

The Antigen-Binding Characteristics of Antibody Pools of Different Relative Affinity

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Summary. The interstrain differences in relative affinity of mouse anti-HSA antibody previously shown by studies at equilibrium are also observed when rates of association and dissociation and avidity indices are measured. Marked differences are demonstrated in the binding characteristics of high and low relative affinity antibody to both protein and hapten antigens. These results are explained on the basis of heterogeneity of antibody-binding affinity.

INTRODUCTION

Antibody affinity, K , can be measured only when the antigen used in the assay is a simple well-defined substance such as a hapten (DNP-lysine or NIP amino caproic acids). Such measurements assume that each antibody molecule can bind two molecules of hapten. Measurement of anti-protein antibody affinity in this sense is not possible because of the multiplicity and heterogeneity of antigenic determinants. In antigen excess, the effect of the multiplicity of antigenic determinants is minimized, i.e. under these conditions it is unlikely that more than one antibody-binding site will be bound to each antigen molecule. The problem of antigenic heterogeneity remains, and in protein-anti-protein antibody systems only measurements of relative affinity K_R , can be made.

We have previously described a method for the measurement of the relative affinity K_R , of anti-protein antibodies in unfractionated mouse serum using ammonium sulphate globulin precipitation (Steward and Petty, 1972). These determinations involve the extrapolation of the plot of $1/b$ versus $1/c$ (where b is the bound and c the free antigen at equilibrium) in the equation:

$$\frac{1}{b} = \frac{1}{K} \cdot \frac{1}{c} \cdot \frac{1}{Ab_t} + \frac{1}{Ab_t}$$

to obtain a measure of antibody concentration, Ab_t (expressed as $\mu\mu$ moles of antigen-binding sites). However, because of the heterogeneity of antibody affinity this relationship deviates from linearity. We suggested that this deviation from linearity resulted from proportionately greater binding of antigen by the high K_R antibody when antigen concentration is low, and that the reverse effect, i.e. proportionately lower binding at antigen excess, would result in an opposite deviation from linearity. In this paper we present data concerning the shape of the binding curves of mouse anti-protein and antihapten antibody systems which demonstrate that for low K_R antibody there is a marked deviation

from linearity in antigen excess. In relative antibody excess, the degree of deviation from linearity is found to be much greater for low K_R antibody than for high K_R antibody. Using methods for comparing K_R which are independent of Ab_t determinations, we present data which confirm the validity of affinity measurements derived from extrapolation of the linear part of such binding curves.

MATERIALS AND METHODS

Antigens

Human serum albumin (HSA) was obtained from the Lister Institute of Preventive Medicine and purified by gel filtration through Sephadex G-200. Radioiodination was carried out by the method of McFarlane (1958). DNP-HSA was prepared by the method of Eisen (1967). H^3 - ϵ -DNP-L-lysine was prepared by the method of Eisen, Simms and Potter (1968).

Mice

Inbred strains of mice were immunized with antigen in saline (0.5 mg i.p.) weekly for 4 weeks and bled out 2 weeks later. Pools of low K_R anti-HSA antibody were prepared from sera from C₅₇B1 and B10D2 new strain mice. Pools of high K_R anti-HSA antibody were obtained from Simpson mice. Low K_R anti-DNP antibody was obtained from SWR/J mice. High K_R rabbit anti-DNP antibody was obtained from a rabbit immunized with DNP-HSA in Freund's complete adjuvant.

Determination of relative affinity

A modification of the previously described method (Steward and Petty, 1972) was used. The volume of serum used for each of ten antigen concentrations was reduced to 10 μ l and the final volume adjusted to 50 μ l. Bound antigen was determined by precipitation with an equal volume of saturated ammonium sulphate and a binding curve constructed by plotting arithmetically the reciprocal of bound antigen versus the reciprocal of free antigen at each antigen concentration. Ab_t was determined by extrapolation of this plot and is the reciprocal of $1/b$ when $1/c = 0$. K_R was obtained from the equation:

$$\log \frac{b}{Ab_t - b} = a \log K + a \log c$$

where a = heterogeneity index

c = free antigen concentration (moles/litre)

by plotting logarithmically $\frac{b}{Ab_t - b}$ versus c . K_R is given by the reciprocal of c when

$$\frac{b}{Ab_t - b} = 0.$$

Avidity index

The relative avidity of mouse anti-HSA antibody was measured for pools of high and low K_R by the method of Celada, Schmidt and Strom (1969). The volumes of antiserum required to bind 50 per cent of the available antigen at a range of antigen concentrations were determined by construction of binding curves. When the value obtained at each antigen concentration was plotted logarithmically against the amount of antigen used for

each serum a straight line was obtained, the slope of which was taken as the index of avidity.

Dissociation rates

The relative avidity was estimated by measuring the rate of dissociation of antigen-antibody complexes (Grey, 1962). Radioiodinated HSA was incubated with sufficient antiserum to bind 30 per cent of it in sealed tubes at 37° for 2 hours and overnight at 4°. Duplicate aliquots were removed and the per cent antigen bound to globulin was estimated by ammonium sulphate globulin precipitation. Unlabelled HSA was then added to give a concentration 250 times that of the ¹²⁵I-HSA present. Aliquots were removed at intervals thereafter and the globulin-bound antigen immediately determined. The dissociation rate is expressed as the ratio P_x/P_E where P_x is globulin-bound antigen at time x after addition of unlabelled antigen and P_E is globulin-bound antigen at equilibrium. Values for the $t_{\frac{1}{2}}$ of the dissociation were determined graphically from the plot of $\log \frac{P_x}{P_E} \cdot 100$ versus time. The dissociation rate k_d is given by

$$k_d = \frac{\ln 2}{t_{\frac{1}{2}}} \text{ sec}^{-1} \text{ (Talmage, 1960).}$$

Association rates

A volume of each antiserum pool sufficient to bind approximately 30 per cent of the antigen (10 μg) at equilibrium was mixed with the antigen. At time intervals after mixing, duplicate aliquots were removed and equal volumes of saturated ammonium sulphate were added immediately to determine the proportion of the antigen bound to globulin. Values for the $t_{\frac{1}{2}}$ of association were determined graphically from a plot of

$$\frac{P_E - P_x}{P_E} \cdot 100 \text{ versus time.}$$

Where P_E = per cent antigen bound at equilibrium
and P_x = per cent antigen bound at time x after mixing.

The association rate fka , where f = valency of antigen (unknown) was calculated (according to the method of Talmage, 1960) using Ab_t values for the serum as a measure of the concentration of free antibody sites at time $t = 0$. The equilibrium constant fK was calculated from the ratio $\frac{fka}{k_d}$.

RESULTS

BINDING CURVES FOR LOW AND HIGH RELATIVE AFFINITY ANTIBODIES

The relationship between antibody-bound antigen and free antigen at a wide range of antigen concentrations was studied in pools of low and high K_R mouse anti-HSA and anti-DNP sera. Deviation from linearity at very low antigen concentrations is seen in both high and low K_R pools of mouse anti-HSA antisera (Fig. 1). The deviation is more marked in the low K_R pool. Deviation from linearity in extreme antigen excess occurs in the low K_R pool but not, at the antigen concentrations used, in the high K_R pool. Similar deviation

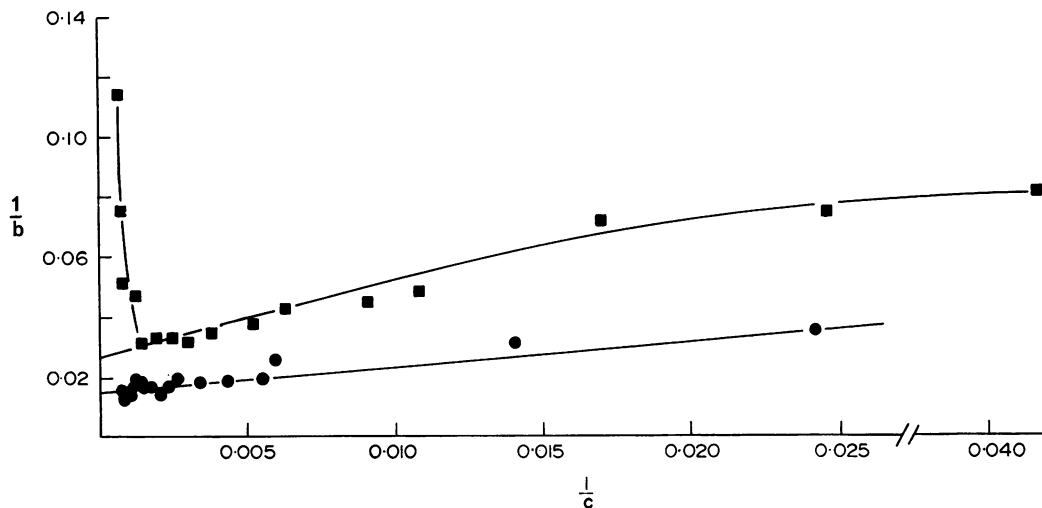


FIG. 1. Binding curves for mouse anti-HSA antibody. (■) Low K_R pool; (●) High K_R pool.

from linearity was observed in mouse low affinity anti-DNP antiserum and rabbit high affinity anti-DNP antiserum. (Difficulty was experienced in obtaining mouse anti-DNP antiserum of sufficiently high affinity; for this comparison, rabbit high affinity anti-DNP antiserum was used) (Fig. 2).

Thus the binding curves of low and high relative affinity antibody differ in three respects: 1. The slope of the linear part of the curve is greater for low than for high K_R antibody. 2. There is much greater deviation from linearity of low K_R antibody than of high K_R antibody at low antigen concentrations. 3. For low K_R antibody, there is opposite deviation from linearity in great antigen excess.

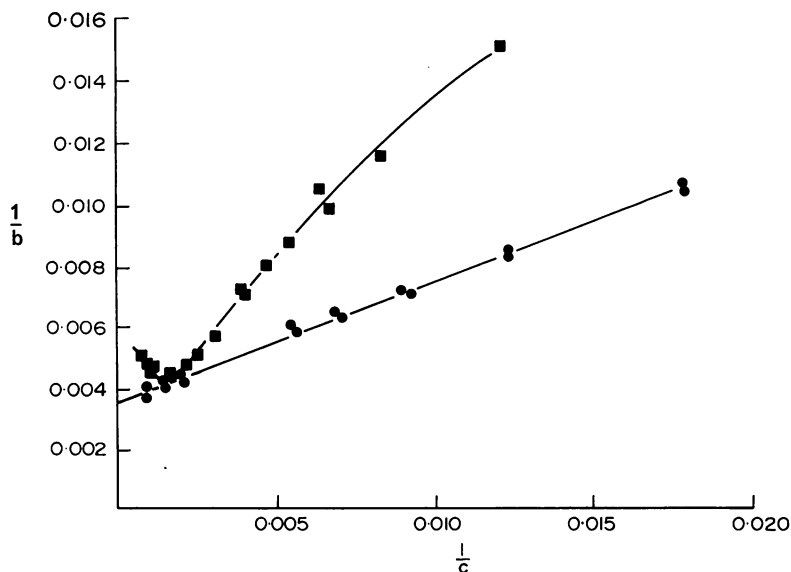


FIG. 2. Binding curves for anti-DNP antibody. (■) Low affinity pool; (●) High affinity pool.

Comparison of binding affinity obtained by three methods

The relative affinities and the indices of avidity of three pools of mouse anti-HSA anti-serum are shown in Table 1. The pools were ranked similarly by the two methods. The rates of dissociation of antigen-antibody complexes in antigen excess with antibody of high K_R (2.5×10^6 L/M) and of low K_R (1.4×10^5 L/M) were determined. The rates of association of the same antibody pools were also measured. The complexes made by low K_R anti-HSA were more readily dissociated in antigen excess than were those complexes made by high K_R anti-HSA. The rate of association of the low K_R pool was slower than that of the high K_R pool. These results, together with values for the equilibrium constant

TABLE 1
THE RELATIONSHIP BETWEEN RELATIVE AFFINITY
AND INDEX OF AVIDITY OF MOUSE ANTI-HSA
ANTIBODIES

Serum pool	Relative affinity (L/M)	Index of avidity
A	5.0×10^5	0.63
B	1.9×10^6	0.85
C	3.5×10^6	0.91

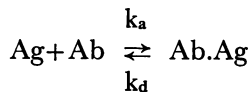
TABLE 2
COMPARISON OF RATES OF ASSOCIATION AND DISSOCIATION OF ANTIBODY POOLS OF
DIFFERENT RELATIVE AFFINITY

Pool	Relative affinity L/M	$t_{1/2}$ Dissocia- tion (sec)	kd (sec ⁻¹)	$t_{1/2}$ Associa- tion (sec)	fka L/mole/sec ⁻¹	fK L/mole
D	2.5×10^6	3000	2.3×10^{-4}	500	2.2×10^3	9.0×10^6
E	1.4×10^5	1200	5.85×10^{-4}	1150	1.9×10^2	3.3×10^5

fK (where f = the unknown antigenic valency) are shown in Table 2. Thus the three methods used for classifying the anti-HSA serum according to its binding characteristics—measurement of relative affinity, the measurement of the index of avidity and the measurement of dissociation and association rates, are in agreement.

DISCUSSION

In this paper we have presented evidence that deviation from linearity exists in the relationship between free and bound protein and hapten antigens in both antibody excess and antigen excess. The deviation from linearity in antibody excess is presumably due to greater binding of antigen by the relatively higher affinity antibody present in the system and, as expected, is more noticeable in the antibody preparation with the lowest average affinity. At high antigen concentrations, high and low K_R antibody bind antigen competitively. At low antigen concentrations high K_R antibody will bind antigen preferentially. Hence the average k_a of the pool of bound antibody molecules is increased, the average k_d is decreased and the ratio k_a/k_d increases. Thus in the reactions



proportionately more antigen is bound and $1/b$ is lower than expected. In the low K_R pool, deviation from linearity in antigen excess also occurs. No marked decrease in bound antigen was demonstrated using the same antigen concentrations with the high K_R antibody, but presumably at even higher concentrations, some deviation would occur. Such deviation may be due to increased dissociation, in antigen excess, of complexes formed by the low K_R antibody. Thus in the determination of affinity of antibody in unfractionated mouse serum to protein and hapten antigens utilizing a measure of Ab_t by extrapolation of the plot of $1/b$ versus $1/c$, such deviations from linearity must be taken into account.

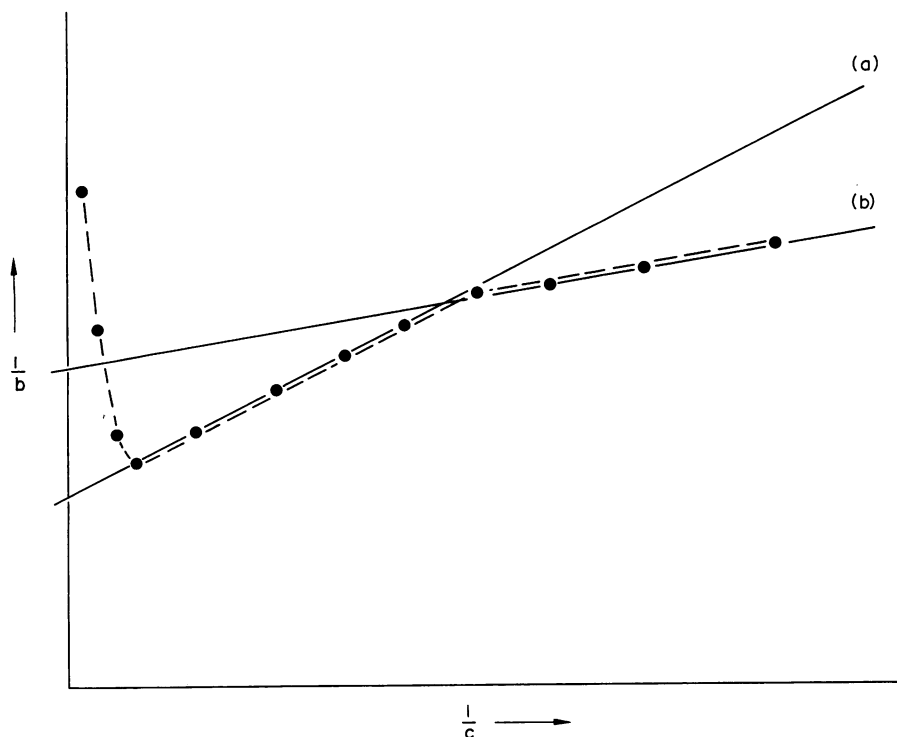


FIG. 3. Determination of Ab_t by extrapolation of the binding curve, (---) of a low affinity pool. (a) Valid extrapolation. (b) Invalid extrapolation.

A sufficient range of antigen concentrations must be employed to ensure that extrapolation is carried out from the linear portion of the curve, particularly when the affinity of the antibody is low. Fig. 3 illustrates the possible error in determination of Ab_t (intercept $1/b$ when $1/c = 0$) which may be made when insufficient points are used to construct the binding curve. Affinity measurements obtained using the technique described have been validated using methods for comparing antiprotein antibody affinity which are independent of the determination of Ab_t . The $t_{\frac{1}{2}}$ values of dissociation and association of the high and of the low affinity pools differ as expected (Table 2). Dissociation rate determinations are independent of Ab_t but association rates are not. However, using the

Ab_t values calculated for these sera and bearing in mind the unknown antigenic valency factor, *f*, the equilibrium constants obtained (Talmage, 1960) confirm the designation of high and low affinity of the two pools. Thus, the measurement of relative affinity by the ammonium sulphate globulin precipitation method provides a measure of the affinity of antibody to protein and haptens in unfractionated sera for comparative purposes. It has the advantage that it can be carried out in individual mouse serum samples since a maximum of 100 μ l of serum is required for each assay.

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