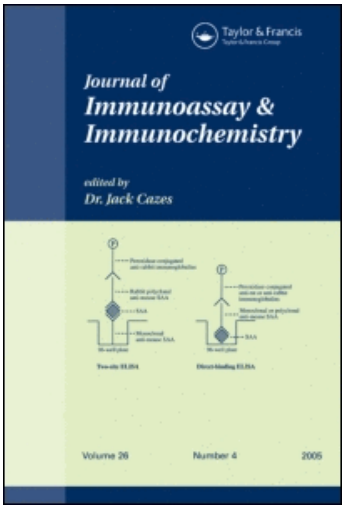

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APPLICATION OF A SOL PARTICLE IMMUNOASSAY TO THE DETERMINATION OF AFFINITY CONSTANTS OF MONOCLONAL ANTIBODIES.

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ABSTRACT

The affinity constants (K_a) of monoclonal antibodies (MAb) for binding to their corresponding antigens (Ag), unlabelled and in buffered solution were determined by the following procedure: 1. Incubation of MAb (fixed concentration) with Ag (concentration dilution series). 2. Rapid bound/free separation by adding immobilized second antibody, followed by centrifugation. 3. Determination of free Ag in the supernatant using a gold sol particle agglutination immunoassay (SPIA) in a microtitration plate format. 4. Calculations and interpretation were based on Scatchard and Sips plots.
K_a values found by this procedure were found to be similar to those obtained by a radio-immunoassay (RIA) procedure. The present method avoids possible artefacts in K_a values introduced by the procedure or chemical modification due to labelling of MAb or Ag. It enables rapid, simultaneous screening of a considerable number of different MAbs under non-specialized (i.e. RIA) laboratory conditions.
(KEY WORDS: Affinity; Monoclonal antibody; human chorionic gonadotrophin; sol particle immunoassay)

INTRODUCTION

Monoclonal antibodies are widely used as primary tools for identification, assay and purification of a variety of antigens. The affinity of an antibody for its corresponding antigen is of crucial importance in the

performance of immunoassays (1, 2, 3, 4). The affinity of an antibody is determined at the equilibrium state and is quantified by an affinity constant. This affinity constant (K_a) is a measure of the strength of the interaction between antigen and antibody, and is a suitable parameter for the specificity of the antibody (5).

The law of Mass Action provides the basis for the calculation of antibody affinity constants in a solution phase assay. A variety of mathematical equations have been developed to facilitate experimental calculations of affinity constants. The most commonly employed are the approaches described by Scatchard (6) and Sips (7). Utilizing these approaches, a number of experimental methods have been described for the measurement of the affinity constants of antigen-antibody interactions.

While equilibrium dialysis has become accepted as the reference method for the determination of affinity constants, it is not applicable to macromolecular antigens. Similarly, other techniques as radioimmuno- and solid-phase assays are less favourable since labelling or immobilization of the proteins may induce structural modification (8, 9).

Friguet (10) introduced a method for the determination of affinity constants, that characterizes the interaction between non-labelled antibodies and macromolecular antigens. However, relatively large amounts of antigen are required and the affinity of binding sites on intact IgG is underestimated (11, 12).

The purpose of the present work was to develop a method for the determination of antibody affinity without applying labelled compounds in the primary (Ag-Ab) system. In order to study the interaction of native human chorionic gonadotrophin (hCG) and anti-hCG, the applicability of U.V.

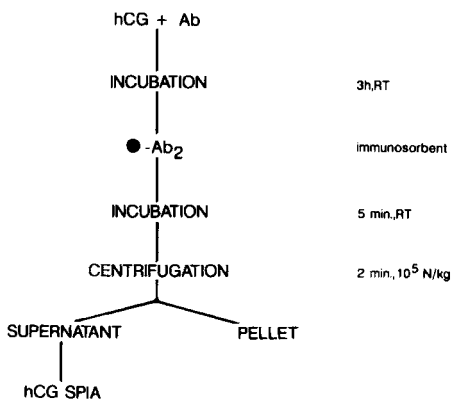

FIGURE 1. Flow chart illustrating the principle of the method. In the first step a constant amount of MAb is incubated with various concentrations of Ag. In the second step a rapid separation of Ag bound and free is achieved by adding immobilized second antibody. After centrifugation the free Ag in the supernatant is determined using a gold sol particle agglutination assay.

spectrophotometry, circular dichroïsm and high performance size exclusion chromatography (HPSEC) were examined. The first two techniques could not be used, however, to investigate hCG/anti-hCG interactions since no significant spectral changes occurred due to complex formation. The exclusion ranges of the commercially available columns and the low molar absorbance of non-labelled hCG at 206 nm were the limiting factors for HPSEC.

Therefore, a technique was developed for the determination of antibody affinity (see Fig.1.) based on the SPIA agglutination procedure in combination with an existing bound/free separation technique (immunosorbent). The SPIA procedure was chosen above other immunoassays because of its reproducibility, low coefficient of variation (≤5%) and easy performance (one step) (13). The obtained affinity values were compared with a standard RIA procedure.

MATERIALS AND METHODS

Reagents

The monoclonal antibodies used in this study were of the mouse IgG1 subclass and were directed against hCG, a glycoprotein with a molecular mass of 38 kD (14, 15). A detailed description of production of the monoclonal antibodies in hollow fibre dialysis modules has already been described (16). The MAbs were purified from dialysis culture supernatants by a 20% sodium sulphate precipitation (17) followed by a Sephacryl S-200 gelfiltration (Pharmacia K26/100 column, eluent 0.15 M Tris/HCl pH 7.6 + 0.2 g/l sodium azide). The isolated IgG fractions contained monomeric IgG (>98%) based on HPSEC (Zorbax GF-250 Dupont) and sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS PAGE).

HCG (12 100 IU/mg) was obtained from Diosynth Oss, The Netherlands. All other chemicals were of p.a. reagent grade quality.

Preparation of the Immunosorbent

The ("second") antibodies (rabbit anti-mouse) were coupled to regenerated cellulose by means of the reactive azo-dye procedure as described by Gribnau (18).

The immunosorbent (DASP[1]: 0.03 mg IgG/mg aminoaryl cellulose) was stored in 0.1 M Na_2HPO_4/KH_2PO_4, 20 mM NaCl, 5 mM EDTA, 1 g/l Thiomersal pH 7.0 buffer at 4°C (dry weight: 24 mg/ml). Before use in binding analysis, the DASP suspension was washed four times with the assay buffer (0.05 M HEPES[2], 0.2 M NaCl, 0.1 g/l Thiomersal and 4 g/l BSA (Boseral-PUR, Organon Teknika) pH 7.3).

1) DASP; double antibody solid phase
2) HEPES; N-2-hydroxyethyl-piperazine-N'-ethanesulphonic acid

Determination of the Optimal DASP Concentration

The amount of DASP (rabbit anti-mouse (RaM) or goat anti-mouse (GaM) Fc specific) needed to separate free Ag from the free first MAb and the complex was determined according to the following procedure:

A constant amount of MAb anti-hCG (0.3 μg in 125 μl) was incubated in a total volume of 350 μl with different amounts of the DASP suspension. After incubation during 5 min. and centrifugation (2 min. 100 000 N/kg), the total mIgG in the supernatant was determined using the SPIA agglutination procedure.

Sol Particle Immunoassay

The concentrations of hCG (i) and mouse IgG (ii) in samples of the supernatants were determined by the SPIA agglutination procedure as described elsewhere (19, 20). The quantitative SPIA uses gold sol particles coated with anti-hCG MAbs (i) or rabbit anti-mouse immunoglobulin (ii). The test was performed as follows:

100 μl sample or standard solution of hCG (i) or MAb (ii) and 50 μl buffered solutions (i) : 0.3 M Tris/HCl, 3 g/l BSA, 1.3 M NaCl, 0.1 g/l Thiomersal, 74 g/l PEG 8 000, pH 7.4 or ii) 0.3 M Tris/HCl, 1.3 M NaCl, 0.1 g/l Thiomersal, 11 g/l PEG 8 000, pH 7.4) were pipetted into 96 well-microtitration plates. After agitation, 50 μl buffered dispersions of i) Au-(anti-hCG) or (ii) Au-(anti-mIgG) conjugate with A_{540nm} = 9.0 in 5 mM Tris/HCl, 0.05 g/l PEG 20M, 160 g/l sucrose, 0.1 g/l Thiomersal pH 8, was added and mixed by a plate shaker. After a 30 min. incubation at RT, the extent of agglutination was determined by measuring the absorbance at 540 nm using a Titertek Twinreader. Wells filled with 200 μl Tris buffer were taken as blank.

Competitive Radio-immunoassay

Affinity constants (K_a) of the antibody-antigen interactions were determined in a sodium phosphate buffer solution (10 mM sodium phosphate, 150 mM NaCl, 2.5 mM $CaCl_2$, 0.1% bovine gamma globulin (BGG) Cohn fraction II (Sigma), pH 7.3) and by using labelled ^{125}I-hCG (Dupont NEX 106 specific activity 2.47 MBq/μg). 500 μl of the antibody preparation (diluted to a concentration which bound 30% of 2 fmol ^{125}I-hCG added as determined in a titration experiment) was incubated with 100 μl of labelled hCG (10 000 cpm, 4 pM) and 100 μl of seven different amounts of unlabelled hCG (0 to 400 pM) for 16 h at RT. Separation of bound and free radioactivity was achieved using RaM DASP immunosorbent. Non-specific binding was assessed by competing the tracer with 1 000 fold excess of unlabelled hCG (Pregnyl 3 300 IU/mg, Organon, The Netherlands), without changing the total incubation volume. All experiments were performed in duplicate.

Determination of Ka by the Procedure using SPIA

MAb anti-hCG, at a constant concentration, was incubated with highly purified hCG at various concentrations (0-55 nM) in 0.25 ml of the assay buffer (0.05 M HEPES, 0.2 M NaCl, 0.1 g/l Thiomersal and 4 g/l BSA, pH 7.3). After incubation at RT (20-22°C) for 3 h to reach equilibrium, an excess of the second antibody immunosorbent (DASP RaM) was added. The reaction mixtures were incubated for 5 min. and continuously agitated. Subsequently, the reaction mixtures were centrifuged at 100 000 N/kg for 2 min. using a Heraeus Biofuge centrifuge. The concentration of free Ag in each supernatant was determined in four fold by the SPIA agglutination procedure.

Protein Determinations

Protein concentrations were determined by absorbance at 280 nm using the following extinction coefficients: 1.45 $(cm \cdot mg/ml)^{-1}$ for IgG and 0.39 $(cm \cdot mg/ml)^{-1}$ for highly purified hCG. The validity of the values were confirmed by combination of absorbance measurements and amino acid analysis. The molecular mass of IgG and hCG were taken as 150 kD and 38 kD (21) respectively.

Calculations

The "modified" Scatchard equation (22) was used to determine affinity constants.

$$\frac{r}{[Ag]} = -rK_a + nK_a \qquad (1)$$

where r = moles of antigen bound per moles of antibody present; n = antibody valence (in this case 2); [Ag] = free antigen concentration; K_a = the affinity constant.

A plot of r/[Ag] versus r over a wide range of antigen concentrations yields a straight line for homogeneous binding and the intercept on the x-axis represents the antibody valence n. The value of K_a can be determined from the slope, which is equal to - K_a. If linearity is not observed over a defined range of antibody binding site saturation, the K_a value is not meaningful (23, 24).

Based on the Sips distribution function for the affinity constant, the following equation was derived (25)

$$\log \frac{r}{n - r} = \alpha \log[Ag] + \alpha \log K_0 \qquad (2)$$

A plot of log(r/n-r) versus log[Ag] yields a straight line, and the index of heterogeneity, α, is given by the slope. The intrinsic affinity constant (K_o) is given by 1/[Ag] when log(r/n-r) = 0. When the index $\alpha = 1$, there is no apparent heterogeneity. A value of $\alpha < 1$ corresponds to a Scatchard plot which is concave ("negative cooperativity") and $\alpha > 1$ is suggestive of "positive cooperativity" (26, 27). Since r is defined as moles of bound Ag per moles of Ab present, the result depends on accurate knowledge of the Ab concentration.

RESULTS

The interaction between anti-hCG MAbs and hCG was studied by the procedure using SPIA. In this procedure Ab-Ag complexes in the equilibrated mixtures were separated from free Ag by using an excess of a second antibody immunosorbent (DASP). An adequate separation was achieved by the addition of 2.4 mg DASP as shown in Fig.2.

The non-specific adsorption of Ag to the reaction vial wall and immunosorbent was suppressed by the amount of 0.4% (w/v) BSA in the HEPES buffer. Antigen binding was determined by measuring the concentration of free Ag after incubation of various amounts of Ag with a constant concentration of Ab. The fraction of bound Ag was calculated by substracting the free Ag concentration from the total antigen concentration.

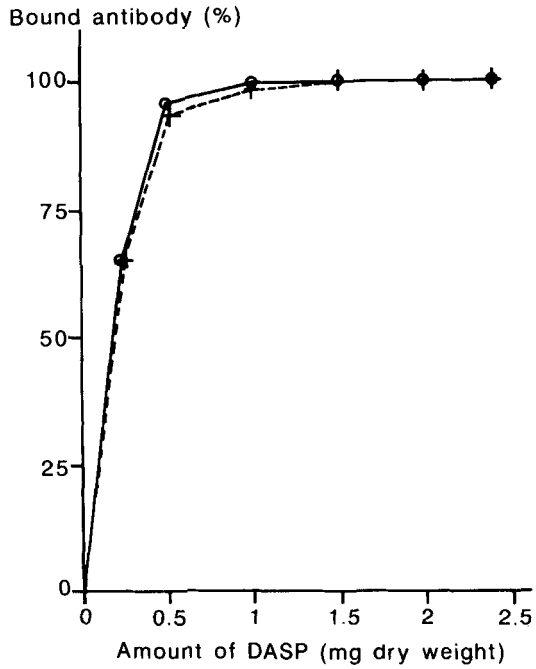

FIGURE 2. The amount of DASP needed to achieve complete binding of MAb OT-7B at a concentration of 7 nM : (o) RaM and (+) GaM Fc specific. Similar results were obtained for other MAbs directed against hCG.

It was found that incubation during 3 h at RT was sufficient to reach equilibrium as illustrated for MAb OT-0A in Fig.3.

Affinity constants for anti-hCG MAbs, determined by the procedure using SPIA, were compared with those obtained by a competitive radio-immunoassay. The results, based on Scatchard (6) and Sips analysis (7), were found to be similar and are summarized in Table 1. The heterogeneity indices are not significantly different from one, which is in agreement with the proposed model of a homogeneous hCG/anti-hCG interaction.

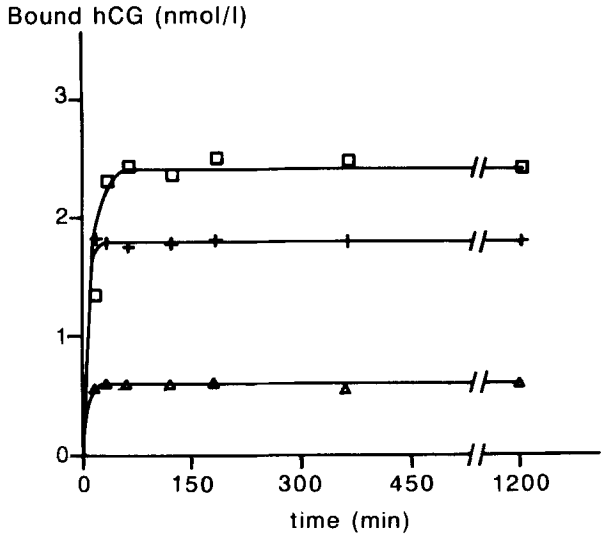

FIGURE 3. The time for 1.3 nM MAb OT-0A to reach equilibrium binding at RT was measured with 0.72 nM (Δ), 2.16 nM (+) and 4.32 nM (□) of hCG.

In addition to linear Scatchard plots (characteristic for a homogeneous interaction) also convex Scatchard plots characteristic for cooperative interactions were obtained (see Fig.4a). The Sips plots of these (hCG/anti-hCG) binding data, as shown in Fig.4b., also indicated that an apparent positive (cooperative) site-site interaction was present, since the heterogeneity index was significantly larger than 1.

Contribution of Potential Artefacts

Deviation from linearity could be due to artefacts. This was ruled out by the following systematic investigations:

TABLE 1

Comparison of Affinity Values Obtained by the Procedure Using SPIA and a Competitive RIA.

MAb[a]	Procedure using SPIA			Competitive RIA		
	Scatchard[b]	Sips[b]		Scatchard[b]	Sips[b]	
	$K_a \times 10^8$ (l/mol)	$K_o \times 10^8$ (l/mol)	Index	$K_a \times 10^8$ (l/mol)	$K_o \times 10^8$ (l/mol)	Index
OT-6A	0.1 ± 0.01	0.1 ± 0.01	0.98 ± 0.04	0.1[c]	0.1[c]	0.99[c]
OT-7B	19 ± 1	18 ± 1	0.98 ± 0.05	11 ± 2	12 ± 2	1.02 ± 0.03
OT-0A[c]	55	57	1.05	76	74	1.04
OT-3A-I	103 ± 7	100 ± 8	1.0 ± 0.04	90 ± 10	89 ± 9	1.05 ± 0.05

[a] The MAb concentration in the new procedure was about 1 nM and in the competitive RIA 0.2 nM. A concentration of 20 nM was used for MAb OT-6A.

[b] The results represent the mean value ± standard deviation of two experiments and are based on linear regression analysis.

[c] The values indicate the results of one experiment.

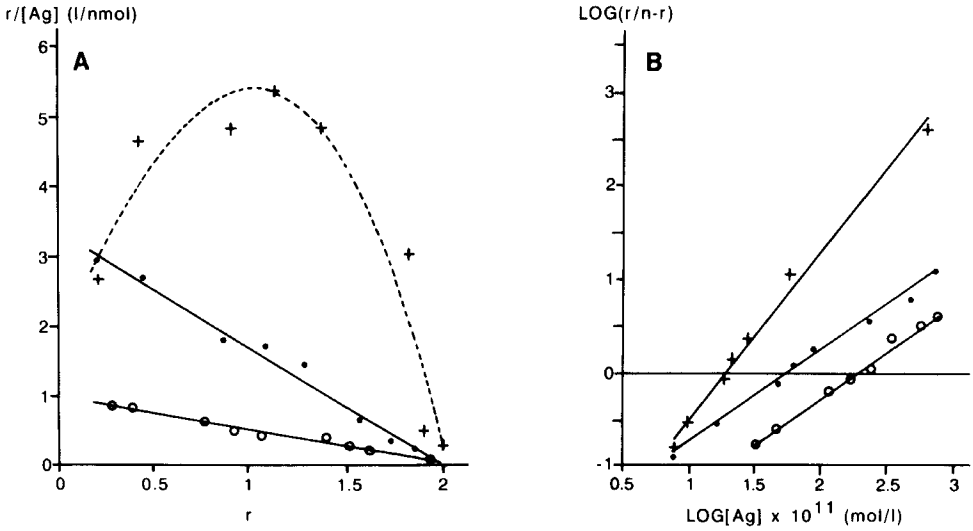

FIGURE 4. Scatchard plot (A) and Sips plot (B) of hCG binding to MAb OT-7B (●); OT-9A (o) and OT-3A (+) at RT. The MAb concentrations were 7 nM, 7.5 nM and 7 nM respectively. Numerical values for K_a, K_o and α are given in Table 3.

Starting materials. These observed convex Scatchard plots could not be due to aggregates in the starting materials since they were monomeric (>98%) as determined by HPSEC and SDS-PAGE.

Non-specific binding. Binding experiments, with both labelled and non-labelled hCG in the assay buffer containing 4 g/l BSA or 1 g/l BGG, showed that the non-specific binding was less than 4% of total binding. Since the convex binding characteristics were independent of correction for non-specific binding, it is very unlikely that the convex Scatchard plots were produced by low non-specific hCG binding. Moreover, imprecise estimation of non-specific binding result usually in concave Scatchard plots (28).

TABLE 2

Disturbance of the Equilibrium during the DASP Incubation Period

DASP[a] incubation time (min.)	MAb OT-7B		MAb OT-3A	
	Sips[b] $K_o \times 10^9$ (l/mol)	Index α	Sips[b] $K_o \times 10^9$ (l/mol)	Index α
2	1.80	1.03	N.D[c]	N.D[c]
5	1.73	1.01	4.7	1.65
10	1.79	0.97	5.1	1.72
15	1.68	1.01	4.7	1.80
30	1.83	1.04	4.8	1.68

[a] 2.4 mg of DASP was used and each hCG concentration was tested in triplicate.
[b] Results are based on equation (3) and linear regression analysis.
[c] not determined.

Inadequate separation. In order to achieve adequate separation in 5 min. of bound and free Ag, an amount of 2.4 mg DASP was used (see Fig.2). Especially in the case of convex Scatchard plots the amount of DASP added to the same Ab concentration was increased. However, no significant decrease in convexity was observed. The heterogeneity index (α) using 2.4 mg DASP was 1.7 ± 0.1 and for 6 mg DASP; 1.65 ± 0.1. Also after substituting RaM DASP by GaM Fc specific, the convexity remained unchanged.

Disturbance of the equilibrium. Dissociation of bound Ag from the Ab-Ag complex during separation was investigated by increasing the DASP incubation time. No differences in K_o and convexity could, however, be

TABLE 3

The Reliability and Reproducibility of the Procedure Using SPIA.

MAb	No. measure-ments[a]	conc. (nM)	Scatchard[b] $K_a \times 10^8$ (l/mol)	Sips[b] $K_o \times 10^8$ (l/mol)	Index α
OT-7A	5	20	0.06 ± 0.007	0.06 ± 0.007	0.98 ± 0.04
OT-6A	5	20	0.1 ± 0.02	0.1 ± 0.02	1.01 ± 0.06
OT-7B	6	7	18 ± 1	18 ± 1	1.00 ± 0.03
OT-9A[c]	5	7.5	4.8 ± 0.4	5.1 ± 0.4	1.02 ± 0.04
OT-3A	6	7	N.D.[d]	48 ± 4	1.75 ± 0.1

[a] Each measurement was performed in triplicate
[b] The results represent the mean value ± standard deviation of the number of measurements and are based on linear regression analysis. (regression coefficients were 0.99)
[c] Equilibrium binding analysis were performed in 0.15 M Tris/HCl, 0.2 M NaCl,4 g/l BSA, 0.1 g/l Thiomersal pH 7.4.
[d] not determined

observed for up to a 30 min. DASP incubation time. The results are shown in Table 2.

Reproducibility of the Procedure using SPIA

The reproducibility and precision of the method at RT was tested by repeated determination of the antibody affinities. The mean affinity of several anti-hCG MAbs were calculated from five or six independent determinations. Each test being performed on a different day and in triplicate. The overall results are summarized in Table 3.

DISCUSSION

The data presented in this paper demonstrate the reliability and reproducibility of the technique for the determination of antibody affinities in the range of 10^6 - 10^{10} M^{-1}. The values of K_a determined by the method using SPIA are similar to the values obtained by a competitive RIA. Moreover, the measured affinity constants of MAbs against hCG are in the range ($1x10^7$ - $4x10^{10}$ M^{-1}) currently described in the literature (29, 30, 31).

This technique has a number of advantages compared with other commonly used techniques. First, no labelling of either antibody or antigen are required and the initial antigen-antibody reaction is carried out in solution. This means no alteration of antibody affinity due to labelling or immobilization. Furthermore, the SPIA agglutination procedure is easy to perform (one step assay) and has a low coefficient of variation ($\leq$ 5%) (13).

The occurrence of convex Scatchard plots (cooperativity) for some individual anti-hCG MAbs in solution is remarkable. Theoretical analyses have indicated that a number of experimental artefacts may explain the nonlinear Scatchard plots. Recently Kermode (28) has described various artefacts together with their usual effects on Scatchard plots. He stressed that essentially all binding studies are affected by some or all of these described artefacts. The magnitude of their impact on the Scatchard plots, however, differs considerably from one study to another.

The study reported here excludes a significant contribution of the described potential artefacts that cause nonlinearity of the Scatchard plots and demonstrates that the interactions of some MAbs are highly ("positive") cooperative.

The phenomenon of cooperative interactions has already been described for antisera (32, 33) and mixtures of MAbs directed against hCG and other antigens (29, 34, 35). However, the mechanisms proposed by these authors with respect to positive cooperative interactions are not directly applicable to our binding studies in which we use one particular MAb directed against one single antigenic site on hCG.

In this study concentration effect(s) may contribute significantly. This has been investigated further in relation to the apparent cooperative interaction (36).

REFERENCES

1. Van Heyningen, V., Barron,L., Brock, D.J.H., Crihton, D. and Lawrie S. Monoclonal antibodies to human α-foetoprotein: analysis of the behaviour of three different antibodies. J. Immunol. Methods. 1982; 50: 123-31.

2. Steward, M.W. and Lew, A.M. The importance of antibody affinity in the performance of immunoassays for antibody. J. Immunol. Methods. 1985; 78: 173-90.

3. Nimmo, G.R, Lew, A.M., Stanley, C.M. and Steward, M.W. Influence of antibody affinity on the performance of different antibody assays. J. Immunol. Methods 1984; 72: 177-87.

4. Devey, M.E. and Steward M.W. The role of antibody affinity in the performance of solid phase assays In: D.M. Kemeny and S.J. Challacombe (Eds.), Elisa and Other Solid Phase Immunoassays. John Wiley & Sons Ltd, 1988: 135-53.

5. Metzger., D.W., Chong, L.-K., Miller, A. and Seracarz, E.E. The expressed lysozyme-specific B cell repertoire. I. Heterogeneity in the monoclonal anti-hen egg white lysozyme specificity repertoire, and its difference from the in situ repertoire. Eur. J. Immunol. 1984; 14: 87-91.

6. Scatchard, G. The attraction of proteins for small molecules and ions. Ann. N.Y. Acad. Sci. 1949; 51: 600-72.

7. Sips, R. On the structure of a catalyst surface. J. Chem. Phys. 1948; 16: 490-5.

8. Friguet, B., Djavadi-Ohaniance, L., Pagès,J., Bussard, A. and Goldberg, M.E. A convenient enzyme-linked immunosorbent assay for testing whether monoclonal antibodies recognize the same antigenic site. Application to hybridomas specific for the β_2-subunit of escherichia coli tryptophan synthase. J. Immunol. Methods. 1983; 60: 351-8.

9. Radicella, J.P., Calvo, J.C. and Charreau, E.H. Effects of radioiodination on hormone binding ability. J. Receptor Res. 1985; 5: 349-65.

10. Friguet, B., Chaffotte A.F., Djavadi-Ohaniance, L. and Goldberg M.E. Measurement of the true affinity constant in solution of antigen-antibody complexes by enzyme-linked immunosorbent assay. J. Immunol. Methods. 1985; 77: 305-19.

11. Harper, M., Lema, F., Boulot, G. and Poljak R.J. Antigen specificity and cross-reactivity of monoclonal anti-lysozyme antibodies. Molec. Immun. 1987; 24: 97-108.

12. Stevens F.J. Modification of an elisa-based procedure for affinity determination: correction necessary for use with bivalent antibody. Mol. Immunol. 1987; 24: 1055-60.

13. Leuvering, J.H.W., Thal, P.J.H.M., Waart, M. v.d., Schuurs, A.H.W.M. Sol particle agglutination immunoassay for human chorionic gonodotrophin. Fresenius Z. Anal. Chem. 1980; 301: 132.

14. Bellisario, R., Carlsen, R.B. and Bahl, O.P. Human chorionic gonadotropin. Linear amino acid sequence of the subunit. J. Biol. Chem. 1973; 248: 6796-09.

15. Carlsen, R.B., Bahl, O.P. and Swaminathan, N. Human chorionic gonadotropin. linear amino acid sequence of the ß subunit. J. Biol. Chem. 1973; 248: 6810-27.

16. Schönherr, O.T., Van Gelder, P.T.J.A., Van Hees, P.J., Van Os, A.M.J.M. and Roelofs, H.W.M. A hollow fibre dialysis system for the in vitro production in mice. Develop. Biol. Stand. 1987; 66: 211-20.

17. Tijssen, P. In: R.H. Burdon and P.H. Knippenberg (Eds.), Laboratory Techniques in Biochemistry and Molecular Biology, Vol. 15, Practice and theory of enzyme immunoassays. Amsterdam: Elsevier, 1986: 96.

18. Gribnau, T., van Lith, Th., van Sommeren, A., Roeles, F., van Hell, H. and Schuurs, A. Synthesis and application of immunosorbents based on halopyrimidine or reactive azo-dye activated polysaccharides- influence of ligand modification on the performance of immunosorbents. in Egly J.M. (Ed.) Affinity Chromatography and Molecular Interactions, INSERM, Paris, 1979: 175-85.

19. Leuvering, J.H.W., Thal P.J.H.M., Waart, M. v.d. and Schuurs, A.H.W.M. Sol particle immunoassay (SPIA). J. Immunoassay. 1980; 1: 77-91.

20. Leuvering, J.H.W., Goverde, B.C., Thal P.J.H.M., and Schuurs, A.H.W.M., A homogeneous sol particle immunoassay for human chorionic gonadotrophin using monoclonal antibodies. J. Immunol. Methods. 1983; 60: 9-23.

21. Moyle, W.R., Anderson, D.M. and Ehrlich P. A circular antibody-antigen complex is responsible for increased affinity shown by mixtures of monoclonal antibodies to human chorionic gonadotropin. J. Immunol. 1983; 131: 1900-5.

22. Feldman, H.A. Mathematical theory of complete ligand binding systems at equilibrium. Some methods for parameter fitting. Anal. Biochem. 1972; 48: 317-38.

23. Klotz, I.M. Numbers of receptor sites from Scatchard graphs: facts and fantasies. Science. 1982; 217: 1247-9.

24. Connors, K.A. Binding constants: The measurement of molecular complex stability. New York: John Wiley & Sons. 1987: 59.

25. Karush, F. Immunologic specificity and molecular structure. Adv. Immunol. 1962; 2: 1.

26. Rodbard, D. and Feldman, H.A. Theory of protein-ligand interaction. Methods Enzymol. 1975; 36: 10-5

27. Byers L.D. Probe-dependent cooperativity patterns in Hill-plots. J. Chem. Ed. 1977; 54: 352-4.

28. Kermode J.C. The curvilinear Scatchard plot. Biochem. Pharmacol. 1989; 38: 2053-60.

29. Ehrlich, P.H., Moyle W.R, Moustafa, Z.A. and Canfield, R.E. Mixing two monoclonal antibodies yields enhanced affinity for antigen. J. Immunol. 1982; 128: 2709-13.

30. Berger, P., Kofler, R. and Wick, G. Monoclonal antibodies against human chorionic gonadotrophin (hCG): II. Affinity and ability to neutralize the biological activity of hCG. Am. J. Reprod. Immunol. 1984; 5: 157-60.

31. Singh, V. and Singh, O. A comparative study of the methods for determination of association constant of antigen-antibody complexes. Biol. Mem. 1985; 10: 131-40.

32. Weintraub, B.D., Rosen, S.W., Mccammon, J.A. and Perlman, R.L. Apparent cooperativity in radioimmunoassay of human chorionic gonadotrophin. Endocrinol. 1973; 92: 1250-5.

33. Matsukura, S., West, C.D., Ichikawa, Y., Jubiz, W., Harada, G. and Tyler, F.H. A new phenomenon of usefulness in the radioimmunoassay of plasma adrenocorticotropic hormone. J. Lab. Clin. Med. 1971; 77: 490-500.

34. Tosi, R., Tanigaki, N., Sorrentino, R., Accolla, R. and Corte, G. Binding of one monoclonal antibody to human Ia molecules can be enhanced by a second monoclonal antibody. Eur. J. Immunol. 1981; 11: 721-6.

35. Ehrlich, P.H.,and Moyle W.R. Cooperative immunoassays: ultrasensitive assays with mixed monoclonal antibodies. Science. 1983; 221: 279-81.

36. Erp, R. v., Gribnau, T.C.J., Sommeren, A.P.G. v., and Bloemers, H.P.J. (1990) Affinity of monoclonal antibodies: Interpretation of the positive cooperative nature of anti-hCG/hCG interactions. J. Immunol. Methods. submitted for publication.