Fundam. appl. Nematol., 1992, **15** (1), 55-61.

Quantitative ELISA for the detection of potato cyst nematodes in soil samples

Arjen SCHOTS[1][2][3], Fred J. GOMMERS[2] and Egbert EGBERTS[1]

[1] *Department of Experimental Animal Morphology and Cell Biology, Agricultural University, P.O. Box 338, 6700 AH Wageningen, The Netherlands, and*

[2] *Department of Nematology, Agricultural University, P.O. Box 8123, 6700 ES Wageningen, The Netherlands.*

Accepted for publication 30 January 1991

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.

Résumé — ***Méthode quantitative pour détecter les nématodes à kystes de la pomme de terre,* Globodera rostochiensis *et* G. pallida, *dans les échantillons de sol*** — Des anticorps monoclonaux (McAbs) ont été typés à partir de caractéristiques physiques uniformes. Le comportement des McAbs dans un immunoessai a pu donc être prévu une fois que les principaux paramètres de liaison aient été déterminés. Les auteurs décrivent une méthode pour caractériser un ELISA indirect en vue de la détermination qualitative et quantitative des nématodes à kystes de la pomme de terre *Globodera rostochiensis* et *G. pallida*. Ils établissent l'utilisation d'anticorps monoclonaux sur des constantes de liaisons (K's) parfaitement déterminées. Les implications pratiques d'une telle caractérisation sont démontrées, en prenant particulièrement en compte le phénomène de réaction croisée. Une étude expérimentale a été effectuée pour déterminer la portion de la courbe sigmoïde utilisable de façon routinière. Les conditions nécessaires pour une utilisation correcte de la méthode sont discutées.

Key-words : *Globodera*, determination, antibodies, potato.

Since the development of the hybridoma technique by Köhler and 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 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*. 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 several other cyst nematodes and (3) McAb WGP 1, which binds pre-

[3] *Present address : Laboratory for Monoclonal Antibodies, P.O. Box 9060, 6700 GW Wageningen, The Netherlands.*

* Abbreviations : ELISA, enzyme-linked immunosorbent assay; McAb, monoclonal antibody; K, binding constant; OD, optical density; TSPaP, thermostable protein antigens isolated from *Globodera pallida*; TSRoP, thermostable protein antigens isolated from *Globodera rostochiensis*.

ISSN 1164-5571/92/01/55 07 $ 2.70 / © Gauthier-Villars - ORSTOM

ferentially 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 a McAb with preferent specificity for TSRoP. McAb WGP 1 is used solely to exclude the possibility that thermostable 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.*, unpubl.).

In this report, a theoretical validation of the proposed assay is described. With the theory of Rodbard and Bertino (1973) and the previous determined binding constants of the monoclonal antibodies (Table 1; from Schots *et al.*, 1989), the antibody quantities are calculated that bind at varying concentrations of antigen, or vice versa. From these data it is possible 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.* (1989). 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).

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.*, 1989).

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

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 h, 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 h at 37 °C the plates were washed, and 100 µl affinity purified goat-antimouse IgG (H + L) - HRP (Bio-Rad, Richmond, Ca.), diluted 1:2000 in incubation buffer, were added to each well. After 2 h 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 min 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 when the increasing concentration of the antigen is expressed on a logarithmic scale. Such a curve can be described by the four parameter logistic function (De Lean *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.

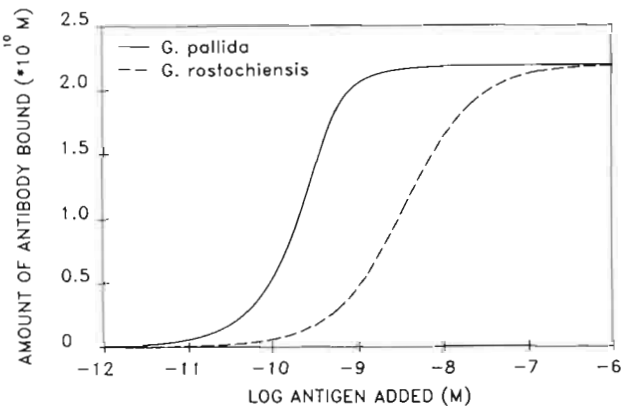

Fig. 1. Theoretical curves for the binding of antibody WGP 2 at $2.24^* 10^{-10}$ M to varying concentrations of TSPaP or TSRoP. The data for these curves were calculated using the computer program KINETICS. On the *X*-axis the quantities of TSPaP or TSRoP applied to the microtitre plate are given, on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

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

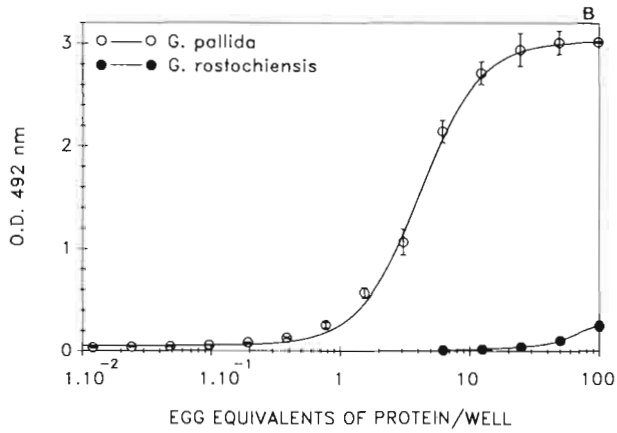

Fig. 2. Reactivities of antibody WGP 2 at 2.24* 10^{-10} M with varying quantities of protein antigens isolated from either *G. rostochiensis* or *G. pallida*. The curves were fitted using the computer program ALLFIT. On the *X*-axis the antigen quantity corresponding to the number of eggs from which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

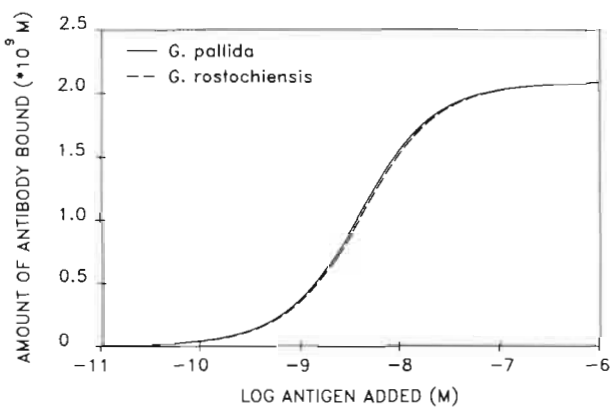

Fig. 3. Theoretical curves for the binding of antibody WGR 2 at 2.09* 10^{-9} M with either TSPaP or TSRoP. Axes and data calculation as in Figure 1.

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 (Fig. 4).

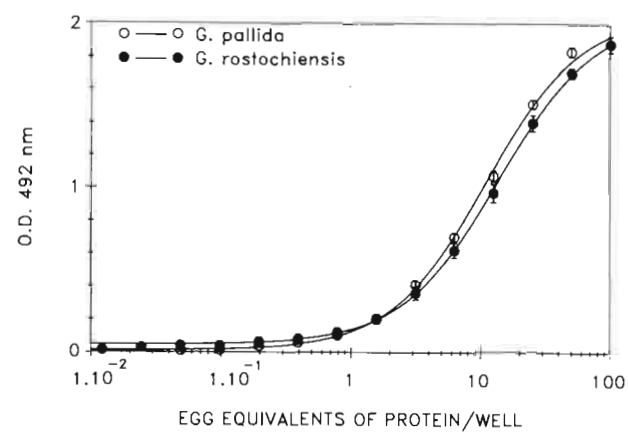

Fig. 4. Reactivities of antibody WGR 2 at 2.09* 10^{-9} M with antigens isolated from *G. rostochiensis* or *G. pallida*. The curves were fitted using the computer program ALLFIT. Axes as in Figure 2.

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.*, 1989). 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 0.5-1* 10^{8} M WGP 1 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 (Fig. 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.

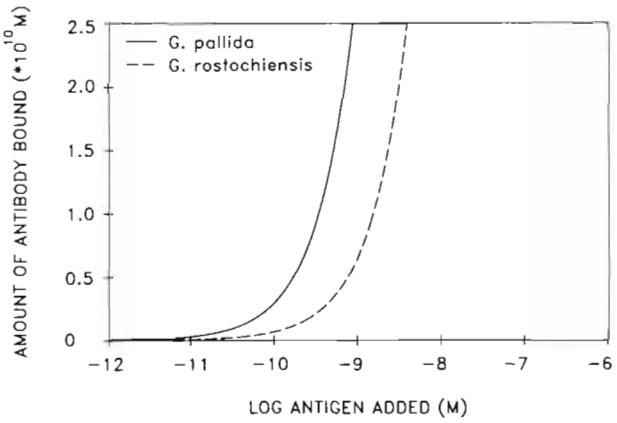

Fig. 5. Theoretical curves for the binding of antibody WGP 1 at 7.70* 10^{-9} M with either TSPaP or TSRoP. Axes and date calculation as in Figure 1.

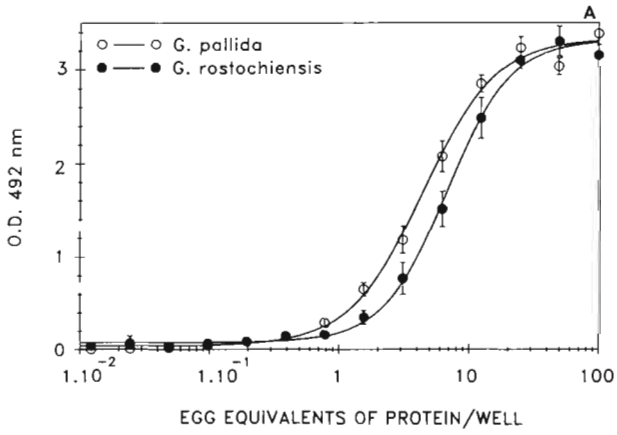

Fig. 6. Reactivities of antibody WGP 1 at 7.70* 10^{-9} M with antigens isolated from *G. rostochiensis* or *G. pallida*. The curves were fitted using the computer program ALLFIT. Axes as in Figure 2.

Discussion

The reactivity pattern of a McAb with an antigen in an immunoassay can be predicted with the computer programme 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). 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 (Figs 1, 3, 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 (Fig. 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 and Bertino (1973). The following conditions have to be met : *i)* the affinity of the McAb should be determined properly; *ii)* The antibody and antigen quantities used should be within limits defined by the mass action law, and *iii)* 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 microtitre-plate might decline. The method, as outlined, can be validated using an appropriate immunoassay and a suitable curve fitting routine, *e.g.*, ALLFIT.

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 (pratical)
WGP 2 on TSPaP	1.54	1.61 (4.0)
WGR 2 on TSRoP and TSPaP	1.12	1.12 (2.6)

References

CANTARERO, L. A., BUTLER, J. E., & OSBORNE, J. W. (1980). The adsorptive characteristics of proteins for polystyrene and their significance in solid-phase immunoassays. *Analyt. Biochem.*, 105 : 375-382.

DE LEAN, A., MUNSON, P. J., & RODBARD, D. (1978). Simultaneous analysis of families of sigmoidal curves : application to bioassay, radioligand assay, and physiological dose-response curves. *Am. J. Physiol.*, 235 : E97-E102.

FELDMAN, H. A. (1972). Mathematical theory of complex ligand-binding systems at equilibrium. Some methods for parameter fitting. *Analyt. Biochem.*, 48 : 317-338.

GUARDABASSO, V., RODBARD, D., & MUNSON, P. J. (1987). A modelfree approach to estimation of relative potency in doseresponse curve analysis. *Am. J. Physiol.*, 252 : E357-E364.

KLEIN, M. (1985). Purification of immunoglobulins. *Pract. Meth. Clin. Immunol.*, 8 : 93-120.

KÖHLER, G., & MILSTEIN, C. (1975). Continuous cultures of fused celles secreting antibody of predefined specificity. *Nature*, 256 : 495-497.

LARSSON, P. H., JOHANSSON, S. G. O., HULT, A., & GOETHE, S. (1987). Covalent binding of proteins to grafted plastic surfaces suitable for immunoassays. I. Binding capacity and characteristics of grafted polymers. *J. immunol. Meth.*, 98 : 129-135.

MALAN, P. G. (1986). Immunoassay detection limits. *Commun. Lab. Med.*, 2 : 40-45.

MUNSON, P. J., & RODBARD, D. (1980). LIGAND : a versatile computerized approach for characterization of ligand-binding systems. *Analyt. Biochem.*, 107 : 220-239.

MUNSON, P. J., & RODBARD, D. (1984). Computerized analysis of ligand binding data : basic principles and recent developments. *In :* Rodbard, D. & Forti, G. (Eds). *Serono Symposia publications from Raven Press Vol. 14. Computers in Endocrinology.* New York, Raven Press : 117-145.

NYGREN, H., & STENBERG, M. (1985). Calibration by ellipsometry of the enzyme linked immunosorbent assay. *J. Immunol. Meth.*, 80 : 15-24.

RODBARD, D., & BERTINO, R. E. (1973). Theory of radioimmuno-assays and hormone-receptor interactions. II. Simulation of antibody divalency, cooperativity and allosteric effects. *In :* O'Malley, B. W. & Means, A. R. (Eds). *Receptors for reproductive hormones. Adv. Exp. Med. Biol.*, 36 : 327-341.

SALONEN, E. M., & VAHERI, A. (1979). Immobilization of viral and mycoplasma antigens and of immunoglobulins on polystyrene surface for immunoassays. *J. Immunol. Meth.*, 30 : 209-218.

SCHOTS A., BAKKER, J., GOMMERS, F. J., BOUWMAN-SMITS, L., & EGBERTS, E. (1987). Serological differentiation of the potato cyst nematodes *Globodera pallida* and *G. rostochiensis :* partial purification of species specific proteins. *Parasitology*, 95 : 421-428.

SCHOTS, A., VAN DER LEEDE, B. J., DE JONGH, E., & EGBERTS, E. (1988). A method for the determination of antibody affinity using a direct ELISA. *J. immunol. Meth.*, 109 : 225-233.

SCHOTS, A., HERMSEN, T., SCHOUTEN, S., GOMMERS, F. J., & EGBERTS, E. (1989). Serological differentiation of the potato cyst nematodes *Globodera pallida* and *G. rostochiensis.* II. Preparation and characterization of species specific monoclonal antibodies. *Hybridoma*, 8 : 401-413.