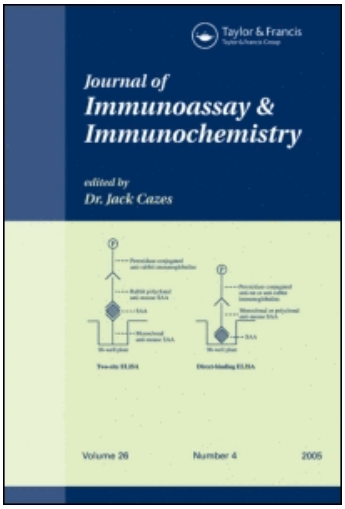

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Theoretical Analysis of the Reaction of Moltivalent Antigen with Heterogeneous Antibody: A Model of Soluble Phase Antibody Assays

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THEORETICAL ANALYSIS OF THE REACTION OF MULTIVALENT ANTIGEN WITH HETEROGENEOUS ANTIBODY: A MODEL OF SOLUBLE PHASE ANTIBODY ASSAYS

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ABSTRACT

The theoretical characteristics of soluble phase antibody assays were analyzed using a computer model of antigen-antibody interaction which allowed antibody to be heterogeneous and antigen multivalent. Studies show that when antibody is heterogeneous with respect to affinity, the high affinity antibody will usually mask the low affinity antibody. If the reaction of multivalent antigen with multispecific antibody is studied in antigen excess, the reaction closely approximates a one to one binding reaction. Several characteristics of the soluble phase antibody assay are determined by the affinity-antigen product or the product of the antibody affinity constant multiplied by the molar antigen concentration. When the product is high, the sensitivity of the assay will vary with antigen concentration and titers will be independent of antibody affinity. If the affinity-antigen product is low, sensitivity is dependent upon antibody affinity and titers will reflect antibody affinity as well as antibody concentration.

(KEY WORDS: ANTIBODY, ANTIGEN, ANTIBODY AFFINITY).

INTRODUCTION

Measurement of antibody directed against specific antigens is important in understanding the mechanisms of many infectious, neoplastic and autoimmune diseases. One of the most widely used methods for measuring antibody is the soluble phase primary binding assay (1). In this procedure antibody and radiolabeled antigen react in a soluble phase system. A series of tubes, each containing a fixed concentration of antigen and a variable dilution of antibody, is prepared. After incubating long enough to reach equilibrium, antigen bound to antibody is separated from free antigen by a variety of methods including electrophoresis, column chromatography or differential precipitation. The data, after correction for non-specifically bound antigen, give an antibody dilution curve which defines the fraction antigen bound to antibody as a function of antibody dilution. A given antibody is characterized in terms of the highest dilution, or "titer", which can bind the antigen under conditions of the assay.

A theoretical model of soluble phase antibody assays has recently been developed (2). Studies with

this model, which is based upon the assumptions of simple univalent antigen and homogeneous antibody, have defined some important features of the assay. The present study was performed to extend theoretical understanding of the soluble phase antibody assay to cases where antigen is multivalent and antibody heterogeneous. Since antigens and antibodies of these types occur in many immunologic systems, the results have relevance to many important biologic phenomena.

THEORY

Each antigen molecule possesses n distinct, non-repeating epitopes designated e_1 , e_2 ...e_n . Since the epitopes are unique, the concentration of each epitope is equal to the total antigen concentration denoted TAG.

The antibody which reacts with the antigen molecule can be subdivided into n subpopulations denoted b_1 , b_2 ...b_n . The subpopulation b_i has specificity only for the correspondingly numbered epitope e_i . The total concentration of antibody binding sites, TAB, is given by the expression

$$TAB = \Sigma\ TAB_i \qquad (1)$$

where TAB_i is the total concentration of binding sites of the i^{th} antibody subpopulation. Each antibody site b_i reacts with an epitope e_i according to the reaction:

$$e_i + b_i \rightleftharpoons e_i b_i \qquad (2)$$

Assuming the reactions are independent and that cyclic compounds do not form, the fraction of any epitope which is bound to antibody, denoted F_i can be calculated as the negative root of the equation: (See Appendix I).

$$K_i (TAG)F_i^2 - (1+K_i(TAG)+K_i(TAB_i))F_i + K_i(TAB_i) = 0 \quad (3)$$

where F_i is the fraction of the i^{th} epitope which is bound to antibody and K_i is the equilibrium binding constant of the i^{th} reaction.

Soluble phase antibody assays measure antigen-antibody interaction by separating free antigen from bound antigen. These assays are unable to determine exactly how many antibody molecules are attached to an antigen molelcule. Free antigen is accurately

measured, but other species, such as $AgAb_4$, $AgAb_2$ and AgAb, are all grouped together as "bound antigen". These antibody assays can be viewed as assays for free antigen. This idea can be expressed symbolically as follows:

$$P\{\text{antigen free}\} = P\{\text{all epitopes free}\} \quad (4)$$

where P { } denotes the probability of the stated event occurring. Since the binding of the antibody to each epitope is an independent event, it follows that

$$P\{\text{all epitopes free}\} = \Pi\, P\{e_i \text{ free}\} \quad (5)$$

The probability that an individual epitope is free can be expressed by the relationship

$$P\{e_i \text{ free}\} = 1 - P\{e_i \text{ bound}\} \quad (6)$$

From 3 we can say that

$$P\{e_i \text{ bound}\} = F_i \quad (7)$$

substituting we have

$$P\{\text{antigen free}\} = \Pi\, (1-F_i) \quad (8)$$

The fraction of antigen molecules with one or more antibodies attached, denoted F, is given by the relationship:

$$F = 1 - P \{\text{antigen free}\} \qquad (9)$$

PARAMETER SELECTION: Soluble phase antibody assays measure the fraction of a fixed amount of antigen which is bound to antibody. A small, constant antigen concentration is generally used in the assay. Antibody concentration is varied by diluting the antibody. To simulate these conditions TAG was held constant while TAB was progressively decreased. In most simulations the antigen concentration was chosen to be 10^{-9}M because this particular concentration is often used in antibody assays. The number of epitopes on the antigen was varied from one to seven. The binding constants were varied between 10^6 and $10^{10}M^{-1}$. In simulations of the reaction of multivalent antigen with homogeneous antibody all of the antibody subpopulations were assumed to have the same binding constant. The starting antibody binding site concentration was 10^{-5}M. To simulate commonly used dilution experiments TAB was progressively decreased. For simulations of the reaction between multivalent

antigen and antibody of homogeneous affinity, the concentration of the individual antibody subpopulations was calculated by the relationship

$$TAB_i = TAB/n$$

The fraction antigen bound, F, was calculated at a series of decreasing total antibody concentrations.

The behavior of antibody heterogeneous with respect to affinity was studied using two antibody subpopulations. The primary population reacted with epitope e_1 and had a binding constant of $10^{10}M^{-1}$. The secondary subpopulation reacted with epitope e_2; the binding constant was varied between 10^6 and $10^{10}M^{-1}$. Antibody dilution curves for a combination of the two antibodies were simulated using a fixed antigen concentration. The starting concentration of each antibody subpopulation was $10^{-5}M$. The concentration of both antibodies was decreased proportionately through a series of dilutions. F was calculated at each antibody concentration as described above.

Another simulation of heterogeneous antibody-antigen interaction was performed to determine what concentration of secondary antibody would be required to increase the observed F value from 0.10 to 0.19.

This was done by determining the concentration of primary antibody, acting alone, which was required to bind ten percent of the antigen. The concentration of secondary antibody which would have to be added to the primary population in order to increase the overall F value to 0.19 was then determined.

Derivation of the sensitivity of the assay for homogeneous and heterogeneous antibody is shown in Appendix II.

RESULTS

MULTIVALENT ANTIGEN AND HOMOGENEOUS ANTIBODY: The binding curves for the reaction of homogeneous antibody with univalent and trivalent antigens are compared in Figure 1. The total amount of antibody is the same in each case. For trivalent antigen one third of the antibody is assumed to react with each epitope. Results for three different binding constants are presented. The curves vary with the affinity-antigen product (the product of antibody affinity times the antigen concentration) of the system. When the system is balanced, with an affinity-antigen product near unity, the two curves are very similar (Figure 1a). If the system has a high or low affinity-antigen product, the curves are

similar at F values below 0.5 but vary somewhat at higher values (Figures 1b and 1c).

The effect of varying valence on the fraction antigen bound (F) is shown in Figure 2. The change in fraction antigen bound produced by increasing valence reaches a plateau value above N equals two.

MOLTIVALENT ANTIGEN AND HETEROGENEOUS ANTIBODY: The effect of introducing antibody heterogeneity on the antibody dilution curve was studied by adding a secondary low affinity subpopulation to a primary high affinity subpopulation with a binding constant of $10^{10} M^{-1}$. The antibody dilution curves for each antibody alone and for the mixture of the two antibodies is shown in Figure 3. The results again vary with the affinity-antigen product. When the product for both antibodies is high (Figure 3a) both populations increase the fraction antigen bound. When the product for the secondary antibody is low (Figure 3b), the addition of the low affinity antibody has very little effect on the antibody dilution curve.

The increase in fraction antigen bound produced by the addition of a secondary antibody population of variable affinity is shown in Figure 4. Both antibody populations had the same concentration. Antigen

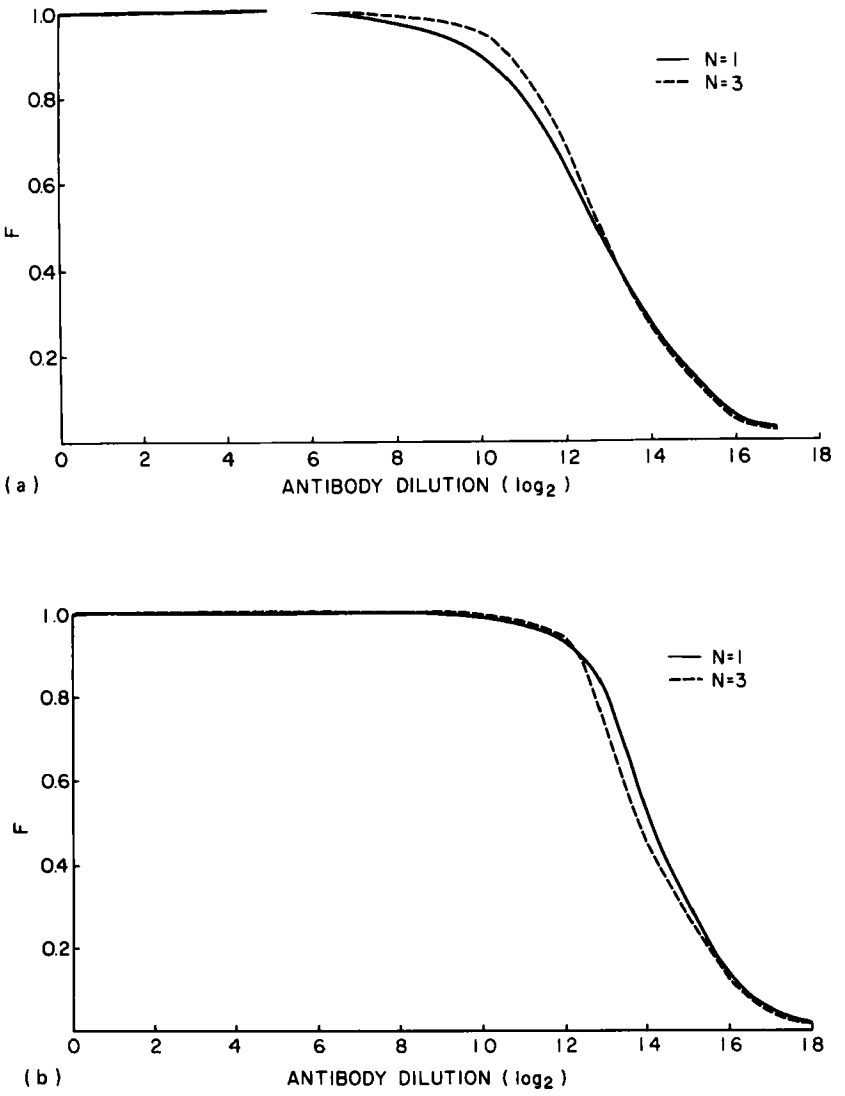

Figure 1. Antigenic Multivalence: Comparison of antibody dilution curves for univalent and trivalent antigen. F is the fraction antigen bound. N is the antigenic valence. Antigen concentration is the same for each curve, but in the trivalent case one third of the antibody reacts with each epitope. Binding constants are as follows: Figure 1a $K=10^{9}M^{-1}$, Figure 1b=$10^{10}M^{-1}$, Figure 1c $K=10^{7}M^{-1}$.

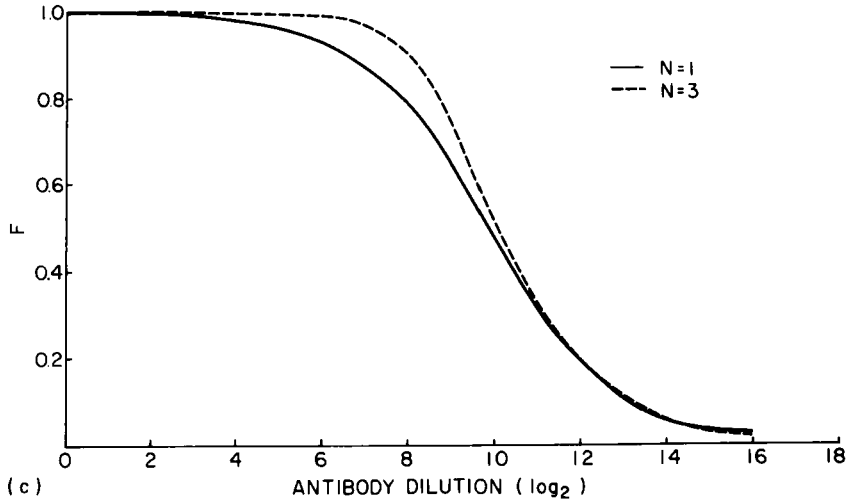

Figure 1c (continued)

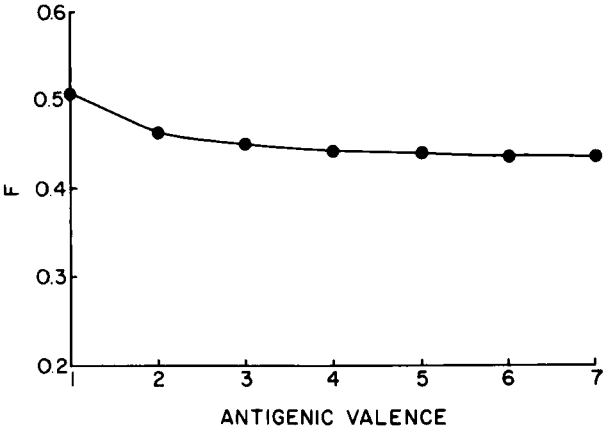

Figure 2. Antigenic Multivalence: The effect of increasing antigen valence on the fraction antigen bound. Antigen concentration is 10^{-9} M. Binding constant equals 10^{10} M^{-1}. Antibody concentration is 0.6×10^{-9} M.

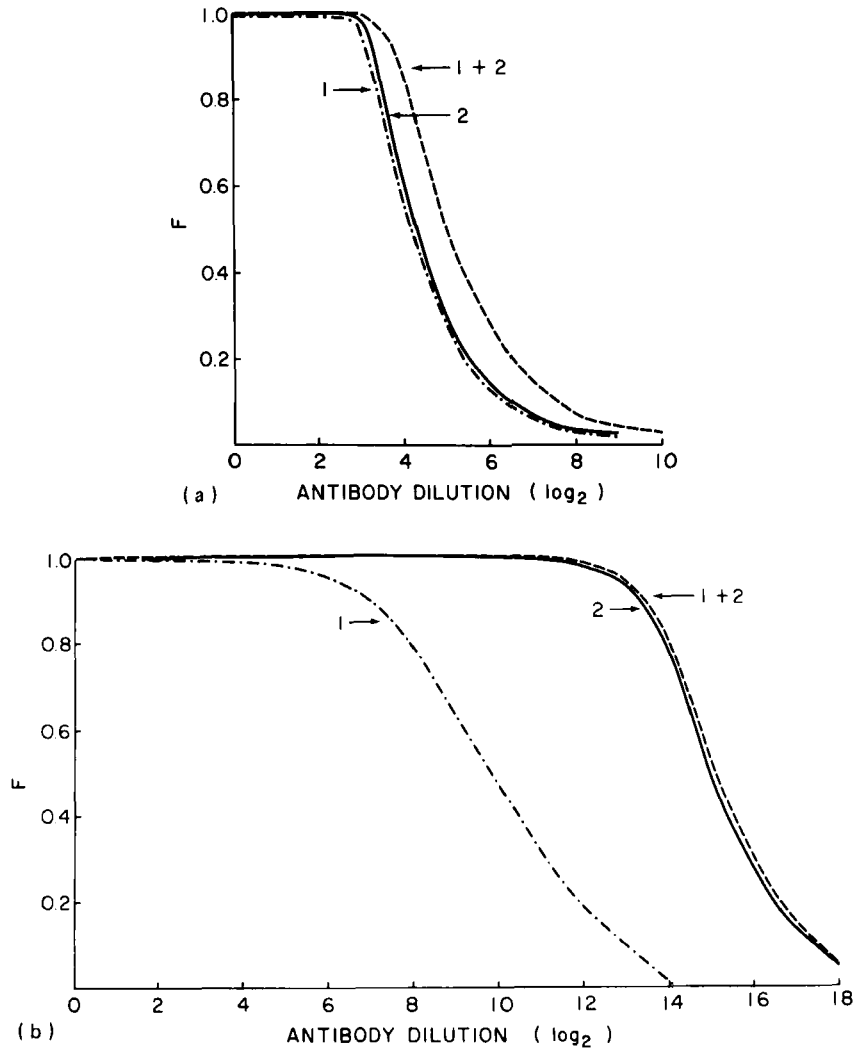

Figure 3. Antibody Heterogeneity: The effect of antibody heterogeneity on the antibody dilution curve. The curve labelled "1" shows the primary antibody population alone ($K=10^{10}M^{-1}$). Curve "2" shows the secondary antibody subpopulation alone ($K=10^{6}M^{-1}$). The antibody dilution curve for the combination of the two antibody subpopulations is labelled"1+2". In Figure 3a the antigen concentration is $10^{-6}M$. In figure 3b the antigen concentration is $10^{-9}M$.

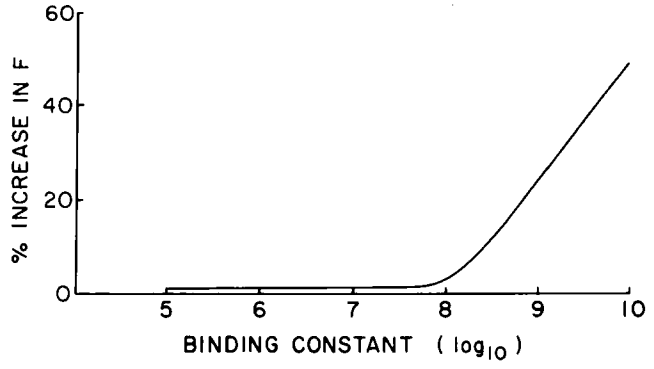

Figure 4. Antibody Heterogeneity: The effect of adding a secondary low affinity antibody subpopulation, of equal concentration but variable affinity, on the fraction antigen bound (F). The affinity of the primary antibody subpopulation is $10^{10}M^{-1}$.

concentration was $10^{-9}M$. The affinity of the primary population was $10^{10}M^{-1}$. The secondary population produces insignificant changes in F unless the affinity of the secondary population approaches the affinity of the primary population.

Figure 5 shows the concentration of secondary low affinity antibody required to increase the overall F value seen with a high affinity primary population ($K=10^{10}M^{-1}$) from 0.1 to 0.19. When the antigen concentration is low, secondary populations must be present in extremely high concentrations compared to the primary population in order to have a significant effect on the antibody dilution curve.

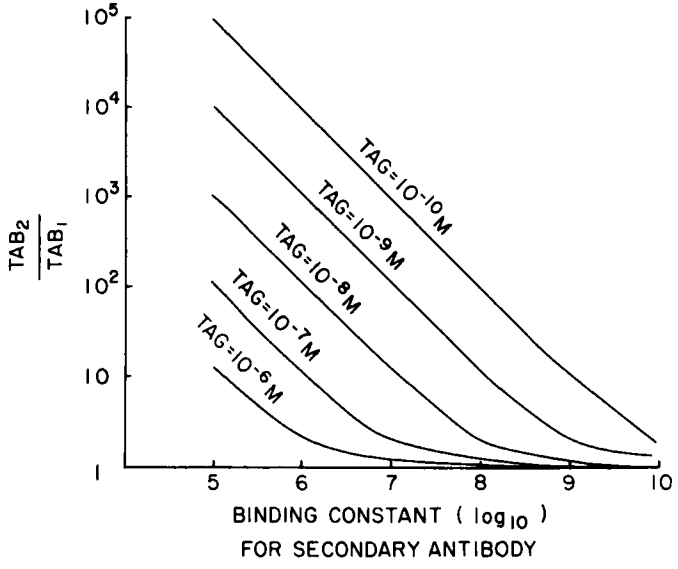

Figure 5. Antibody Heterogeneity: The amount of secondary antibody required to increase the fraction bound (F) from 0.1, for the primary antibody subpopulation alone, to 0.19 for the combination of the two antibody subpopulations, is shown at different antigen concentrations. The affinity of the primary antibody subpopulation is $10^{10}M^{-1}$. Antigen concentration is shown on the curves.

Low affinity secondary subpopulations are easier to detect when high antigen concentrations such as $10^{-6}M$ are used in the assay. In general, any secondary antibody subpopulation with an affinity-antigen product of one or less will be difficult to define unless it is present in very high concentrations compared to the primary high affinity subpopulation.

DISCUSSION

Multivalence of antigen can produce extremely complicated reactions with antibody. For example, the reaction of multivalent antigen with polyclonal antibody may produce very large complexes which precipitate spontaneously. Under conditions of antigen excess, however, the reaction of multivalent antigen may be simplified. In antigen excess, multivalent antigen will behave like univalent antigen. This finding is similar to mathematical analysis of Otterness and Karush (5). They also concluded that multivalent antigen would behave like a univalent ligand in antigen excess.

Many important characteristics of a soluble phase antibody assay can be predicted by the affinity-antigen product. This term is the product of the antigen concentration multiplied by the affinity constant of the antibody species. When the affinity antigen product is near one, multivalent antigen will behave like univalent antigen over a wide range of antigen excess. The sensitivity of the assay will be given as shown in Table I. When the affinity-antigen product is much less the one, the assay will have the characteristics listed in Table II. In this case titer and sensitivity vary with affinity of

TABLE I

CHARACTERISTICS OF A SOLUBLE PHASE ANTIBODY ASSAY WITH AN AFFINITY-ANTIGEN PRODUCT NEAR UNITY

1. Multivalent antigen behaves like univalent antigen over most of the curve.

2. The sensitivity of the assay is given by the expression

$$S = \frac{\alpha}{(1 - \alpha)K} + \alpha(TAG)$$

3. Antibody titers are dependent upon antibody affinity as well as antibody concentration.

the antibody. When the affinity-antigen product is much greater than one, titers will be independent of affinity and will be related to antibody concentration. The sensitivity of the assay will be determined by antigen concentration and the minimum measurable value of fraction antigen bound (Table III).

The molar concentration of antigens used in published soluble phase antibody assays is shown in Table IV. These concentrations were calculated from data presented in materials and methods section of the papers. In nearly all routine antibody assays

TABLE II

CHARACTERISTICS OF STANDARD SOLUBLE PHASE ANTIBODY ASSAYS WITH A LOW AFFINITY-ANTIGEN PRODUCT

1. Multivalent antigen behaves like univalent antigen in antigen excess when F is below 0.5.

2. The sensitivity of the assay for antibody, (S), is given by:

$$S = \frac{\alpha}{(1 - \alpha)K}$$

where α is minimum fraction antigen bound which which can be measured and K is the binding constant.

3. Antibody titers are dependent on antibody affinity as will as antibody concentration.

the concentration of antigen is low, being in the range of 10^{-9}M. The principle exception to this rule is the studies of Mulligan, Osler and Rodriguez (3) who developed a modified Antigen Binding Capacity test (mABC) for anti-BSA. In the mABC assay high antigen concentrations, in the range of 10^{-5}M, were used to saturate all of the antibody binding sites. Since antibody affinity constants vary between 10^{6}

TABLE III

CHARACTERISTICS OF A SOLUBLE PHASE ANTIBODY ASSAY WITH A HIGH AFFINITY-ANTIGEN PRODUCT

1. Multivalent antigen behaves like univalent antigen in antigen excess when F is below 0.5.

2. The sensitivity of the assay is given by the expression

$$S = \alpha\ (TAG)$$

where α is the minimum fraction antigen bound which can be measured and TAG is total antigen concentration.

3. Antibody titers are independent of affinity and reflect antibody concentration.

and $10^{10}M^{-1}$ for most systems, the affinity-antigen product for standard soluble phase antibody assays with an antigen concentration of $10^{-9}M$ will usually be low. Standard assays will have the characteristics listed in Table II. The occasional assay done at high antigen concentrations, such as the mABC, will have features in Table III. The value

TABLE IV
MOLAR ANTIGEN CONCENTRATIONS USED IN SOLUBLE PHASE ANTIBODY ASSAYS

ANTIGEN	ANTIGEN CONCENTRATION	REFERENCE
Hemogloblin	2×10^{-9}M	Garver & Talmage (7)
DNA	2×10^{-9}M	Aarden, Lakmaker &Feltkamp (8)
Acethylcholine Receptor	2×10^{-10}M	Lindstrom et al (9)
Glomerular Basement Membrane	3×10^{-10}M	Mahieu, Lambert & Miescher (10)
Bovine Serum Albumin	10^{-8} to 10^{-9}M	Farr (1)
	10^{-5}M	Mulligan et al (3)

of α , the lowest measurable fraction antigen bound, will range from 0.05 to 0.1 depending upon the magnitude and variability of the non-specific binding in the assay.

Antibody which contains a mixture of heterogeneous species with different binding constants appears after immunizing a host with antigen. These antibodies, generally termed polyclonal in nature, frequently have unimodal or bimodal affinity distributions, with one or two major subpopulations of different affinities (4). If

antibody is heterogeneous with respect to affinity, but the affinity-antigen product for all species is high, then all species will be measured in the assay. If, however, some species have high products and others low, the higher affinity antibody will usually mask the low affinity populations. The principle exception to this rule will occur when the concentration of low affinity antibody is much higher than the concentration of high affinity antibody.

The model used in this study allows antigen to be multivalent and antibody to be heterogeneous. The epitopes were generally assumed to be distinct and capable of reacting independently with antibody. Gandolfi & Strom (6) have used a similar model of antigen-antibody interaction. One important difference is that the model of Gandolfi & Strom assumes that the epitopes are identical. The assumption of epitope similarity is valid when synthetic conjugates of hapten carrier molecule are studied. It is not valid for most natural protein antigens which have distinct epitopes. Otterness & Karush (5) have used a model similar to the one used in this paper to analyze antigen-antibody interaction. In the present study, antibody heterogenity was analyzed assuming that the

antibodies with different specificities have different affinities. It would be possible to have antibodies of different affinity reacting with the same epitope. In this type of reaction the low affinity antibody subpopulations would also be masked. The masking effect would even be greater because the low affinity antibody would have to compete with the high affinity populations for the same epitope.

The model used in this study, and the models mentioned above, assume that epitopes bind to antibody independently. Although independence of binding is a reasonable assumption for many systems, there may be cases where the reactions are not independent. The binding of one antibody to one epitope may sterically hinder the binding of a second antibody to an adjacent epitope on the same antigen. If the antigen molecule is very large, the second paratope on a bound antibody molecule may be unable to bind another antigen because of steric hindrance. Complete steric hindrance will reduce the apparent reactant concentrations but shouldn't change the binding affinity of the reaction. Partial steric hindrance might lower the binding energy. If the epitopes of the antigen are similar and

strategically placed, then both arms of the antibody might be able to bind to the same antigen molecule. In this type of chelation binding, the effective antibody site concentration will appear reduced and the binding constant will be higher by about 500 times. If epitopes happen to be strategically placed, cyclic compounds may form. In this case binding between antigen and antibody should be in the ratio of approximately one antigen per antibody molecule. The binding constant will also be enhanced about 500 times over the simple binding reaction when only one of the antibody paratopes binds to the antigen molecule. Thus a simplified, one to one binding reaction model with modified parameters could also be used for more complex reactions provided that antigen excess conditions are maintained.

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APPENDIX I

Derivation of Equations for Soluble Phase Antibody Assay

The formulas governing the reaction of univalent antigen with antibody are as follows:

$$K = \frac{(AGAB)}{(AG)(AB)}$$

$$(TAG) = (AGAB) + (AG)$$

$$(TAB) = (AGAB) + (AB)$$

$$(AGAB) = F(TAG) \qquad 0 < F < 1$$

where K = binding constant, (AGAB) is concentration of complex, (AG) is the concentration of free antigen, (AB) is the concentration of free antibody, (TAG) is total antigen concentration, (TAB) is total antibody site concentration and F is fraction antigen bound. From these equations it is possible to derive the following relationship (11).

$$K = \frac{F}{(1-F)(TAB - F(TAG))}$$

With simple algebraic rearrangement it is possible to derive the following second order polynomial which can be solved for F.

$$K(TAG)F^2 - (1 + K(TAG) + K(TAB))\ F + K(TAB) = 0$$

If K_i is substituted for K, F_i for F and TAB_i for TAB, the relation (3), which governs the reaction of each antibody subpopulation with its corresponding epitope, is apparent.

APPENDIX II

Measurements of the minimum amount of antibody which can be detected by an antibody assay will be done at a very low fraction antigen bound. In most cases, 33% or less of the antigen will be bound to antibody. Under these conditions the behavior of multivalent antigen can be approximated using a model of univalent antigen.

The equation in Appendix I for calculating the binding constant can also be rearranged to give an expression for TAB.

$$TAB = \frac{F}{(1 - F)K} + F(TAG)$$

If S denotes the minimum detectable antibody concentration and α denotes minimum fraction antigen bound which can be distinguished from non-specific binding then it is apparent that

$$S = \frac{\alpha}{(1 - \alpha)K} + \alpha (TAG)$$

If the affinity-antigen product (TAG*K) is high (i.e., or greater than 10) then the equation reduces to

$$S = \alpha (TAG)$$

If the affinity-antigen product is low (i.e., less than 1/10) then the equation reduces to

$$S = \frac{\alpha}{(1 - \alpha)K}$$

A reasonable value for α is in range of 0.05 to 0.1. If α is 0.1 then S will be 0.1 TAG for systems with a high affinity-antigen product and 0.11/K for systems with a low product.

Sensitivity for a second antibody subpopulation of lower affinity also depends upon the affinity-antigen product. If the product is high for both species then the sensitivity for the second antibody is the same as for the first. If the products are low and α is the same for both antibodies then the minimum concentration required for the second antibody, S_2 is given by

$$S_2 = \frac{K_1 S_1}{K_2}$$

where K_1 binding constant for first antibody, K_2 is binding constant for second antibody and S_1 is minimum detectable concentration for the first antibody.