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A method for the determination of antibody affinity using a direct ELISA

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Antibody affinity is of major significance in 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 program LIGAND. Five of the antibodies studied bound to their antigen with two classes of antigen binding site, 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.

Key words: Enzyme immunoassay; Antibody affinity; Monoclonal antibody

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 such as SP-RIA, ELISA, Farr assay, complement-mediated haemolysis, and precipitation reactions are insensitive to antibodies of very low affinity. Also, in an extensive review, Steward and 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

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Abbreviations: β, cooperativity parameter; BSA, bovine serum albumin; cIg, carp immunoglobulin; $E^{1\%}_{1cm}$, extinction of a 1 mg/ml solution; ELISA, enzyme-linked immunosorbent assay; GAM, goat anti-mouse immunoglobulin; HPLC, high performance liquid chromatography; HRP, horseradish peroxidase; McAb, monoclonal antibody; K, binding constant; OD, optical density; PBS, phosphate-buffered saline; R, binding capacity; SP-RIA, solid-phase radioimmunoassay; TSPaP, thermostable protein isolated from *Globodera pallida*; Var, variance; WCI, monoclonal anti-carp immunoglobulin antibodies; WCT, monoclonal anti-carp thymocyte antibodies; WGP, monoclonal anti-TSPaP antibodies.

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 and Tacey, 1978; Munson and 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 relatively 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 and 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 horseradish peroxidase (GAM-HRP, Bio-Rad).

TABLE I

THE MONOCLONAL ANTIBODIES USED FOR THE AFFINITY BINDING STUDIES

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

Conjugation to HRP

Purified antibodies were conjugated to horseradish peroxidase (HRP) using the two-step glutaraldehyde method of Avrameas and Ternynck (1971). After conjugation, the remaining unconjugated peroxidase 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.

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 two-fold dilution series of a conjugate in PBS/0.05% Tween 20/0.1% BSA were introduced into eight wells of a microtitre plate (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. Microtitre plates 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 microtitre plate reader (SLT-labinstruments, Gröding, Austria). The specific activities of the McAb-HRP conjugates were calculated from these results as OD units produced per mol 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-coating buffer 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 eight 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 and Rodbard (1980). LIGAND version 2.3.12 was used which includes the possibility of fitting a cooperativity parameter β (Munson and Rodbard, 1984). Input files were made using LOTUS 1-2-3. The general model $\mathrm{Var}(Y) = 0.0001Y^2$ (Rodbard et al., 1976) was used as a weighting model (constant percent error). LIGAND was run on an Olivetti M24 personal computer. With each data file a fit for a one-site and a two-site model with or without cooperativity was made. When more then one fit is successful, 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/l) 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 which is 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 microtitre plate, 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, whether 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 successful fits were obtained with more than one model, the program compares the fits using an F-test.

Six of the 11 antibodies tested showed a successful 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 (Fig. 1). However, four of the 11 antibodies tested (WCI 7, WCT 5, WGP 2 and WGP 3) showed a reaction with two apparent classes of binding sites. In Fig. 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 shown by the F-test (the F-value was 28.61 with a critical level (P) of 0.000).

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

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.

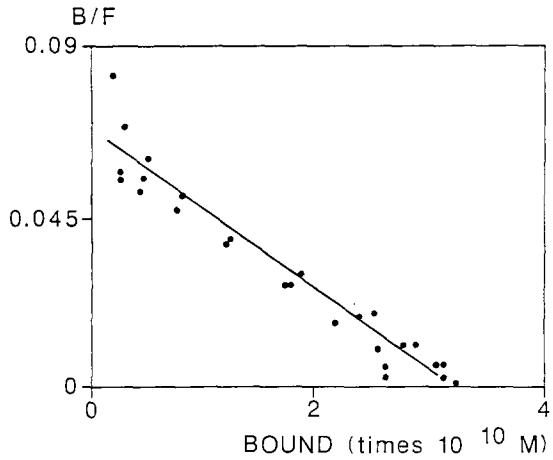

Fig. 1. Scatchard plot for antibody WCT 26, a monoclonal antibody reacting with carp immunoglobulin. The antigen shows one binding site to which the antibody can bind. On the x-axis the concentration of bound antibody is given in mol/l, on the y-axis the bound to free ratio for the antibody is given.

and accuracy of the estimated parameters. The fits obtained with a one-site model without the assumption of cooperativity (Fig. 3*A*) and with the assumption of positive cooperativity (cooperativity parameter $\beta_1 > 1$, Fig. 3*B*) were the least accurate. The assumption of a one-site model with negative cooperativity (cooperativity parameter $\beta_1 < 1$, Fig. 3*C*) or a two-site model without cooperativity (Fig. 3*D*) resulted in two fits of nearly the same accuracy. However, the two-site model

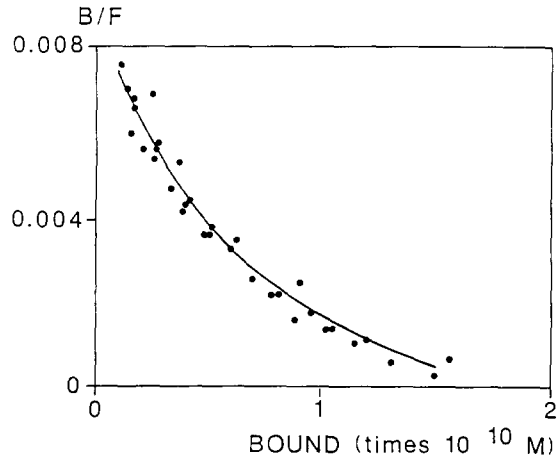

Fig. 2. Scatchard plot for antibody WCT 5, a monoclonal antibody binding to carp immunoglobulin apparently at two sites. Axes as in Fig. 1.

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. 3*E*) or both binding sites (Fig. 3*F*). 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. 3*E*).

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 microtitre plate. 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 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 and 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

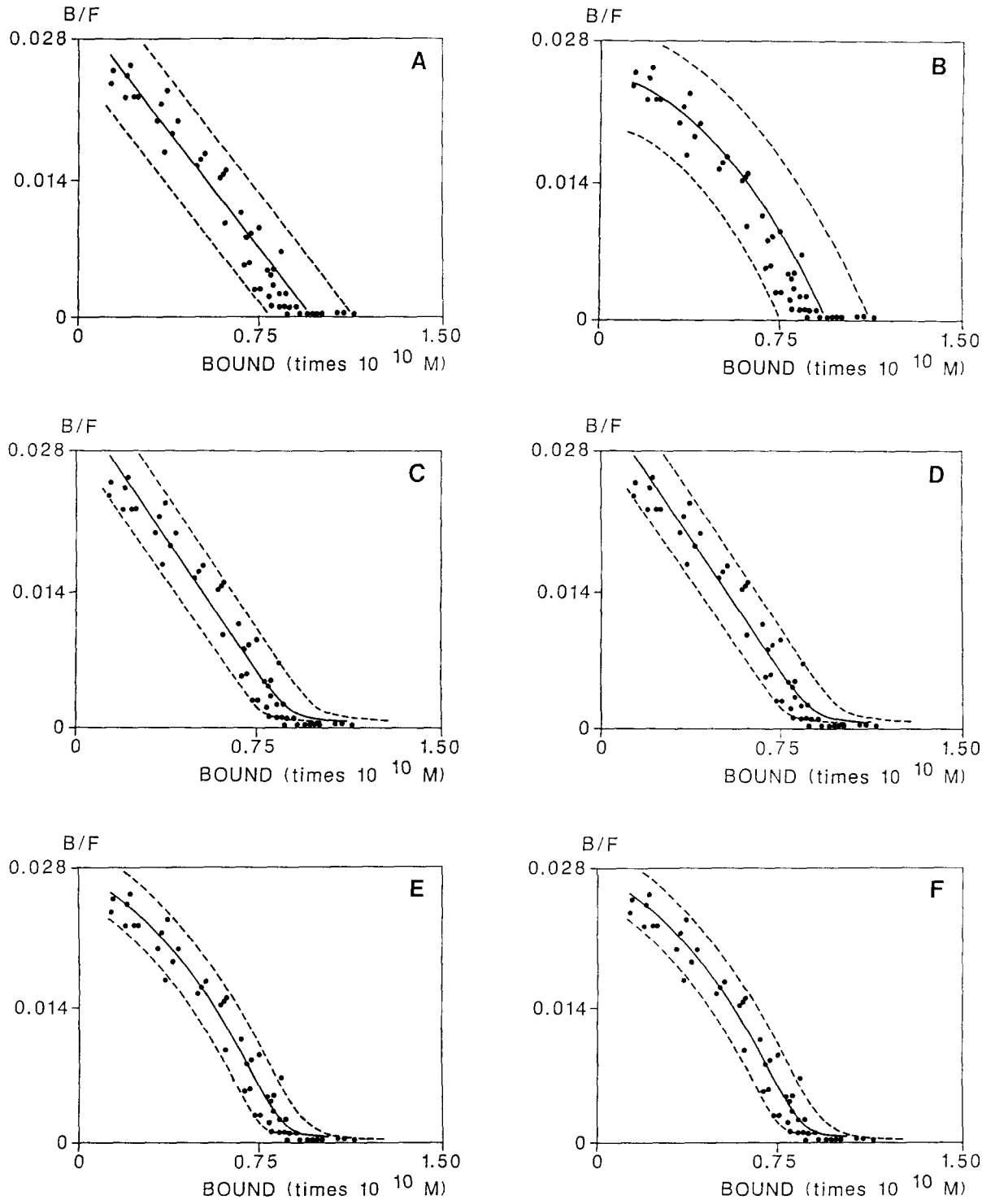

Fig. 3. Scatchard plots with different fits for antibody WCI 12 with confidence limits. *A*: One-site model. *B*: One-site model with cooperativity parameter $\beta_1 > 1$. *C*: One-site model with $\beta_1 < 1$. *D*: Two-site model. *E*: Two-site model with positive cooperativity for K_1. *F*: Two-site model with cooperativity for both apparent binding sites. Axes as in Fig. 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		

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. 3*E* 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 and Lyles, 1982; Lemke and Hämmerling, 1982; Lubeck and 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 labelled antigen was found by Matsukura et al. (1971) and Rodbard and Bertino (1973) developed a theory to describe this phenomenon of cooperativity in 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 and 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 cannot 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 and Rodbard, 1980, 1984) does provide such a system, and it can be of great help for the estimation of binding constants 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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