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Homotropic Cooperative Binding of the First Component of Guinea Pig Complement to Rabbit IgG–Erythrocyte Complexes: A Possible Allosteric Effect*

(binding sites/2,4-dinitrophenol/sensitized erythrocytes/binding domain)

JAMES J. THOMPSON AND LOUIS G. HOFFMANN

Department of Microbiology, College of Medicine, University of Iowa, Iowa City, Iowa 52240

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ABSTRACT **Binding of the activated first component of guinea pig complement to immune complexes formed between dinitrophenylated erythrocytes and rabbit IgG antibody to 2,4-dinitrophenylhapten has been studied quantitatively. Cooperative binding was observed; it involves no interactions between the domains on the erythrocyte surface that bind the activated first component of complement, or between the activated complement molecules in solution. By curve-fitting methods, we find that the data are consistent with an allosteric model, which assumes 10 binding sites per domain, a low allosteric equilibrium constant, and virtually exclusive binding to one of the isomers.**

In spite of speculations about a possible role of conformational transitions in immunologic phenomena (1, 2), attempts to establish that such transitions occur have so far met with little success (3–5). These negative results were obtained in studies that sought to ascertain whether a conformational change occurs in antibody molecules upon combination with antigen or hapten. We now report experimental results that are compatible with a conformational transition associated with the binding of the activated first component of complement to antigen–antibody complexes. This binding shows homotropic cooperative interactions between molecules of activated complement 1 that may be interpreted by an allosteric mechanism, such as that proposed by Monod *et al.* (6) for regulatory enzymes. Two alternative interpretations of our results have been excluded.

MATERIALS AND METHODS

Reagents. The preparation of veronal buffers of various ionic strengths (μ), sensitized erythrocytes that carry at least one molecule of complement 4, the second component of complement, guinea pig serum in EDTA buffer, and Type B guinea pig complement (activated first component), have been described (7).

Rabbit IgG Antibody to 2,4-Dinitrophenyl (Dnp) Hapten. In initial experiments, we used an ammonium sulfate fraction of the antiserum to Dnp–bovine gammaglobulin (Dnp–BGG), prepared in the rabbit as described by Campbell *et al.* (8). This preparation contained a β-globulin contaminant, which was detected by immunoelectrophoresis, but contained no high molecular weight aggregates or contaminants when examined by Schlieren optics in a model E ultracentrifuge, at concentrations as high as 15.6 mg/ml [assumed extinction coefficient at 280 nm = 1.36 ml/mg; Small and Lamm (9)]. In later experiments, we used an immunospecifically purified anti-Dnp–BGG fraction prepared from rabbit serum by precipitation at equivalence with trinitrophenylated bovine-serum albumin (Tnp-BSA), dissolution of the precipitate in 0.1 M 2,4-dinitrophenol (pH 7.5), and removal of hapten and Tnp–BSA on a three-layer Sephadex G-25–DEAE-Sephadex–Dowex 1 column, to which 0.1 M paranitrophenol had been added immediately before the addition of the antibody–hapten mixture (10). No discernible contamination was detected by immunoelectrophoresis, ultracentrifugation, or polyacrylamide disc electrophoresis in the antibody preparation.

Sensitized Erythrocytes (Anti–Dnp–Dnp-Erythrocytes). Dinitrophenylated sheep erythrocytes (Dnp-erythrocytes) were prepared by the addition of 0.2 ml of fluorodinitrobenzene, diluted in acetone (1:165) to 20 ml of 5% erythrocyte suspension, v/v, in borate–KCl buffer (pH 9.5) (11). Anti-Dnp antibody concentration varied as indicated in the individual experiments. Dnp-erythrocytes and antibody were mixed in veronal buffer with 10 mM EDTA to prevent contamination of the complexes with rabbit complement 1. Erythrocytes were washed in veronal buffer with divalent cations, resuspended, and standardized to the desired final cell concentrations.

Binding Assay. Fig. 1 shows the conditions used for assay of bound and free activated complement 1. The initial reaction between sensitized erythrocytes and the activated complement 1 occurred in μm = 0.094 buffer, above the apparent solubility limit of the complement alone (12). This had two effects: (*a*) Carry-over of the activated complement 1 by unsensitized erythrocytes in the assay was less than that at lower ionic strength, and (*b*) anomalous binding curves, apparently due to precipitation at high inputs of complement 1, were avoided.

After centrifugation, both the cells and supernatant fluids were diluted in μ = 0.094 buffer, without washing. We

Abbreviations: BGG, bovine gammaglobulin; BSA, bovine-serum albumin.

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find that washing removes specifically-bound activated complement 1 (13). 0.5 ml of each dilution was then added to separate tubes, which contained sensitized erythrocytes that carry at least one molecule of C4, to give a final ionic strength of 0.136 for transfer. After incubation at 30°C to complete the transfer of activated complement 1 to the sensitized erythrocytes with complement 4 (ref. 7), the ionic strength was lowered to 0.073 by the addition of complement 2 in μ = 0.009 buffer for optimal sensitivity in the steady-state portion of the assay (13). Incubation in guinea pig serum in EDTA buffer (as a source of terminal complement components) completed the reaction. Percentage lysis was converted to site-forming units per cell by the one-hit theory of immune hemolysis, with appropriate control corrections (14).

The number of site-forming units of activated complement 1 bound specifically to anti-Dnp–Dnp-erythrocytes was calculated by subtraction from the total number of site-forming units bound to the sensitized erythrocytes, the number of site-forming units bound to unsensitized Dnp-erythrocytes at the same activated complement 1 input; this nonspecific "carryover" amounted to an average of 5.5% of the activated complement 1 input, and was independent of it. Recovery of the activated complement was virtually complete (average = 94.8%, SD = ±7.5%), independently of the ratio of bound to free activated complement 1.

RESULTS AND DISCUSSION

The binding data are plotted directly, as site-forming units bound per cell (R) versus free activated complement 1 concentration (C), in Fig. 2*A*, and according to Scatchard (15) in Fig. 2*B*. The direct plot is sigmoid rather than hyperbolic, as would be expected for simple binding, and the Scatchard plot passes through a maximum. These features are characteristic of homotropic cooperative binding (16). They can be explained only by postulating that the surface of each cell contains a number, Z, of binding domains, each capable of binding several molecules of activated complement 1; let this number be n. The homotropic cooperative effect can be described by stating that successive molecules of activated complement 1 that interact with the same binding domain are bound more firmly than the first molecule adsorbed to that domain. Similar cooperative effects have been described in other systems, e.g., the well-known oxygen dissociation curve of hemoglobin or the binding of ligands to regulatory enzymes (6). In our system, the postulated binding domain corresponds to a single molecule of hemoglobin or enzyme, and the activated complement 1 corresponds to the ligand. A binding domain may be thought of as consisting of an area on the cell surface with several attached IgG molecules.

Monod *et al.* have proposed an allosteric model (6) to account for homotropic cooperative interactions in other systems, according to which the fraction of binding sites occupied by ligand ($\bar{Y}$) is given by the equation

$$\bar{Y} = [Lc\alpha(1 + c\alpha)^{n-1} + \alpha(1 + \alpha)^{n-1}]/[L(1 + c\alpha)^n + (1 + \alpha)^n]$$

where n is as defined above; L is the equilibrium constant for the interconversion of the two isomeric states, R and T, of each binding domain; $c = K_R/K_T$; K_R = the intrinsic dissociation constant of activated complement 1 bound to a binding site in a domain in the R state; K_T = the analog

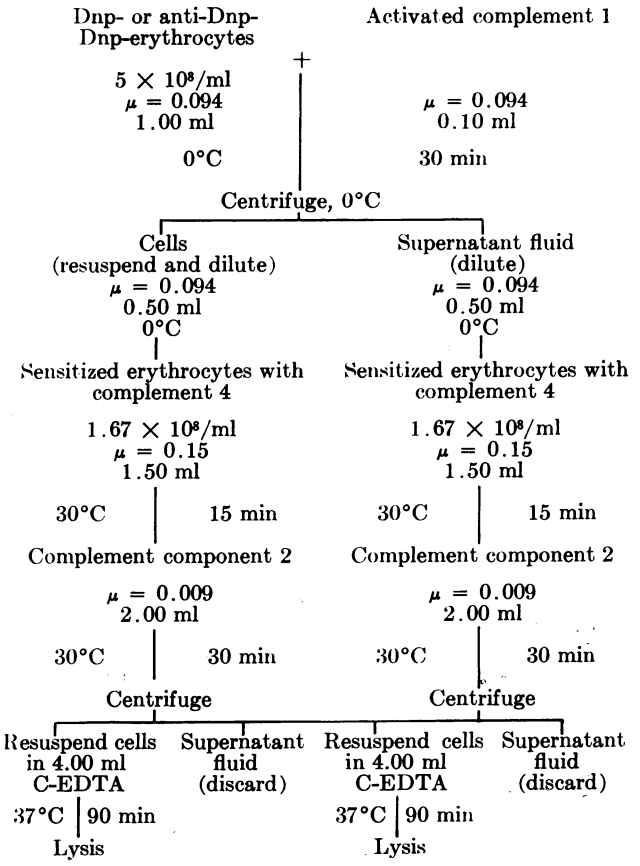

FIG. 1. Schematic representation of a binding experiment. In some of the experiments, the concentration of Dnp-erythrocytes or anti-Dnp-Dnp erythrocytes was reduced to 5 × 10^7/ml. C-EDTA, guinea pig serum in EDTA-buffer as a source of terminal complement components.

of K_R for the T state; K_T is defined as greater than K_R; $\alpha = C/K_R$, and C is as defined above.

To evaluate the applicability of this model to our data, we have constructed a *normalized Scatchard plot* of the data from three separate experiments (Fig. 3) on the basis of the following considerations. The number of binding sites per cell is obtained by multiplying the number of binding domains per cell by the number of binding sites per domain, i.e., nZ. The fraction of occupied binding sites, $\bar{Y}$, can then be calculated by dividing the number of site-forming units of activated complement 1 bound per cell, R, by nZ:

$$\bar{Y} = R/nZ,$$

where nZ may be estimated from the intercept of the Scatchard plot (Fig. 2*B*) with its abscissa. As shown by Changeux *et al.* (16), the ordinate intercept of the straight line which the Scatchard plot approaches asymptotically is nZ/K_R; dividing R/C by this quantity yields, in view of the above definitions, $\bar{Y}/\alpha$:

$$\bar{Y}/\alpha = (R/C)/(nZ/K_R).$$

A normalized Scatchard plot shows $\bar{Y}/\alpha$ as a function of $\bar{Y}$. It permits the pooling of data from several experiments in which nZ and K_R may differ, the former because of variations in the amount of antibody per cell, and both because of day to day variations in assay sensitivity (17), since all activated

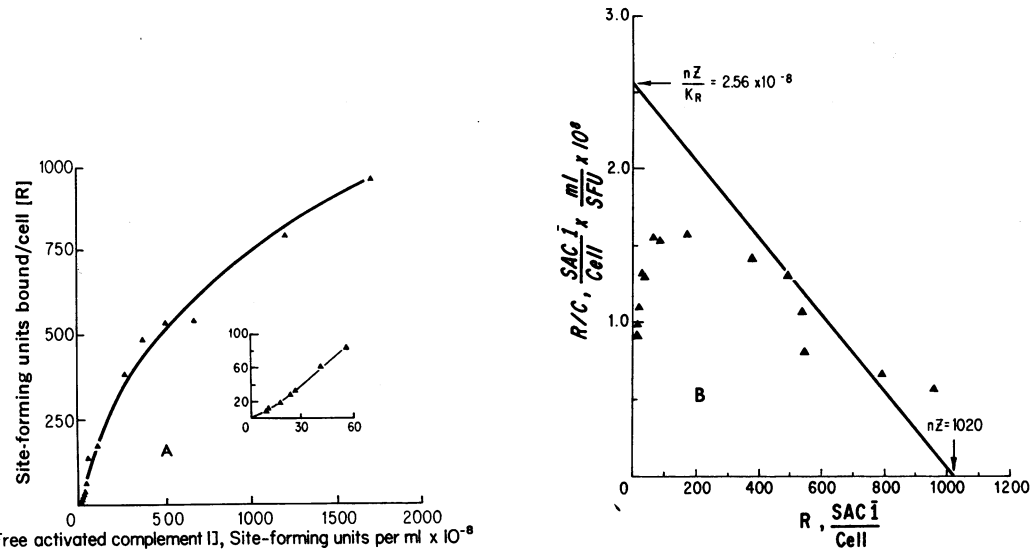

FIG. 2 (*A*) Binding curve for anti-Dnp-Dnp erythrocytes prepared with pure IgG antibody. The experimental protocol is shown in Fig. 1; the concentration of the sensitized erythrocytes was 5×10^7/ml. R = number of site-forming sites of activated complement 1 bound per cell; $[C\bar{1}]_f$ = free activated complement 1 concentration. Insert axes are the same units as those on the larger-scale graph. (*B*) Scatchard plot of the data in Fig. 2*A*. R is defined as in Fig. 2*A*; C = [activated complement $\bar{1}$]$_f$; Z = number of activated complement 1-binding domains per cell; n = number of activated complement 1-binding sites per domain; K_R = dissociation constant of the more-firmly binding isomer of the binding domain. $1/K_R = 2.51 \times 10^{-11}$ ml/site-forming unit.

complement 1 concentrations are expressed in terms of activity units rather than weight. The other parameters in the equation, namely, n, c, and L, are not affected by such variations, and one might expect a single set of values for these parameters to fit the data from all of the experiments, if the model is correct.

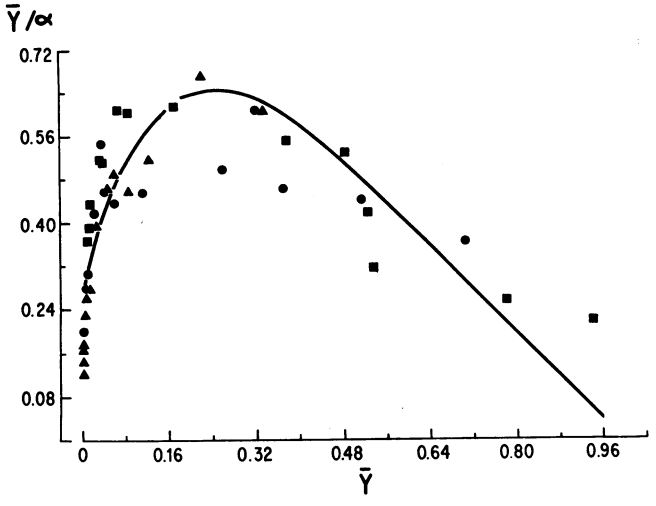

FIG. 3. Normalized Scatchard plot of data from three separate binding experiments, with theoretical line calculated from the allosteric equation for the values of n, L, and c indicated. $\bar{Y} = R/nZ$; $\alpha = C/K_R$; A/E = relative amount of antibody per cell—low A/E is $^1/_5$ that of high A/E. The experiments labeled "AS PPT" were performed with $(NH_4)_2SO_4$-precipitated IgG antibody and a cell concentration of 5×10^6/ml; the data for pure IgG are the same as those shown in Fig. 2. *Squares* represent pure IgG, $nZ = 1020$, $1/K_R = 2.51 \times 10^{-11}$; *triangles* represent AS PPT, high A/E, $nZ = 1430$, $1/K_R = 2.05 \times 10^{-11}$; *circles* represent AS PPT, low A/E, $nZ = 53.6$, $1/K_R = 1.83 \times 10^{-11}$. $n = 10$, $L = 3$, $c = 0.0$.

Theoretical values of $\bar{Y}$ and $\bar{Y}/\alpha$ were calculated on an IBM 360/65 computer for a large number of combinations of values for n, c, and L, and a range of values of α from 10^{-2} to 10^3. The results of these calculations were plotted as normalized Scatchard plots on a CALCOMP plotter. The line shown in Fig. 3 was generated in this manner and fits the data reasonably well. Theoretical curves for n less than 8 did not fit; values of n greater than 10 were not tried. Somewhat better fit might be obtained by a slightly different choice of nZ and/or K_R for some of the data sets, but valid estimates for all the parameters can be obtained only by statistical methods. A computer program to accomplish this task is under development.

The fact that the data cannot be fitted, even to a first approximation, by values of n less than 8 implies that each binding domain comprises at least 8 (and probably 10) functionally identical subunits (i.e., protomers), each of which can bind one molecule of activated complement 1. The value of $L = 3$, with which the line in Fig. 3 was obtained, represents a fairly sharp optimum: $L = 1$ and $L = 4$ give distinctly poorer fit to the experimental points. This implies that in the absence of activated complement 1, neither of the two postulated conformational states of the antigen-antibody complexes that comprise the binding domains is strongly favored, i.e., the free energy change associated with the allosteric transition is small. Finally, the value of $c = 0$, which produces distinctly better fit than $c = 0.01$, indicates that binding of activated complement 1 is confined almost exclusively to the "*R*" form of the antigen–antibody complex; the dissociation constant for the "T" form is at least 100 times greater.

Two alternatives to an allosteric model can be eliminated on the basis of present data: interaction between univalent binding domains, and concentration-dependent interaction between free activated complement 1 molecules in solution. If the observed cooperative binding were due to interaction between binding domains, a reduction in the number of

binding domains per cell should suppress the cooperativity. The data marked "AS PPT" in Fig. 3 show that this is not the case: both sets of data were obtained with the same preparation of $(NH_4)_2SO_4$-precipitated IgG antibody; the only difference between them is a 5-fold difference in the amount of antibody per cell. As observed by others (18), this results in a 25-fold difference in the number of activated complement 1 binding sites per cell, but the degree of cooperativity is not affected to a detectable extent.

To determine whether the activated complement molecules interact in solution in the concentration range relevant to our experiments, we measured the sedimentation coefficient of our purified activated complement 1 preparation by the method of Yphantis (19), under exactly the same conditions as prevailed in the binding experiments, using a Kel-F fixed partition cell, at 30,000 rpm. The results showed that the sedimentation coefficient of the complement is independent of concentration over a 200-fold range.

One alternative to an allosteric model remains to be examined: that an activated complement 1 molecule that is bound to an antigen–antibody complex undergoes a conformational change that results in the creation of one or more new complement-binding sites on the bound complement molecule, that have a higher affinity for activated complement 1 than the original antigen–antibody complex. Results obtained by others would suggest that this possibility should be considered, since their evidence indicates an antigenic change in the activated complement 1 bound to sensitized erythrocytes (2). Since our binding data are sigmoid (i.e., they approach a finite asymptote at high activated complement 1 concentrations), such a model would have been subject to the constraint that the process of association between activated complement 1 molecules at the antigen–antibody complex cannot continue indefinitely; the least arbitrary assumption that would satisfy such a constraint is that only an activated molecule that is bound directly to an antigen–antibody complex can acquire new complement 1-binding sites. Such a model has not yet been explored in detail.

Finally, it should be emphasized that binding site heterogeneity, either in the antigen–antibody complexes or in the activated complement 1, cannot account for our results. Such heterogeneity would result in a Scatchard plot that is curved in the opposite sense, i.e., one which is convex to the abscissa rather than concave.

In conclusion, our data show that binding of activated complement 1 to antibody–antigen complexes of IgG on the surface of erythrocytes involves a homotropic cooperative interaction between the activated complement molecules. This effect cannot be explained in terms of either a concentration-dependent aggregation of free activated complement molecules or an interaction between binding domains. The results are compatible with a model that postulates that the cooperative binding is mediated by an allosteric change in the antigen–antibody complex.

NOTE ADDED IN PROOF

An extensive series of experiments, performed after submission of this manuscript and to be described in detail elsewhere, has convinced us that the occurrence of a substantial number of points *below* the ordinate intercept of the theoretical line shown in Fig. 3 is a consistent result and cannot be dismissed as due to random error. We have thus been led to revise our estimates of the parameters for the allosteric equation for this system to $n = 20$, $L = 3.5$, and $c = 0$.

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