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Binding properties of monoclonal anti-IgG antibodies: analysis of binding curves in monoclonal antibody systems

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Summary. The binding properties of an immune complex-forming system comprising human IgG and mouse monoclonal antibodies against human IgG have been studied. A refined binding assay has been applied directly on ascitic fluid containing monoclonal antibody. Complete sets of binding data of a series of different monoclonal antibodies were collected and analysed by various graphical and statistical methods. Special attention was given to methods which allow determination of specific monoclonal antibody concentration as well as antibody affinity. It was found that the formation of genuine antigen:antibody complexes *per se* gives rise to deviations from expected linearity in commonly used binding equations. Good correlation was found between the antibody concentrations obtained by various graphical approaches, whereas the size of the association constant seemed to depend on the method in use. The binding pattern was found to be dependent on the concentration of antibody. Most reliable parameters were obtained if the product of the antibody concentration and the association constant was below 10.

INTRODUCTION

Antigen–antibody interactions constitute a type of biochemical binding reaction with distinctive properties. An oligovalent antigen and a bivalent antibody form together a distribution of different complexes, individually termed Ag_iAb_j, where i as well as j can be larger than one. The characterization of an antibody in terms of its association constant therefore encounters two problems, namely that the composition of the distribution of complexes depends on the reaction mixture, and that the measurable quantity, the concentration of bound antigen, is not directly related to the number of bonds created. As an example, the complex Ag_3Ab_2 contains three antigen molecules, but four bonds are required for the formation of this complex (Steensgaard & Johansen, 1980).

In the present work we have studied the binding properties of a series of mouse monoclonal antibodies directed against human IgG. Human IgG is expected to present each epitope twice due to its symmetrical structure, and the present model system for antigenic binding of antibodies is therefore the simplest possible system for studying the formation of genuine immune complexes. A series of different graphical approaches have been used to analyse the binding data, and it has been found that the data consistently gives rise to deviations from linearity in linear transforms of simple binding equations. It seems that antibody concentrations can be obtained fairly well by all methods, but difficulties arise in the estimation of association constants.

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MATERIALS AND METHODS

Materials

The antigen, a human IgG1 kappa paraprotein, and ascitic fluids containing monoclonal antibodies were kindly provided by Dr R. Jefferis, Department of Immunology, University of Birmingham, U.K. The production of these monoclonal antibodies has been described in detail by Lowe *et al.* (1981) and Partridge *et al.* (1982). The following monoclonal antibodies were used: 3e10, OF3, J10d, xIa11, We10, xIe4, 49/2c3 (all anti-pFc); QF1, K1d, HIb, 9a6 (all anti-Cγ2). Most of these monoclonal antibodies are available through Seward Laboratory, UAC House, Blackfriars, London, U.K. An anti-human kappa light-chain preparation was obtained from Bethesda Research Laboratories (BRL), Maryland, U.S.A. The antigen was iodinated with ^{125}I by the chloramine-T method (Hallaba & Drouet, 1971). Rabbit IgG against mouse IgG was obtained from Dako a/s, Copenhagen, Code No. Z 109.

Binding experiments

The binding experiments were performed with a refined version of a previously described immune precipitation method for affinity determination of affinity-purified monoclonal antibodies (Jacobsen, Frich & Steensgaard, 1982). The procedure was as follows. The ascitic fluids were initially diluted to an absorption of 0·2 at 280 nm. Equal volumes, 350 μl, of ^{125}I-labelled antigen (10 dilutions in the range 6–120 μg/ml) and of ascitic fluids in phosphate-buffered saline pH 7·4 with 0·1% BSA and 0·01% Tween 80, were gently agitated 30 min at 37°. Aliquots, 2 × 100 μl, were withdrawn for radioactivity counting. Rabbit IgG against the Fc-portion of mouse IgG, 250 μl, typically diluted 1:30, was added and agitation continued for another 30 min before centrifugation 30 min at 3200 ***g***. The required concentration of rabbit IgG was determined by difference turbidometric measurements (Jacobsen & Steensgaard, 1979). Aliquots (2 × 100 μl) were withdrawn for radioactivity counting. The correlation between c.p.m. and nM antigen concentration in the incubation solution was obtained from a standard curve. Bound antigen concentration was calculated as the difference between total free and unprecipitated antigen concentrations.

Theoretical methods

The experimental data were analysed by using various plot systems according to the binding equations listed in Table 1. The direct binding plot (eqn. I) is obtained when bound Ag (b) is plotted directly as a function of free antigen ($[Ag]$). A numerical approximation of the binding data to equation I gives K, (Ab) and a as described previously (Jacobsen *et al.* 1982). The exponent a is taken as a mathematical device to improve the curve fitting, only. If a is equal to 1, the general binding equation can be transformed to linear equations, which allow determinations of total antibody concentration, (Ab), and K. These are listed as equations II–IV in Table 1. The straight lines in all figures were obtained by linear regression analysis. Equation II is frequently named the Steward–Petty plot (Steward & Petty, 1972). If equation IV is divided throughout with (Ab) it is identical to the Scatchard equation (Scatchard, 1949). Equation V is the Scatchard equation. The 6th equation is Sips

Table 1. The binding equations used in the analyses of binding data

Direct:
(I) $b = n\,(Ab)\,K\,[Ag]^a/(1 + K\,[Ag]^a)$

Linear transformations:	y-axis	x-axis	y-axis intercept	slope
(II) $1/b = 1/(n\,K\,(Ab)\,[Ag]) + 1/(n(Ab))$	$1/b$	$1/[Ag]$	$1/(n(Ab))$	$1/(n\,K(Ab))$
(III) $[Ag] = n\,(Ab)\,[Ag]/b - 1/K$	$[Ag]$	$[Ag]/b$	$-1/K$	$n(Ab)$
(IV) $b = -b/([Ag]K) + n(Ab)$	b	$b/[Ag]$	$n\,(Ab)$	$-1/K$
(V) $r/[Ag] = -r\,K + n\,K$	$r/[Ag]$	r	$n\,K$	$-K$
(VI) $\log(r/(n-r)) = a\log[Ag] + \log K$	$\log(r/(n-r))$	$\log[Ag]$	$\log K$	a

b is bound Ag concentration; n is the valency of Ab (i.e. 2); (Ab) is the total Ab concentration; K is the association constant; $[Ag]$ is the equilibrium concentration of Ag and $r = b/(Ab)$.

transformation which allows determination of K and a, provided (Ab) is known (Sips, 1948).

RESULTS

Effect of antibody concentration

Figure 1 shows the binding data of the anti-kappa-BRL monoclonal antibody plotted graphically in six different ways and for two different concentrations. The highest concentration of antibody reflects a 100-fold dilution of the original ascitic fluid, and the lowest concentration a 200-fold dilution. The specific antibody concentrations and the corresponding association constants as estimated by the different approaches are given in Table 2. Figure 1(a) shows binding according to equation I. The best fitting curves were obtained for $a = 0{\cdot}6$ for the highest concentration of antibody, and 0·8 for the lowest antibody concentration, respectively. In Fig. 1(b), the same data has been plotted according to equation II, the Steward–Petty approach. Although the data approaches a linear shape, they exhibit a clear convex curvilinearity. In Fig. 1(c), the data is plotted according to equation III. The regression analysis gave correlation coefficients very close to one, as seen in Table 2, in either case. It deserves to be mentioned that this approach consistently yielded the highest correlation coefficients with all antibodies tested. In Fig. 1(d), the data are plotted according to equation IV. Both dilutions of antibody give rise to deviations from linearity. Although the correlation coefficient is rather high (cf. Table 2) the slope of the line is clearly dependent on the range of antigen concentrations, and illustrates the importance of a broad range of antigen concentrations. In Fig. 1(e), the data have been plotted the Scatchard way using the antibody concentrations obtained by the direct approximation. Although the points by eye appear to belong to the same concave curve, the linear regression analysis gives two different lines. Both of these lines, however, intercept the x-axis correctly at a value of two. Finally, in Fig. 1(f), the data are plotted according to the Sips approach, again using the antibody concentration as determined by the direct method. Again two different lines appear with a estimated to 0·91 and 0·96, respectively. These values do not agree with those found by the direct binding method, and it was found for all the antibodies tested that the value of a determined by the Sips method was higher than the value of a determined by the direct binding method.

The estimated antibody concentrations and association constants obtained for this antibody are summarized in Table 2. It can be seen that the estimated association constants depend on the antibody concentration used, and that they appear to be lowest with the lowest antibody concentration.

Estimation of antibody concentration

Figure 2 shows the correlation between the antibody concentrations obtained by the different approaches. Data from all 12 monoclonal antibodies is included, and 49/2c3 and the BRL antibody have been used at more than one concentration. There is clearly a good correlation between the values of the antibody concentrations obtained by the different approaches, and there is no favouring of a particular approach.

Estimation of association constants

Figure 3 shows the correlation between the value of the association constants obtained by the various approaches. The correlation between the different set of values is clearly poor. The best correlation ($r^2 = 0{\cdot}96$) is between the values obtained by the direct approach with optimized a and with $a = 1$, respectively. The poorest correlation is seen between the direct method with optimized a and the Sips plot (eqn.(6), $r^2 = 0{\cdot}58$). The correlation coefficients of the other plot approaches against the direct approach were 0·92, 0·66, 0·91 and 0·90, respectively, corresponding to equations II, III, IV and V, respectively.

As the results shown in Fig. 1 indicated that the estimated value of the association constant apparently depended on the antibody concentration in use, this phenomenon was studied further by plotting the product of the association constant and the antibody concentration against a, the index of ideality. The results are shown in Fig. 4. It appears from this figure that a clearly has the lowest value for the highest value of the product, and that a approaches unity when the product of the association constant and the antibody concentration is as low as technically possible.

DISCUSSION

In the study of monoclonal antibodies it is important to be able to estimate the active antibody concentration as well as the association constant of the antibody. The general principle herein is to measure the

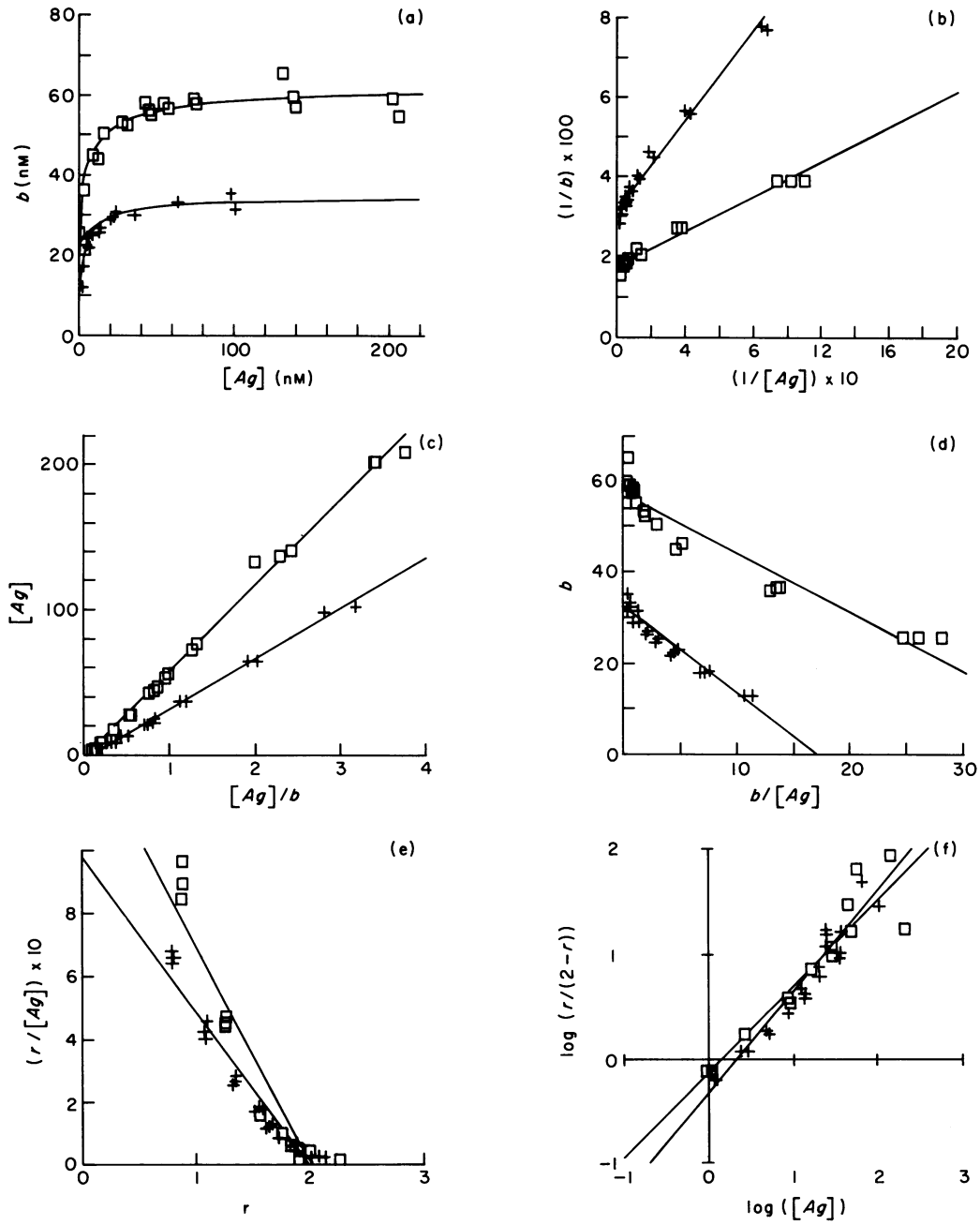

Figure 1. The binding data of the anti-kappa BRL monoclonal antibody plotted graphically in six different ways and for two different concentrations. The highest concentration of antibody reflects a 100-fold dilution of the original ascitic fluid (□), and the lowest concentration a 200-fold dilution (+). Plots a, b, c, d, e and f correspond to equations I, II, III, IV, V and VI in Table 1, respectively.

Table 2. Specific antibody concentrations and corresponding association constants estimated by the different approaches shown in Fig. 1

	b vs [*Ag*]	1/*b* vs 1/[*Ag*]	[*Ag*] vs [*Ag*]/*b*	*b* vs *b*/[*Ag*]	*r*/[*Ag*] vs *r*	log(*r*/(2−*r*)) vs log([*Ag*])
$K \times 10^{-8}$ (M^{-1})	6·9	8·2	6·2	7·6	6·9	6·0
(*Ab*) (nM)	31	28	29	28	—	—
r^2		0·97	0·996	0·908	0·908	0·96
$K \times 10^{-8}$ (M^{-1})	4·5	5·6	3·3	5·2	4·9	4·7
(*Ab*) (nM)	16	16	17	16	—	—
r^2		0·98	0·997	0·942	0·942	0·95

The monoclonal antibody was anti-kappa-BRL diluted 100- and 200-fold, respectively; r^2 is the linear correlation coefficient.

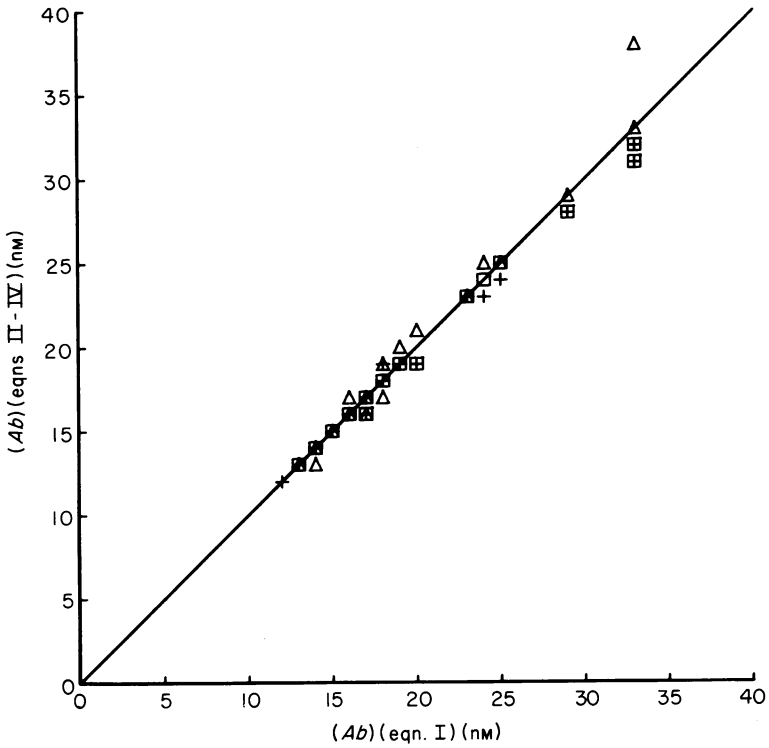

Figure 2. Correlation between antibody concentrations determined from the various plot systems. The abscissa is (*Ab*) obtained from equation I taking $a = 1$; (+), (△) and (□) are (*Ab*) obtained from equations II, III and IV, respectively.

concentration of free and bound antigen, respectively, for a series of antigen:antibody mixtures that have reacted and have reached chemical equilibrium. The resulting data are then treated by graphical or statistical methods to obtain the desired parameters of the antibody. In the present work we have used and compared six different approaches for estimation of antibody concentration and association constants using data from 12 different monoclonal antibodies against human IgG. The antibodies have previously been characterised by other means (Lowe *et al.*, 1981; Lowe *et al.*, 1982; Partridge *et al.*, 1982; Jefferis *et al.*, 1982; Steensgaard *et al.*, 1982).

All generally used binding equations, including

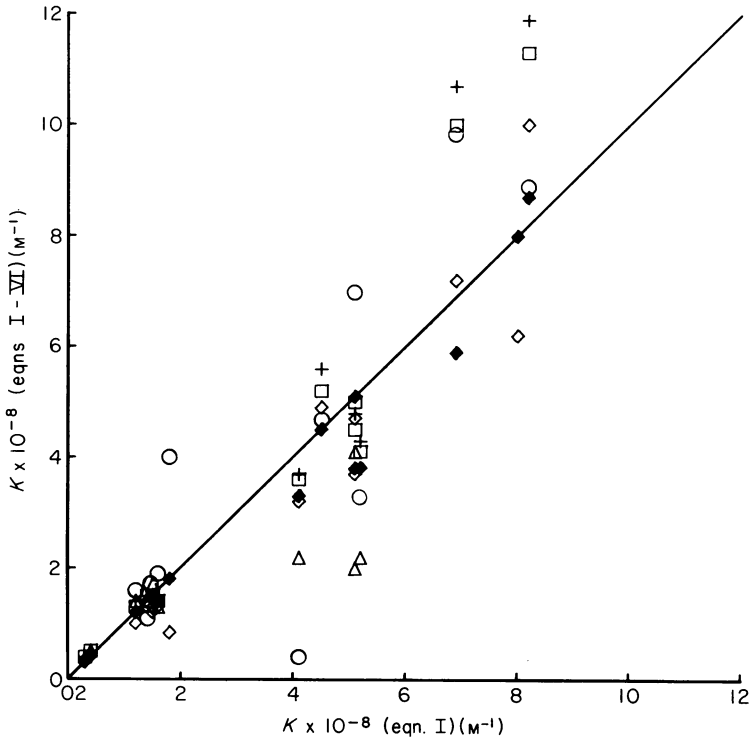

Figure 3. The affinities (as K) obtained from the various plot systems. The abscissa is K obtained from equation I optimized with regard to a; (♦). K obtained from equation I with $a = 1$; (+), (△), (□), (◊) and (○), K values obtained from the reciprocal plot equations II, III, IV, V and VI, respectively.

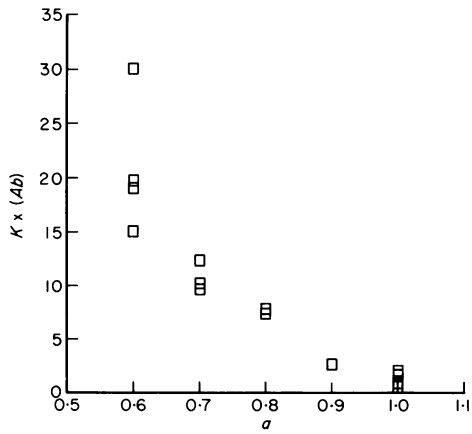

Figure 4. The product of K and (Ab) versus a. The values K, (Ab) and a were all obtained from the direct binding plot equation I for the series of monoclonal antibodies.

those shown in Table 1, are based on the assumption that the number of bound antigen molecules exactly matches the number of bonds created in antigen–antibody complexes. In the present model system as well as in many other antigen:antibody interactions this does not always hold, as larger complexes are structurally possible. The results shown in Fig. 1 reveal that the actual binding reaction is more complicated than assumed in the common binding equations listed in Table 1. In the direct binding approach a is typically lower than one. The index a thus reflects how much the actual system deviates from ideal binding. If a equals or approaches one the system behaves as a simple ligand binding system following a simple theoretical pattern. Values of a below one is traditionally attributed to antibody heterogeneity (Nisonoff & Pressman, 1958), but in the present case the individual antibodies as well as the antigen are homogeneous. Further physico-chemical reasons for finding low values of a are negative cooperativity between the two binding sites and the formation of genuine complexes. The question on cooperativity between the antibody binding sites has not been studied here, but the way the various binding plots diverge from simplicity found experimentally here is in agreement with theoretical predictions made by computer simulations (Steens-

gaard, Steward & Frich, 1980). We therefore assume that the low values of a are due mainly to the formation of a mixture of complexes exemplified by Ag_3Ab_2 in which the number of bonds do not match the number of bound antigen molecules.

It is in this connection an interesting finding that the index of ideality, a, decreases with increasing concentrations of antibody and antigen, but maintaining the same range of antigen excess. The basic theory does not account for any concentration dependency. It is, however, in agreement with predictions from our previously published computer model of antigen–antibody interactions (Steensgaard *et al.*, 1980). According to this model, an increase in antibody concentration, but with the same antigen:antibody ratio, will lead to the formation of relatively larger complexes, thus yielding more of those complexes that have more bonds than bound antigen molecules. Thus, the formation of genuine antigen–antibody complexes is a concentration-dependent process. The least deviation from expected linearity is achieved for the lowest possible antibody concentration, more precisely when the product of association constant and antibody approaches one. It is, however, our experience that it can be difficult or impossible to use very low antibody concentrations, because non-specific binding of the antigen may give rise to unfortunate high background binding values.

The deviations from linearity in the linear transforms of the binding equation were found to be consistent. The inherent tendency to curvilinearity, especially of data plotted according to equations II and IV means that the estimated slope is highly dependent on the range of antigen concentrations used. The more narrow the range is, the steeper the line will be, and this may be one main part of an explanation of the lack of correlation between the association constant obtained in different ways. It was found that plot of data according to equation III throughout gave the best lines as judged from the correlation coefficients. However, the intercept on the y-axis is too close to zero to provide a good method for estimation of the association constant.

The Scatchard and Sips methods require knowledge of antibody concentration for estimation of the association constant. It is, however, found that the measured binding data consistently gives rise to curvilinearity, leaving these approaches only a doubtful applicability on systems where genuine antigen–antibody complexes can be formed. Thus, the Steward–Petty approach may be preferred due to its simplicity. We have in the present context used the direct binding approach as our reference model, because it gives both antibody concentration and association constant, as well as a measure, a, for the apparent ideality of the system. Also this approach shows if saturation of the antibody binding sites are reached, and the association constant is to be taken as the reciprocal of that antigen concentration which provide half saturation of the binding sites. The method, however, requires substantial computation, and the value of the resulting numbers are not necessarily improved by computer manipulations.

Finally, it was found that the average antibody concentration in ascitic fluids was $1{\cdot}7 \pm 0{\cdot}7$ (SD) mg/ml and that the average value of the association constant was $4{\cdot}3 \pm 3{\cdot}0 \times 10^8$ (SD) M^{-1}, reflecting the results of the immunization procedure.

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