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Theoretical and ultracentrifugal analysis of immune complex formation between monoclonal antibodies and human IgG

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Summary. Immune complex formation of four different mouse monoclonal antibodies against human IgG has been studied using analytical zonal centrifugation. A theoretical model has been used to depict thermodynamic ideal immune complex formation of monoclonal antibodies. It was found that the four monoclonal antibodies differed very much with respect to immune complex formation with human IgG. One of the monoclonal antibodies formed immune complexes in agreement with the theoretical model. Another was strongly related thereto. A third monoclonal antibody formed only a single complex and might exhibit a positive co-operativity between its two sites. A fourth formed an excess of a possibly cyclic complex. Thus monoclonal antibodies differ substantially with respect to physical properties adding a new aspect to the problems of antibody heterogeneity. It is moreover found that analytical zonal centrifugation can be used to estimate the number of antigenic determinants and antibody-binding sites thereby assuring whether or not a particular IgG monoclonal antibody has two binding sites.

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INTRODUCTION

To fully exploit the potential of monoclonal antibodies it is necessary to define the parameters which determine the interaction of individual antibodies with antigen. In the present study we have examined interactions of monoclonal antibody to human IgG by analysis of the immune complexes formed. Such studies may contribute to the refined application of monoclonal antibodies, and give insights into immune complex formation, *in vivo*, which is a feature of a number of pathological conditions.

The symmetry of the IgG molecule results in each epitope typically being expressed twice and being spatially disposed such that the molecule is functionally antigenically divalent. Therefore on interaction with a single bifunctional antibody it can form linear or cyclic complexes that will be soluble even in antibody excess and so can be analysed by analytical zonal ultracentrifugation (Steensgaard, Johansen & Jacobsen, 1979).

The present work contains a detailed study of immune complex formation of four different mouse monoclonal antibodies by analytical zonal centrifugation. Because it was found that they all differed in immune complex forming properties, our previously described theoretical model of thermodynamic ideal immune complex formation (Steensgaard, Maw Liu, Cline & Møller, 1977; Steensgaard & Frich, 1979) was

used to serve as an external reference for evaluation of experimentally obtained immune complex distributions.

MATERIALS AND METHODS

Monoclonal antibodies

The principle of Köhler & Milstein (1975) was followed whilst the detailed protocol was as reported by Lowe, Hardie, Jefferis, Lung, Drysdale, Richardson, Raykundalia, Catty, Appleby, Drew & Maclennan (1981). Antibodies 6e1, x3a8 and a55 were affinity-purified by passage of ascitic fluid over a Sepharose-IgG column (with human polyclonal IgG) eluted with 0·02 M phosphate, pH 7·0+0·2 M NaCl. The antibody was eluted with 3 M KSCN and dialysed into saline. Antibody 1a1 did not bind to the Sepharose-IgG under these conditions. However, it was obtained as a distinct retarded peak if a 4% polyethylene glycol (mol. wt 1500) was added to the eluent. Alternatively it could be isolated conventionally from an Affigel-IgG immunosorbent column. Concentrations of antibodies were estimated from the absorbance at 280 nm, taking the extinction coefficient as 14. Thus total IgG is measured rather than active antibody. Affinity purified antibody preparations were stored at −20° with 0·1% azide. All transportations were by air mail. All preparations have been treated in the same way.

Human IgG antigen

A single IgG paraprotein of subclass one bearing kappa light chains was used throughout the study. It was isolated from serum as the breakthrough peak on DEAE-cellulose equilibrated in 0·01 M phosphate, pH 7·0. The human IgG was labelled with I-125 by the chloramine-T method (Hallaba & Drouet, 1971).

Formation of immune complexes

Immune complexes of monoclonal antibodies were made by mixing human IgG (radiolabelled) with the monoclonal antibody in question in a final volume of 2·2 ml. The mixture was incubated for 1 hr at 37° and left overnight at 4°. The actual concentrations of antigen and antibody are given in the text to the figures. In all cases a constant amount of antigen was used, and the concentration of antibody was varied. The principal concentration range was obtained by doubling the antibody concentrations, but some intermediate concentrations were also used. Two millilitres of the immune complex mixture were used as sample in the zonal centrifuge. PBS (pH 7·4) was used as a solvent in all samples.

Zonal centrifugation

Zonal centrifugations were performed on a TZT48 zonal rotor in a Kontron TGA65 ultracentrifuge as previously described in more detail (Steensgaard & Jacobsen, 1979). Two millilitres of sample and 100 ml of overlay were used. The gradient was a 5%–20% (w/w) isokinetic sucrose gradient (Steensgaard, 1970) in phosphate-buffered saline. Centrifugations were carried out at 8° for 6 hr at 48,000 r.p.m. Sixty-five fractions were collected, each of 10 ml.

Sucrose concentration, volume and radioactivity were measured in all fractions and used to calculate the equivalent sedimentation coefficient of all fractions by the computerized method (Steensgaard, Møller & Funding, 1978). Finally, the computed sedimentation coefficients and the measured radioactivities were used to plot the measured activity versus the calculated sedimentation coefficients as in Figs 2, 3, 4 and 5. Correction for sectorial dilution in the zonal rotor was made. The centrifugal patterns were found to be perfectly reproducible, and the mean sedimentation coefficients varied at most 1S. To achieve ideal sedimentation conditions (Steensgaard, Møller & Funding, 1975) very low total protein concentrations were used so that the sedimentation of immune complexes only could be followed by measuring the radioactivity.

THEORETICAL CONSIDERATIONS

The present antigen, human IgG1 (with kappa chains) is believed to have two identical antigenic determinants per monoclonal antibody. The mouse monoclonal antibodies are similarly presumed to possess two independently reacting and identical combining regions. All complexes that are structurally possible can be systematized in the following immune complex matrix:

$$\begin{array}{ccccccc} Ag_2Ab_1 & Ag_3Ab_2 & Ag_4Ab_3 & Ag_5Ab_4 & Ag_6Ab_5 & \ldots\ldots \\ Ag_1Ab_1 & Ag_2Ab_2 & Ag_3Ab_3 & Ag_4Ab_4 & Ag_5Ab_5 & \ldots\ldots \\ & Ag_1Ab_2 & Ag_2Ab_3 & Ag_3Ab_4 & Ag_4Ab_5 & \ldots\ldots \end{array}$$

At chemical equilibrium, all complexes will be in a state of mutual equilibrium with each other, and also with free antigen and free antibody. It has been found that the concentration of an individual complex

Ag_iAb_j at chemical equilibrium can be assessed by use of a computer model (Steensgaard *et al.*, 1977; Steensgaard & Frich, 1979; Steensgaard, Steward & Frich, 1980b). The calculations are, however, simpler in this case because one can suppose that the antigen is divalent and that the antibody is homogeneous. A new program in Basic was written for the present purpose and all calculations were performed on a Tektronix 4052 minicomputer, and the curves presented and drawn directly by the computer using a Tektronix 4662 digital plotter. The program gives two outputs. The first gives the calculated immune complex distributions as a table whereas the second output shows the immune complex distributions as they would appear upon gradient centrifugation.

Table 1 shows three calculated immune complex distributions. Because immune complex formation is dependent on the absolute concentrations of antigen as well as antibody, their valences and their mutual affinity, the outcome of antigen–antibody interactions is not simply related to the antigen:antibody ratio. To simplify the problem dimensionless concentrations are expressed as the product of the molar concentration of the species in question and the association constant. The antigen excess situation is accordingly defined as the situation which gives a rather high concentration of free antigen, and correspondingly a low concentration of free antibody. The antibody excess situation is similarly defined as the situation that ends up with very little free antigen, but an excess of free antibody. The intermediate situation (not equivalence) does not give any of the free species in large excess alone. In the antigen excess situation in Table 1, 70% of the antigen has not reacted at equilibrium. Most complexed antigen is calculated to exist in the form of complexes containing only one antibody molecule (23·5%) and less than 7% of the original antigen is complexed in larger complexes. Complex concentrations rapidly decrease with increasing complex size. In the antibody excess situation (with about 60% free antibody) the complex Ag_1Ab_2 prevails together with the structurally related complex Ag_2Ab_3. Only 2% antigen exists here in free form. Finally, in the calculated intermediate situation about 27% of antigen as well as antibody has not reacted. A richer variety of complexes is formed here, but most of the complexes are rather small complexes and the calculated concentrations of complexes again rapidly decrease with increasing complex size.

To convert these complex distribution patterns to graphs that are more directly comparable with experimental findings a program was written to show the complexes as they would appear upon density gradient

Table 1. Calculated immune complex distribution using equations (3) and (4) as described in Theoretical Considerations.

Total antigen (K·(Ag))	2		2		2	
Total antibody (K·(Ab))	0·5		2		8	
Free antigen (%)	70·1		27·1		2·2	
Free antibody (%)	17·6		27·1		59·9	
Composition	Ag conc. (%)	Ab conc. (%)	Ag conc. (%)	Ab conc. (%)	Ag conc. (%)	Ab conc. (%)
Ag_2Ab_1	17·30	34·61	15·96	7·98	0·91	0·11
Ag_1Ab_1	6·17	24·67	14·71	14·71	10·44	2·61
Ag_3Ab_2	3·20	8·54	7·04	4·70	0·28	0·05
Ag_2Ab_2	1·52	6·08	8·66	8·66	4·36	1·09
Ag_1Ab_2	0·54	4·34	7·98	15·96	50·00	25·00
Ag_4Ab_3	0·53	1·58	2·76	2·07	0·08	0·01
Ag_3Ab_3	0·28	1·13	3·82	3·82	1·36	0·34
Ag_2Ab_3	0·13	0·80	4·70	7·04	20·87	7·83
Ag_5Ab_4	0·08	0·26	1·02	0·81	0·02	0·00
Ag_4Ab_4	0·05	0·19	1·50	1·50	0·38	0·09
Ag_3Ab_4	0·02	0·13	2·07	2·76	6·53	2·18
Ag_6Ab_5	0·01	0·04	0·36	0·30	0·01	0·00
Ag_5Ab_5	0·01	0·03	0·55	0·55	0·10	0·02
Ag_4Ab_5	0·00	0·02	0·81	1·02	1·82	0·57
Ag_7Ab_6	0·00	0·01	0·12	0·11	0·00	0·00
Ag_6Ab_6	0·00	0·00	0·19	0·19	0·02	0·01
Ag_5Ab_6	0·00	0·00	0·30	0·36	0·47	0·14

centrifugation. The molecular weight of a complex is given by its composition. From the molecular weight the equivalent sedimentation coefficient ($s_{20,w}$) can be calculated by use of the formula:

$$s_{20,w} = M^{0.67}\left(\frac{1-\bar{v}\rho}{\sqrt[3]{\bar{v}}}\right)/[\mathcal{N}6\pi\eta\sqrt[3]{0.75/(\mathcal{N}\pi)}\left(\frac{f}{f_o}\right)] \quad (1)$$

where M is the molecular weight of the complex, $\bar{v}$ is the partial specific volume, ρ is the density of the medium (here water at 20°), $\mathcal{N}$ is Avogadro's number, η is the viscosity of the medium, and (f/f_o) is the frictional ratio of the complex (Jacobsen & Steensgaard, 1979). Using zonal centrifugation a single component sediments as a zone that can be described as a Gaussian frequency function (Steensgaard *et al.*, 1975). Thus, the final shape of the zone of any individual complex can be calculated as follows:

$$f(s) = \frac{A}{B\sqrt{2\pi}}\exp[-\tfrac{1}{2}\left(\frac{s-s_M}{B}\right)^2] \quad (2)$$

where A is the area of the zone given by its calculated concentration, B is the band width of the zone given as the standard deviation, and s_M is the position of the mass centre of the zone as calculated from equation (1). The individual zones for all possible complexes are finally summed to give the sedimentation profiles in Fig 1. The areas (A) of the zones were in the present examples calculated from the content of antigen in the complexes to mimic the experimental situation where the antigen only was labelled radioactively. The band width was estimated as the experimental band width of free antigen centrifuged alone.

In the calculations mentioned above neither the partial specific volume nor the frictional ratio is known for individual immune complexes. The effect of these parameters on the calculated sedimentation coefficients of immune complexes of varying composition, assuming a monomer mol. wt of 150,000, a partial specific volume of 0·74 and frictional ratios varying from 1·2 to 1·8 is shown in Table 2.

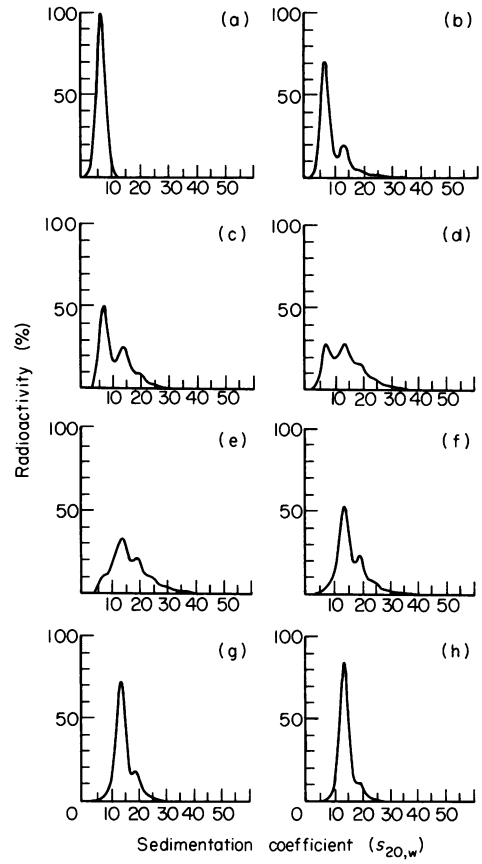

Figure 1. Theoretical sedimentation patterns of calculated immune complex mixtures. The ordinate gives the antigen content (corresponding to the use of labelled antigen), and the abscissa gives the sedimentation coefficient. In (a) the calculated sedimentation of free antigen is shown. In the remaining figures the immune complex distributions stemming from the same concentration of antigen, but increasing concentrations of antibody are shown. In dimensionless units (see Theoretical Considerations) the antigen concentration was 2·0, whereas the concentrations of antibody were as follows: (a) 0·0; (b) 0·5; (c) 1·0; (d) 2·0; (e) 4·0; (f) 8·0; (g) 16·0 and (h) 32·0.

RESULTS

Theoretical immune complex distributions

A series of immune complex distributions was calculated to study and to define the thermodynamic ideal pictures of immune complex formation of monoclonal antibodies from antigen excess to antibody excess. The calculated results are shown in Fig. 1. It appears from Fig. 1 that a distinction can be made between three different situations. Firstly, in antigen excess (b and c) the sedimentation profile is characterized by a large peak containing free and unreacted antigen near 7S. Following the free antigen peak a complex peak appears. These complexes are relatively small, and the majority of complexes have sedimentation coefficients below 20S. Comparison of Table 1 and Fig 1 reveals

Table 2. Calculated sedimentation coefficients of IgG complexes with different frictional ratios. The values were calculated from equation (5) in Theoretical Considerations assuming a partial specific volume of 0·74 and a mol. wt of 150,000 for IgG.

No. of IgGs in the complexes	f/f$_o$ 1·2	1·3	1·4	1·5	1·6	1·7	1·8
	Equivalent *s* values (Svedberg units)						
1	8	7	7	6	6	6	5
2	13	12	11	10	10	9	9
3	17	16	14	13	13	12	11
4	20	19	17	16	15	14	14
5	24	22	20	19	18	17	16
6	27	25	23	21	20	19	18
7	30	27	25	24	22	21	20
8	32	30	28	26	24	23	22
9	35	32	30	28	26	25	23
10	38	35	32	30	28	27	25
11	40	37	34	32	30	28	27
12	42	39	36	34	32	30	28

that the antigen excess complexes are most likely to be the following Ag_2Ab, $AgAb$, Ag_3Ab_2 and Ag_2Ab_2. Secondly, in antibody excess (f, g and h) the picture is dominated by a 13S peak containing the complex $AgAb_2$. Very little free antigen at 7 S is present. The 13S peak is followed by smaller peaks of larger complexes near 18S and 23S. These complexes are thermodynamically likely to be Ag_2Ab_3, Ag_3Ab_4 etc., in the bottom row of complexes in the complex matrix (see Theoretical Considerations). Thirdly, between antigen excess and antibody excess, the intermediate range (d and e), a variety of different complexes are present simultaneously. However, it appears that the smallest complexes are formed in largest amounts, and that the system from a thermodynamic point of view seems to be self-limiting, and the vast majority of complexes have sedimentation coefficients below 30S. Thus, if monoclonal antibodies react as described by the present theoretical model, these three situations should be identifiable in a series of centrifugations with constant amount of antigen and a systematically varied antibody concentration.

Immune complex formation of the monoclonal antibody 6e1

The monoclonal antibody 6e1 was allowed to react with iodinated human IgG as described under Materials and Methods. A series of immune complex mixtures was centrifuged and the results were computationally converted to graphs of the same type as those used for theoretical results. The experimental results are shown in Fig 2. Antigen alone sediments to give a single homogeneous peak with a sedimentation coefficient of 7S. Next (part figures b and c) it appears that in antigen excess complex distributions related to those predicted theoretically are present, though the actual experimental separation of complexes from free antigen is not as good as predicted theoretically. With further increase in antibody concentration (d and e) a picture resembling the intermediate situation is achieved. Even the relative size of the dominating peaks matches convincingly. In antibody excess (f, g and h), a dominating peak near 12S appears in accordance with the theoretical treatment followed by

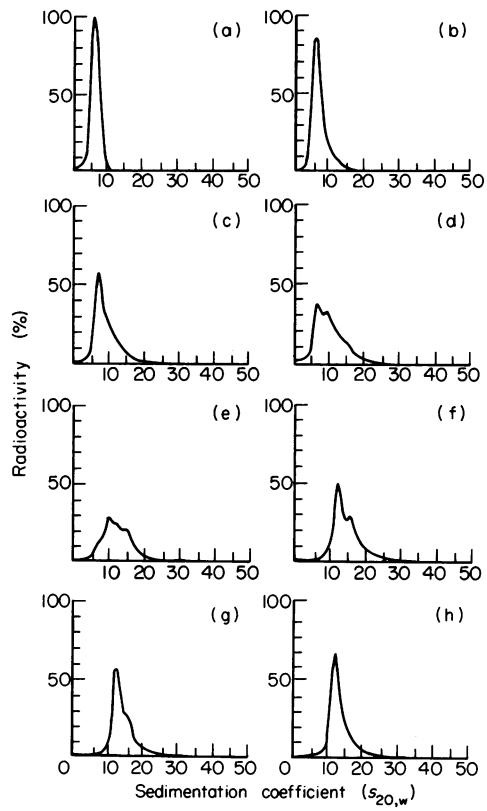

Figure 2. Experimental centrifugation of immune complexes formed of the monoclonal antibody 6e1. The amount of antigen in the sample (2·2 ml) was 15 μg, and the amounts of antibody were as follows: (a) 0 μg; (b) 25 μg; (c) 50 μg; (d) 75 μg; (e) 100 μg; (f) 200 μg; (g) 400 μg and (h) 800 μg.

a decreasing peak of larger complexes. Ultimately in gross antibody excess most of the antigen is included in the 12S peak, corresponding to the complex $AgAb_2$. Thus, as a whole the monoclonal antibody 6e1 appears to form immune complexes in an uncomplicated way as described by the present theory.

Immune complex formation of the monoclonal antibody 1a1

A series of centrifugal studies on the monoclonal antibody 1a1 is shown in Fig. 3. In antigen excess (a, b and c) a picture resembling the theoretically obtained results is seen. In contrast to antibody 6e1 the antigen excess complex has a higher sedimentation coefficient, 13S, and less of the complex AgAb is present, as judged by the separation of the 7S and the 13S peak. In the intermediate situation (e and f), the resolution is rather poor, although the general picture follows the principles of the theoretically ideal immune complex formation. In antibody excess (g and h) the antigen is nearly completely included in an 11S peak, corresponding to the thermodynamically ideal peak of the complex $AgAb_2$. The monoclonal antibody 1a1 can therefore be classified as an antibody with properties in accordance with the ideal thermodynamic antibodies described by the theoretical model, but also as an antibody with slightly deviating properties. Individual immune complexes may have characteristic sedimentation properties. The observation that the complex Ag_2Ab, formed in antigen excess, sediments somewhat faster than the complex $AgAb_2$, formed in antibody excess, although the two complexes have nearly the same molecular weight, can be explained if these complexes have different frictional ratios. Some of the differences between the complexes of 6e1 and 1a1 may also be due to differences in frictional ratios of otherwise identical complexes.

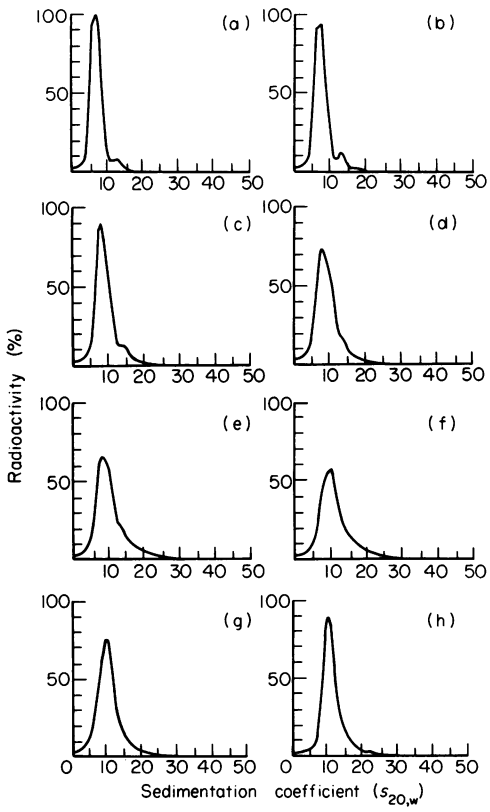

Figure 3. Experimental centrifugation of immune complexes formed of the monoclonal antibody 1a1. The amount of antigen in the sample (2·2 ml) was 75 μg, and the amounts of antibody were as follows: (a) 7 μg; (b) 18 μg; (c) 35 μg; (d) 46 μg; (e) 60 μg; (f) 70 μg; (g) 88 μg and (h) 140 μg.

Immune complex formation of the monoclonal antibody x3a8

The monoclonal antibody x3a8 was studied in a similar series of centrifugations. The results are given in Fig. 4. It is immediately apparent from the figure that the monoclonal antibody x3a8 does not follow the same thermodynamic rules for immune complex formation as the previous two monoclonal antibodies. Throughout the concentration range tested only two peaks occur upon zonal centrifugation. The first of these at 7S contains free antigen, whereas the second peak at about 15S probably contains the complex Ag_2Ab. It can be seen that the 7S peak decreases consistently with increasing addition of antibody, and that the amount of complexes in the 15S peak increases correspondingly. An integration of the curves was performed, and the amount of material in all experiments was found to be the same. There is no simple explanation of how the monoclonal antibody x3a8 forms complexes as observed here.

One possible explanation for absence of the complex AgAb is that the two active binding sites of the monoclonal antibody x3a8 are interactive by a positive co-operation mechanism. Thus each site is of low affinity, but when one site binds antigen the second site is activated to a high affinity binding site. If there is insufficient antigen present to form the complex

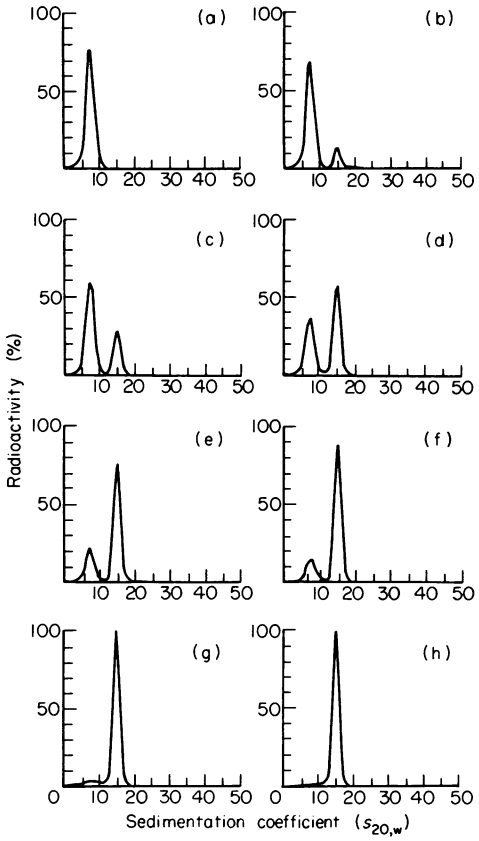

Figure 4. Experimental centrifugation of immune complexes formed of the monoclonal antibody x3a8. The amount of antigen in the sample (2·2 ml) was 150 μg, and the amounts of antibody were as follows: (a) 0 μg; (b) 160 μg; (c) 320 μg; (d) 640 μg; (e) 800 μg; (f) 960 μg; (g) 1120 μg and (h) 1280 μg.

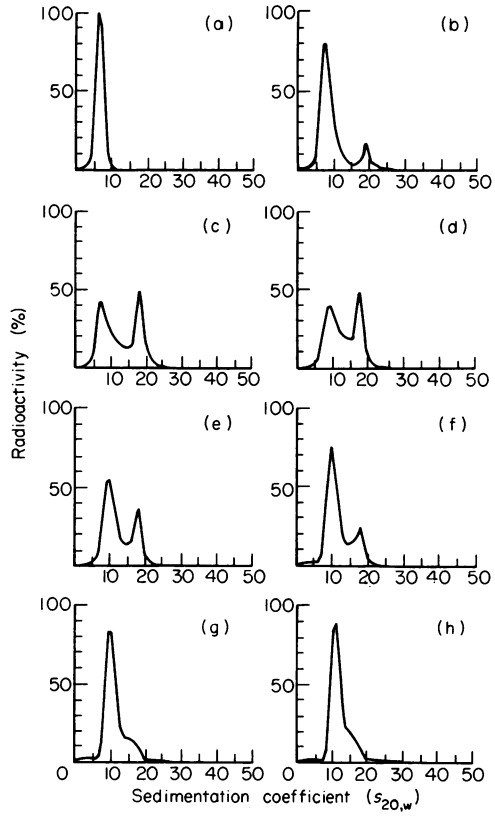

Figure 5. Experimental centrifugation of immune complexes formed of the monoclonal antibody a55. The amount of antigen in the sample (2·2 ml) was 14 μg, and the amounts of antibody were as follows: (a) 0 μg; (b) 7 μg; (c) 16 μg; (d) 32 μg; (e) 64 μg; (f) 128 μg; (g) 256 μg and (h) 512 μg.

Ag_2Ab, then the complex AgAb dissociates again. This antibody is apparantly unable to form larger complexes. Irrespective of the true mechanism of immune complex formation of x3a8, these experiments demonstrate that immune complex formation very much depends on the properties of the antibody in question, and that antibodies exist which do not follow the simple thermodynamic rules of immune complex formation described here.

Immune complex formation of the monoclonal antibody a55

This antibody also gives a different pattern of immune complex formation to that of the thermodynamic ideal. In high antigen excess two peaks appear, see Fig. 5. The first at 7S is free antigen. There is a shoulder on this peak probably composed of the typical antigen excess complexes (mostly Ag_2Ab) as for 6e1. A peak near 18 S also is present, which from Table 1 is likely to contain a complex containing 4 IgG molecules. With increasing addition of antibody the 7S and the 18S peaks decrease, and are finally replaced by the typical 11S peak seen in antibody excess. The changes in complex formation with increasing addition of antibody to the complex mixture ressembles the complex formation of 6e1 and 1a1 for the first peak only, but the appearance of an 18S peak is particular to the antibody a55. The 18S complex is likely to be Ag_2Ab_2. The relatively high proportions of this complex suggest that this species is particularly stable. Such

stability might result from the formation of a cyclic complex. A cyclic complex is likely to possess a special stability and the formation of cyclic complexes disturb the general thermodynamic equilibrium conditions in a way that leads to the formation of rather high amounts of the cyclic complexes. It is consistent with this explanation that the cyclic complexes are formed in antigen excess and in the intermediate situation where the linear complex Ag_2Ab_2 theoretically is formed in largest amount, and the linear version of Ag_2Ab_2 is the precursor of the cyclic complex with the same composition. The experiments with a55 provide additional evidence that immune complex formation depends on the structure of the antigen as well as on the properties of the monoclonal antibody.

DISCUSSION

A normal humoral immune response results in the production of a heterogeneous population of structurally distinct antibodies. One source of heterogeneity is accounted for by the fact that a macromolecular antigen may be composed of many structurally unique epitopes. Additionally, antibodies directed against a single epitope are structurally and functionally heterogeneous exhibiting marked differences in affinity of binding to antigen (Heidelberger & Kendall, 1935; Pauling Pressman & Grossberg, 1944; Steensgaard *et al.*, 1980b). In the present study we have examined the pattern of immune complex formation for a series of four monoclonal antibodies to investigate whether or not individual properties of monoclonal antibodies influence the formation of antigen–antibody complexes.

Theoretical model of immune complex formation

The theoretical model was developed to define thermodynamic ideal immune complex formation, and thus to have an external reference for evaluation of experimental results. Thermodynamic ideal immune complex formation is here defined as immune complex formation between a divalent antigen and a divalent antibody with two equally active and independent sites. Thus formation of cyclic complexes as well as mutual interactions between the two antibody-binding sites is principally excluded in the model. Differences between the predictions of the model and experimental findings therefore show that the antibody in question reacts in a more complicated way than depicted by the theory.

The present model of immune complex formation comprises two parts. The first gives the distribution of immune complexes at equilibrium, whereas the second shows how the calculated distribution of immune complexes will appear upon zonal centrifugation as described under Materials and Methods. The calculation of immune complex distributions has been discussed previously in detail (Steensgaard *et al.*, 1977; Steensgaard & Frich, 1979). It was found in these studies that the theoretical model was able with reasonable accuracy to predict the outcome of polyclonal antigen–antibody interactions in antigen excess where all complexes were soluble. The present model is simpler in dealing exclusively with a divalent antigen, and it is moreover experimentally possible to check immune complex formation from antigen excess to antibody excess because all complexes are completely soluble irrespective of composition. In the second part of the numerical calculations the sedimentation pattern of calculated immune complex distributions is computed. These calculations involve calculation of sedimentation coefficients of individual complexes and a calculation of zone widths. The sedimentation coefficients are calculated from the mol. wt of antigen and antibody, both here taken as 150,000, from the frictional ratio taken as 1·5 and from the partial specific volume, taken as 0·74. It can be seen that the free IgG peak closely approximate the experimental findings, in part substantiating the method of calculation. It is to be expected that the sedimentation coefficient of different individual complexes of the same composition will show variation. However, the good agreement between the calculated results and the experimental findings for the antibody 6e1 demonstrates that monoclonal antibodies exist which form immune complexes according to simple thermodynamic rules, and also that their sedimentation behaviour can be predicted with fair accuracy.

Immune complex formation of individual monoclonal antibodies

Of four monoclonal antibodies against human IgG only one exhibited an immune complex formation which is directly comparable with the thermodynamic ideal immune complex formation, namely 6e1. However, they all share some common features. In antigen excess all four antibodies produced a complex in the 11–15S range (Ag_2Ab, by exclusion of other possible complexes) strongly indicating that they all have two active binding sites. Three of the antibodies, 6e1, 1a1

and a55, produced an approximately 11S complex ($AgAb_2$), in gross antibody excess; indicating that the antigen in each case had two independently reacting antigenic determinants. However, the simulated results in Fig. 1 show this argument is valid only if gradual build up of an 11S peak can be seen with increasing antibody concentration. The antibody x3a8 deviates so much from the ideal behaviour throughout the range of antibody concentrations, that the same conclusion cannot be made for this antibody. It is, nevertheless, important to note that series of centrifugations as shown here can demonstrate whether or not the antibodies in question have two active binding sites, and likewise the number of antigenic determinants can be estimated.

The antibodies studied were selected because they are being applied in assay systems dependent upon immunoprecipitation (Steensgaard *et al.*, 1980a; Jefferis, Deverill, Ling & Reeves, 1980). It is surprising that they differ in immune complex forming properties to the extent they do. One (6e1) follows the thermodynamic pattern of ideal immune complex formation rather strictly. Another (1a1) follows the same rules although less rigidly, but its deviations are likely to be due mainly to very high frictional ratios of the complexes. The third (x3a8) is able to form only one kind of immune complex, Ag_2Ab. The complete absence of the complex AgAb which must be an intermediary in the formation of the complex Ag_2Ab can be explained by assuming that the two sites of x3a8 can interact co-operatively. A mechanism of co-operativity between active sites has been recorded for some enzymes, but not before for an antibody. The last antibody, a55, is able to form cyclic complexes. This has been observed before for divalent haptens (Valentine & Green, 1967; Schumaker, Green & Wilder, 1973), but not directly for antibodies towards a macromolecular antigen. The very clearly expressed peak at 18S of the supposed cyclic complexes is in agreement with the idea that cyclic complexes are thermodynamically more stable than linear complexes of same composition. To explain their predominance it is neccesary to assume that when ring closure has taken place, the cyclic complexes disturb the equilibrium, leading to the formation of an excess of the cyclic complexes. The ability to form cyclic complexes of macromolecular antigens is the result of combined structural properties of the antigen as well as of the antibody, and these findings therefore stress the importance of the antigenic structure for the outcome of antigen–antibody interactions.

The present experimental findings lead to the suggestion that antibodies towards the same antigen not only differ in affinity towards its antigenic determinant, but to a large extent also in physical properties. The latter comprise the possible ability for interactions between binding sites as well as features determining the rigidity of complexes, and hence their frictional ratios. In a wider sense it could apply to all effector functions of antibodies.

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