearly been established that such cultivars are susceptible to all pathotypes of G. pallida in Europe.

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SUMMARY

The potato cyst nematode species G. rostochiensis and G. pallida are a threat to the cultivation of potatoes. Their presence in the soil embodies a potential financial loss to the farmer either because of harvest reduction, or because of rejection of seed potatoes, and other crops with adhering soil, for certification. Together with crop rotation and the use of nematicides, resistant potato varieties are essential for the control of these parasites. A reliable, simple and quick screening test to characterize and monitor field infestations of potato cyst nematodes according to species should improve possibilities for control, by means of cultivating resistant potato varieties. Both parasites are hard to distinguish, and considerable attention has been paid to the identification of these nematode species by methods other than those based on morphological characteristics. Such methods have mostly been based on biochemical entities, but have not proven suitable for routine applications. In this thesis, the outlines are presented of an immunoassay, which is based on species-differentiating monoclonal antibodies, and which can be used for routine purposes.

To raise species-differentiating (monoclonal) antibodies, species-specific antigens must be available. In chapter II the purification of two major groups of heat-stable proteins from homogenates of eggs of G. rostochiensis and G. pallida is described. SDS-polyacrylamide gel electrophoresis revealed two bands specific for G. rostochiensis with apparent molecular weights of 20.7 kD and 18.0 kD, and three bands specific for G. pallida with apparent molecular weights of 21.0 kD, 20.5 kD and 17.0 kD. Two-dimensional gel electrophoresis revealed that actually four thermostable proteins are present in either nematode species, of which two (present in the 17.0 kD and 18.0 kD band respectively) have the same apparent molecular weight but differ in iso-electric point. Conventional antisera made against proteins of either one of the Globodera species were shown to exhibit a strong cross-reactivity with these species-

specific proteins.

Monoclonal antibodies (McAbs) hold the promise of better differentiating reagents since they recognize a single epitope with a possible reduction in the extent of cross-reactivity. The development of such antibodies using thermostable proteins of either nematode species as an antigen source is described in chapter IV. The antibodies produced by three of the hybridomas (WGP 1, WGP 2 and WGP 3) bind preferentially to thermostable protein antigens of G. pallida while two other hybridomas (WGR 11 and WGR 12) produce antibodies which prefer binding to proteins of G. rostochiensis. Most of the hybridomas which were isolated, however, produce antibodies which bind to thermostable proteins from both potato cyst nematode species. To quantitate the differences in affinity, binding constants were determined (chapter IV) according to the method as described in chapter III. In immunoblotting experiments, it was demonstrated that all G. pallida specific, and some aspecific, McAbs bind to the same two proteins in a mixture of four thermostable proteins from either G. pallida alone, or G. rostochiensis and G. pallida. Besides their very similar physico-chemical characteristics this can be interpreted as another indication that these proteins are homologous. In addition, the reactivity with thermostable proteins from other commonly occurring cyst nematodes was also investigated. Three categories of McAbs could be distinguished: i) WGP 2 and WGP 3, which only bind to thermostable proteins from G. pallida, ii) WGP 1, which binds to proteins of both G. pallida and G. rostochiensis, and iii) all other McAbs, which bind to thermostable proteins of potato cyst, and other cyst nematodes. In chapter V, the topological relationship between the antigenic sites on the G. rostochiensis and G. pallida antigens as defined by six of these McAbs was determined with a competition ELISA.

From the McAbs so far characterized, a deliberate choice was made with respect to the development of a routine test for the identification of the potato cyst nematodes G. rostochiensis and G. pallida. In chapter VI, the reactivity of these McAbs in a direct ELISA was predicted and verified with the use of the formerly established binding constants.

Thus, the McAbs obtained and characterized in this study establish a basis for the serological identification of the potato cyst nematode species G. rostochiensis and G. pallida by an immunoassay. In chapter VII, the implications of such an assay for the reduction of nematicide applications, and the utilization of resistant potato varieties is discussed.

SAMENVATTING

In deze samenvatting is getracht de inhoud en de doelstellingen van dit proefschrift ook voor niet-ingewijden duidelijk te maken. Degenen die geïnteresseerd zijn in meer vaktechnische details worden verwezen naar de "Introduction" en "General Discussion".

Aardappelcysteaaltjes, de veroorzakers van aardappelmoeheid, vormen een bedreiging voor de aardappelteelt in Nederland en daarbuiten. Het genencentrum van deze nematoden is gelegen in het Andesgebergte in Zuid-Amerika waar zij co-evolueerden met hun belangrijkste waardplant, de aardappel. Introductie in Europa vond waarschijnlijk plaats in de tweede helft van de vorige eeuw met aardappelen die werden ingevoerd in verband met de veredeling op resistentie tegen de schimmel Phytophtora infestans, de veroorzaker van de aardappelziekte. Momenteel worden aardappelcysteaaltjes aangetroffen in bijna alle gebieden waar aardappelen worden verbouwd. Door de intensieve teelt in de eerste helft van deze eeuw, waarbij geen rekening werd gehouden met vruchtwisselingsschema's, kon het aaltje zich onopgemerkt verspreiden en vermeerderen. In de veertiger en vijftiger jaren werd pas goed duidelijk welk een schade door deze nematoden wordt veroorzaakt en werden, onder andere in Nederland, wettelijke maatregelen getroffen.

Het aardappelcysteaaltje werd in 1923 door Wollenweber beschreven als Heterodera rostochiensis, vernoemd naar de vindplaats Rostock in Duitsland. Sinds 1973 is bekend dat er twee soorten aardappelcysteaaltjes zijn: Globodera rostochiensis en G. pallida. Ofschoon beide soorten reeds miljoenen jaren geleden zijn gedivergeerd, zijn ze op grond van uiterlijke kenmerken niet of nauwelijks van elkaar te onderscheiden.

De bestrijding van aardappelcysteaaltjes in Nederland berust op combinaties van: i) vruchtwisseling, ii) chemische grondontsmetting en iii) het gebruik van resistente aardappelrassen. Er kleven echter meerdere nadelen aan deze bestrijdingsstra-

tegie. Ten eerste beperken verplichte vruchtwisselingsschema's de teelt van aardappelen, en dwingen de boer gewassen te verbouwen die financieel minder opbrengen. Ten tweede vormen de bodemfumigantia een zware belasting voor het milieu. Jaarlijks wordt ongeveer 40.000 ha. ontsmet met 1,3-dichloorpropeen en methylisothiocyanaat. Het aandeel van deze nematiciden bedraagt, uitgedrukt in volume, ongeveer 60% van alle in Nederland toegepaste bestrijdingsmiddelen. Recent onderzoek heeft tevens aangetoond dat na herhaald toepassen de bodemfumigantia op veel percelen versneld worden afgebroken en daardoor minder effectief zijn voor de bestrijding van aaltjes. Dit is het gevolg van een verschuiving in de microflora resulterend in een versnelde omzetting van deze bestrijdingsmiddelen in inactieve verbindingen. Ten derde resistente aardappelrassen zijn niet effectief tegen alle populaties (pathotypen) van aardappelcysteaaltjes. Tot nu toe zijn in Europa acht pathotypen gedefinieerd, vijf binnen de soort G. rostochiensis (Ro_1-Ro_5) en drie binnen de soort G. pallida (Pa_1-Pa_3). De pathotypen worden gekarakteriseerd aan de hand van hun vermogen zich te vermeerderen op toetsplanten met verschillende vormen van resistentie ("differentials").

De bestrijding van aardappelcysteaaltjes met behulp van resistentie rassen zou verbeterd kunnen worden wanneer een snelle en eenvoudige toets beschikbaar is waarmee op praktijkschaal kan worden aangetoond welke van beide soorten aaltjes in een grondmonster aanwezig zijn (kwalitatieve toets) en, in het geval van mengpopulaties, het aandeel van beide soorten kan worden bepaald (kwantitatieve toets).

Identificatie van de beide soorten aardappelcysteaaltjes op basis van morfologische kenmerken is zelfs voor specialisten tijdrovend, en daarom onmogelijk op grote schaal uit te voeren. De aanwezigheid van soorten en hun pathotypen in grondmonsters wordt momenteel vastgesteld in een biotoets, waarin de vermeerderingsfactoren van aardappelcysteaaltjes op aardappelplanten, met verschillende resistentie-eigenschappen in potten in een kas of in gesloten containers, wordt vastgesteld. Deze methoden

zijn tamelijk arbeidsintensief en geven variabele resultaten. Biochemische methoden bieden een beter perspectief voor de identificatie van G. rostochiensis en G. pallida. Met behulp van een aantal gevoelige eiwitscheidingstechnieken (elektroforese) is het mogelijk G. rostochiensis en G. pallida te onderscheiden op basis van soortspecifieke eiwitten. Beide soorten bevatten een aantal eiwitten die in geringe mate, onder andere in grootte en lading, van elkaar verschillen. Nadeel van deze biochemische methoden is dat ze eveneens arbeidsintensief zijn en daardoor te duur voor routine doeleinden.

De laatste decennia zijn in de landbouw serologische technieken in zwang gekomen. Deze zijn vaak goedkoop en zijn bovendien te automatiseren. Voordat een serologische toets kan worden uitgevoerd moet echter een antiserum worden verkregen. Hiertoe wordt een dier (bijvoorbeeld een konijn) ingespoten met een lichaamsvreemde stof, het antigeen (bijvoorbeeld een eiwit). In reactie hierop worden antistoffen gemaakt die aan het eiwit binden. Antistoffen worden door witte bloedcellen gemaakt en uitgescheiden in het bloed. Antiserum verkregen uit dit bloed wordt gebruikt in serologische toetsen. Deze conventionele antisera kunnen echter ook aspecifiek reageren, dat wil zeggen de antistoffen binden ook aan, meestal verwante, eiwitten waartegen de antisera niet opgewekt zijn. Dit is ook het geval met antisera die opgewekt werden tegen eiwitten uit G. rostochiensis. Antistoffen uit deze sera geven eveneens een reaktie te zien met eiwitten van G. pallida. Dergelijke antisera zijn dus onbruikbaar in routinetoetsen voor soortsonderscheiding van aardappelcysteaaltjes.

Een conventioneel (of polyklonaal) antiserum is een mengsel van antistoffen. Op het antigeen komen verschillende oppervlaktestructuren voor, tegen elk waarvan een geïmmuniseerd dier antistoffen maakt. Eén witte bloedcel produceert slechts één type antistof, en één type antistof bindt slechts aan één oppervlaktestructuur. Het is waarschijnlijk dat de soortspecifieke eiwitten van G. pallida en G. rostochiensis naast overeenkomende ook individuele oppervlaktestructuren bezitten, die verschillend

zijn. Antistoffen specifiek voor deze structuren binden wel aan het eiwit uit de ene maar niet aan het eiwit uit de andere soort. Het specifieke karakter van deze antistoffen in een conventioneel antiserum wordt echter gemaskeerd door de in overmaat aanwezige aspecifiek reagerende antistoffen. Indien een witte bloedcel die deze specifieke antistof maakt, geïsoleerd en tot deling gebracht kan worden heeft men de beschikking over een specifiek antiserum. Een techniek om deze (monoklonale) antistoffen te verkrijgen werd in 1975 door Köhler en Milstein ontwikkeld.

Witte bloedcellen die antistoffen maken bevinden zich onder andere in de milt. Door de milt uit het geïmmuniseerde dier te nemen krijgt men de beschikking over veel witte bloedcellen die antilichamen uitscheiden gericht tegen het ingespoten antigeen. Om deze cellen buiten het lichaam in leven te houden worden ze gefuseerd met tumorcellen. De gefuseerde cel, hybridoma, heeft de eigenschappen van beide oudercellen, hij scheidt monoklonale antistoffen uit en kan onbeperkt delen. Op deze wijze verkrijgt men cellijnen (klonen) die antistoffen maken, specifiek voor één oppervlaktestructuur.

Om met behulp van monoklonale antistoffen de beide soorten aardappelcysteaaltjes te onderscheiden is eerst gezocht naar geschikte eiwitten die soortspecifiek zijn en die als antigeen kunnen dienen. In hoofdstuk II zijn dergelijke eiwitten beschreven, alsmede een methode om deze te zuiveren. Na homogenisatie van nematoden en verhitting in kokend water blijven een aantal soortspecifieke eiwitten in oplossing. Deze thermostabiele eiwitten zijn gebruikt om muizen te immuniseren (hoofdstuk IV). Met behulp van de hybridomatechniek zijn cellijnen verkregen die monoklonale antistoffen uitscheiden die gericht zijn tegen soortspecifieke eiwitten van aardappelcysteaaltjes. In hoofdstuk IV is ook bepaald hoe sterk de monoklonale antilichamen aan de antigenen uit G. rostochiensis en G. pallida binden. De methode die voor deze bepaling werd gevolgd is beschreven in hoofdstuk III. Aan de hand van deze bepaling is het mogelijk de specificiteit, of aspecificiteit,

van de antistoffen te kwantificeren. De antistoffen geproduceerd door hybridomakloon WGP 1 binden met voorkeur aan thermostabiele eiwitten afkomstig uit G. pallida, maar ook, in mindere mate, aan hittestabiel eiwit uit G. rostochiensis. Die van WGR 11 en WGR 12 prefereren binding aan antigenen van G. rostochiensis. De antistoffen WGP 2 en WGP 3, specifiek voor G. pallida, bezitten echter ook een enige honderden malen lagere affiniteit voor de eiwitten uit G. rostochiensis. Bij normaal toegepaste concentraties antistof kan geen binding met de eiwitten uit G. rostochiensis worden gemeten. Alle aspecifiek reagerende antistoffen binden even sterk aan de hittestabiele eiwitten uit beide soorten aardappelcysteaaltjes.

Naast aardappelcysteaaltjes kunnen ook andere cysteaaltjes in de bodem worden aangetroffen zoals bietecysteaaltjes, koolcysteaaltjes, grascysteaaltjes of klavercysteaaltjes. Ook deze cysteaaltjes bevatten hittestabiele eiwitten. Alle aspecifieke antistoffen, alsmede WGR 11 en WGR 12, binden ook aan deze eiwitten. WGP 1 is specifiek voor hittestabiele eiwitten uit aardappelcysteaaltjes. De antistoffen die specifiek aan eiwitten uit G. pallida binden (WGP 2 en WGP 3), hebben geen affiniteit voor hittestabiele eiwitten uit andere cysteaaltjes.

Met deze informatie en die uit hoofdstuk V, waarin is onderzocht of antistoffen aan overeenkomstige oppervlaktestructuren van de betrokken eiwitten binden, zijn een aantal antistoffen geselekteerd die geschikt zijn voor het ontwikkelen van een praktijktoets. De opzet van deze praktijktoets is theoretisch geanalyseerd, en experimenteel geëvalueerd, in hoofdstuk VI. In die experimenten is vastgesteld dat het mogelijk is om een soortsidentifikatie van aardappelcysteaaltjes uit te voeren met eiwithoeveelheden uit minder dan één ei. De toets zou er als volgt uit kunnen zien: eerst wordt de aanwezigheid van aardappelcysteaaltjes in het monster bepaald met behulp van WGP 1 (aan/-afwezigheidstoets). Vervolgens wordt met behulp van WGP 2 of WGP 3 het aandeel van G. pallida individuen in het monster vastgesteld. Aan de hand van de uitslag van beide toetsen kan het aandeel van beide soorten aardappelcysteaaltjes in het monster worden berekend (kwantitatieve toets). Eén van de

aspecifiek reagerende antistoffen (WGR 2) kan eventueel worden gebruikt om vast te stellen of ook nog andere cysteaaltjes in het monster aanwezig zijn.

Bij de toepassing van immunologische toetsen in de landbouw heeft men te maken met een grote verscheidenheid aan monstertypes, variërend van relatief eenvoudig in fysische en chemische eigenschappen, zoals grondwater, tot zeer complex, zoals grond of plantesappen. In tegenstelling tot in de klinische chemie, waar vaak serum- of urinemonsters met goed omschreven karakteristieken worden bewerkt, bemoeilijken de omstandigheden in de landbouw het vinden van de juiste condities waaronder het monster moet worden geanalyseerd. Zo moet voor de onderhavige toets:
i) vastgesteld worden hoe een goede homogenisatie van de aaltjes verkregen wordt.
ii) onderzocht worden wat het effect van resten organisch materiaal in het monster is.
iii) bekeken worden in hoeverre de toets beïnvloed wordt door grondontsmettingen.
iv) onderzocht worden op welke wijze de toets gestandaardiseerd kan worden.

Wanneer deze problemen opgelost zijn, krijgt men de beschikking over een praktijktoets waarmee G. rostochiensis en G. pallida snel en routinematig in grondmonsters kunnen worden aangetoond. De voordelen van zo'n serologische soortsidentificatie boven een biotoets zijn:
i) een serologische toets is zeer gevoelig. In een ELISA kan soortsidentificatie worden uitgevoerd met de hoeveelheid thermostabiel eiwit uit één ei of minder.
ii) in geval G. pallida en G. rostochiensis beide aanwezig zijn kan het aandeel van de soorten nauwkeurig worden vastgesteld. Daarenboven kwantificeert deze toets, zonder extra inspanningen, populatiedichtheden.
iii) een serologische toets is onafhankelijk van de diapauze waarin de larven kunnen verkeren en kan dus op elk gewenst moment worden uitgevoerd.

iv) een serologische toets is snel, binnen 24 uur kan de uitslag bekend zijn.
v) een serologische toets is over het algemeen goedkoop en eenvoudig te automatiseren; grote hoeveelheden monsters kunnen in relatief korte tijd worden verwerkt.

Realisatie van deze serologische toets heeft een aantal implicaties voor de praktijk. Na soortsidentificatie kan een gerichter advies gegeven worden voor de teelt van resistente aardappelrassen. Aangezien de toets niet direkt uitsluitsel verschaft over de virulentie-eigenschappen van populaties moet een dergelijk teeltadvies mede gebaseerd worden op andere informatie, zoals gegevens over de verspreiding van pathotypen in een gebied of de voorgeschiedenis van een perceel voor wat betreft de tot dan toe verbouwde vatbare of resistente rassen. Bijvoorbeeld, indien G. pallida aangetroffen wordt, is het niet zinvol om aardappelrassen te verbouwen met resistentie afkomstig uit Solanum tuberosum ssp. andigena of S. vernei 58.1642/4, omdat deze resistenties onwerkzaam zijn tegen alle in Nederland voorkomende pathotypen van G. pallida. In geval een populatie geïdentificeerd wordt als G. rostochiensis zal, indien jarenlang vatbare rassen zijn geteeld, deze populatie zich waarschijnlijk niet of matig vermeerderen op een ras met resistentie afkomstig van S. vernei 58.1642/4. Hoge vermeerderingsfactoren van G. rostochiensis populaties op dergelijke aardappelrassen zijn zelden waargenomen in Nederland. Uit deze voorbeelden blijkt dat soortsidentificatie gebaseerd op een serologische toets in belangrijke mate kan bijdragen aan te ontwikkelen systemen voor perceelsgerichte geleide bestrijding van aardappelcysteaaltjes.

Voorts biedt een snelle soortsidentificatie meer mogelijkheden voor de resistentieveredeling. Op het ogenblik verloopt het ontwikkelen van resistente aardappelrassen met goede landbouwkundige eigenschappen langzaam. Dit is onder meer het gevolg van zware toelatingseisen ten aanzien van resistente aardappelrassen. Bijvoorbeeld, een nieuw ras met resistentie tegen pathotype Pa_2 wordt bij voorkeur toegelaten op de rassenlijst indien het ook resistent is tegen de belangrijke, in

Nederland voorkomende, pathotypen van G. rostochiensis. Zodra routinematig soortsonderscheid gemaakt kan worden kunnen ook rassen met resistentie tegen uitsluitend G. rostochiensis of G. pallida worden gebruikt. Hierdoor kan de ontwikkeling van nieuwe resistente rassen zonder twijfel worden versneld.

Bovendien kan worden ingespeeld op de voorstellen tot wijziging van het Besluit Bestrijding Aardappelmoeheid (B.B.A.). In het gewijzigde B.B.A. wordt de verplichting tot grondontsmetting afgeschaft en berust de bestrijding in hoofdzaak op vruchtwisseling en het gebruik van resistente rassen. Evenwel niet aangegeven is welke resistente rassen men in voorkomende situaties dient te gebruiken, omdat momenteel de mogelijkheden voor het geven van een teeltadvies te beperkt zijn.

CURRICULUM VITAE

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