THE JOURNAL OF IMMUNOLOGY
Vol. 105, No. 1, July 1970
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THE RELATIONSHIP BETWEEN ANTIBODY AFFINITY AND THE EFFICIENCY OF COMPLEMENT FIXATION

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Received for publication September 25, 1969

The efficiency of complement fixation by antigen-antibody complexes is shown for the first time to be directly related to the affinity of IgG antibody for its antigenic determinant. Decreased complement fixation by IgG antibody at 37°C is independent of antibody affinity. The biologic activity of the complexes in terms of complement fixation parallels their activity in the passive cutaneous anaphylaxis reaction. The implications of these observations with respect to the mechanisms of complement fixation by immune complexes formed with IgG antibody are discussed.

A variety of studies have established that antisera produced against a haptenic determinant represent a heterogeneous population of antibodies that differ greatly in their affinity for the determinant. In addition, it is known that affinities may vary within individual sera (1). The antibody-hapten association constant is influenced significantly by differences in the dose of antigen as well as the time at which the antibody populations are isolated after immunization (2–7). Recently, there has been considerable interest in the correlation between the affinities of antibodies and their biologic activities (1, 8, 9). Antibodies specific for the 2,4-dinitrophenyl group (DNP) are used widely in studies of the relationship between antibody affinity and certain biologic factors (e.g., passive cutaneous anaphylaxis, hemagglutination assays) since simple and precise methods are available for the preparation of purified antibody and the measurement of antibody affinities (2, 10, 11). The direct relationship between the increasing affinities of anti-DNP antibodies and the greater strength of allergic skin responses with these antibodies has been studied (1, 8). The sensitivity of a hemagglutination assay method for dinitrophenol specific antibodies has been shown to be dependent upon antibody affinity (9).

In the present work, the relationship of variation in the antibody-hapten association constant to the efficiency of these complexes in fixing complement and the relationship to passive cutaneous anaphylaxis were studied. The efficiency of the antibody-hapten complex in fixing complement was found to vary directly with the association constant of the given antibody. The significance of these findings in understanding the precise mechanisms involved in the fixation of complement by immune complexes is discussed.

MATERIALS AND METHODS

Immunization. Fifteen adult New Zealand albino rabbits (allotype 1144, mixed sexes) were immunized with 0.05 mg to 5.0 mg of dinitrophenylated rabbit γ globulin (DNP_{17}-RGG) prepared and analyzed according to Eisen (12). An emulsion containing the antigen in complete Freund's adjuvant was injected in a volume of 0.25 ml into each footpad and into one subcutaneous site on the back. The animals were exsanguinated on the 25th day following immunization and the sera were pooled.

Preparation of immunoabsorbents. Bovine γ globulin was coupled to Sepharose 2B by the Rejnek *et al.* (13) modification of the method described by Axen *et al.* (14), employing cyanogen halide coupling. The derivative was then prepared with trinitrobenzene sulfonic acid according to Eisen (12). Polyacrylamide beads (Bio-Gel P 300+) were amino-ethylated by the method of Inman and Dintzis (15) to produce a specific capacity of approximately 1 mM/g. The material was then reacted with unrecrystallized dinitrobenzene sulfonic acid at pH 10.0 (12).

Purification of antibodies. Purified antibody was prepared as follows: EDTA was added to 300 ml of pooled rabbit antisera to give a final concentration of 0.001 M and the material was

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passed over trinitrophenylated bovine γ globulin (TNP-BGG) Sepharose immunoabsorbent. The serum containing unbound anti-DNP antibody was then set aside for isolation and purification of "low affinity" antibody. The "high affinity" anti-DNP antibody which adhered to the TNP-BGG Sepharose was eluted with three washes of 25 ml of 0.1 M dinitrophenolate. The first 25 ml of eluent was designated fraction A, high affinity antibody. The remaining eluent was pooled as fraction B. The column was washed with borate saline, pH 8.0, after the elution with dinitrophenolate. Collection was terminated when the wash eluent gave a reading of 0.005 optical density units at 280 nm in a Beckman DU spectrophotometer. This eluent was then pooled as part of fraction B. The two pools were then dialyzed extensively, first against running tap water and then against 0.15 M NaCl, passed through a Dowex 1-X4 column,[1] followed by separation of IgG and IgM components by passage through a G-200 Sephadex gel column. The specifically purified rabbit IgG anti-DNP was used in all the subsequent work.

The serum pool containing "low affinity" antibody which had been through the TNP-BGG Sepharose was then passed over a dinitrophenylated aminoethyl polyacrylamide immunoabsorbent. The column was eluted with 150 ml of 0.1 M dinitrophenolate. The eluent (fraction C) was dialyzed and purified as described above. All three specifically purified antibody fractions were capable of 87% to 95% binding to the DNP immunoabsorbent.

Measurement of antibody-hapten equilibrium constant. Association constants were measured by fluorescence quenching (2, 11, 16). Purified anti-DNP antibody at concentrations ranging from 52 μg/ml to 70 μg/ml in 1 ml of 0.1 M Tris-HCl, pH 7.5, were titrated with 0.025-ml increments of a 2.9 mM/ml solution of ε-DNP-L-lysine until a total added volume of 0.2 ml was reached, and then 0.05-ml increments until a total added volume of 0.4 ml was attained. Fluorescence was measured in an Aminco-Bowman spectrophotofluorometer with incident exciting light of about 290 nm and recording emission of about 350 nm. The average intrinsic association constants (K_0) were calculated as described by Eisen (16), using a digital computer.

Passive cutaneous anaphylaxis. Passive cutaneous anaphylaxis (PCA) was performed according to Ovary (17). Hartley strain guinea pigs weighing about 250 g received 0.1 ml intradermally of varying dilutions of purified antibody in borate-buffered saline, pH 8.0. Individual guinea pigs received multiple injections consisting of varying dilutions of the same antibody as well as equivalent concentrations of antibodies of different average intrinsic association constants (K_0). Three and one-half to four hours later each animal was injected intravenously with 0.5 ml of a 1% solution of Evans blue containing 10 μg of dinitrophenylated bovine γ globulin (DNP_{32}-BGG) prepared according to Eisen (12). The animals were killed 20 to 25 min later and skinned. Reactions were scored on a 0 to 4+ scale according to the extent and intensity of blueing as observed from the flesh side of the pelt.

Complement fixation. Complement fixation tests were performed in two dimensional blocks as described by Kabat (18) with volumes scaled down by a factor of 60. The titration was performed in Microtiter (Cooke) trays. Guinea pig complement was obtained from Suburban Serum Laboratories, Silver Spring, Md. It was absorbed twice with one-twentieth volume of packed washed sheep erythrocytes at 0°C, which were obtained from sheep raised on the National Institutes of Health farm. Veronal-buffered saline (VBS) containing gelatin and appropriate amounts of Ca^{++} and Mg^{++}, optimally sensitized cells and guinea pig complement were employed as described (18). All solutions were kept at 4°C except where indicated. Titrations were carried out as twofold serial dilutions of antibody over the concentration range of 1.333 to 0.1 μg/ml and threefold serial dilutions of antigen (DNP_{32}-BGG) over the range of 0.243 to 0.001 μg/ml. To 50 μl of antibody solution were added 50 μl of antigen. Exactly 3 CH_{50} units of guinea pig complement contained in 25 μl of solution were added to the mixtures and the trays were incubated at 4°C for 18 hr. At the end of the period of incubation, 25 μl containing 3.2×10^8 optimally sensitized sheep erythrocytes were added. The trays were covered with Mystic tape and frequently mixed for 1 hr at 37°C. After incubation the cells were allowed to settle for 1 hr at room temperature and the end point was determined visually on scoring the reaction from 4+

[1] Spectral analysis indicated that less than 6% of the combining sites were occupied by hapten. This calculation was based on comparison of absorbance at 278 and 360 nm (11).

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(complete hemolysis) to 0 (no hemolysis). The results were expressed as the antibody dilution which, in the presence of a minimum antigen concentration, fixed 2 units of CH_{50}, thus leaving residual complement activity to give 2+ hemolysis. In a parallel series of experiments, the antigen, antibody and complement mixture was incubated at 37°C for 1 hr, instead of at 4°C for 18 hr. The remainder of the procedure was then carried out exactly as described.

Controls included tests done with complement alone and complement with either antigen or antibody. No anticomplementary activity was encountered in any of the control preparations.

RESULTS

Relationship of affinity and the concentration of antibody required for PCA response. The minimum concentration of antibody needed to elicit a positive PCA reaction was determined for each of three preparations of antibodies of varying dilutions (see *Materials and Methods*). Each

TABLE I

Relationship of affinity and the concentration of antibody required for passive cutaneous anaphylaxis[a]

Antibody Preparation	K_0[b]	Minimum Antibody Concentration Giving Positive Reaction
	liters/mol × 10^{-6}	*μg/ml*
A	900	31.3, 31.3, 7.8, 31.3
B	10	62.5, 62.5, 31.3
C	1.1	>500, 500, 500

[a] Reaction obtained with 10 μg of DNP_{32}-BGG.
[b] K_0, association constant.

TABLE II

Two dimensional complement fixation tests with purified rabbit anti-DNP and dinitrophenylated bovine γ globulin[a]

DNP_{32}-BGG (μg/ml)	Antibody Dilution (μg/ml)											
	133	66.5	33.3	16.6	8.3	4.2	2.1	1.0	0.5	0.3	0.1	0
Fraction A												
0.243	0[a]	0	0	0	0	0	3	4	4	4	4	4
0.081	0	0	0	0	0	0	1	2	3	4	4	4
0.027	0	0	0	0	0	0	1.5	3	4	4	4	4
0.009	3	3.5	4	4	4	4	4	4	4	4	4	4
0.003	4	4	4	4	4	4	4	4	4	4	4	4
0.001	4	4	4	4	4	4	4	4	4	4	4	4
0.000	4	4	4	4	4	4	4	4	4	4	4	4
Fraction B												
0.243	0	0	0	0	0	0	1	4	4	4	4	4
0.081	0	0	0	0	0	0	0.5	1.5	4	4	4	4
0.027	0	0	0	0	0	0	2	2	4	4	4	4
0.009	3.5	3.5	3.5	3.5	3	3.5	3.5	4	4	4	4	4
0.003	4	4	4	4	4	4	4	4	4	4	4	4
0.001	4	4	4	4	4	4	4	4	4	4	4	4
0.000	4	4	4	4	4	4	4	4	4	4	4	4
Fraction C												
0.243	0	0	0	0	1	4	4	4	4	4	4	4
0.081	0	0	0	0	1	3.5	4	4	4	4	4	4
0.027	1	1	2	3	4	4	4	4	4	4	4	4
0.009	4	4	4	4	4	4	4	4	4	4	4	4
0.003	4	4	4	4	4	4	4	4	4	4	4	4
0.001	4	4	4	4	4	4	4	4	4	4	4	4
0.000	4	4	4	4	4	4	4	4	4	4	4	4

[a] 0, no lysis; 4, complete lysis.

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preparation was titrated in three or four guinea pigs. The animals were injected intradermally with varying dilutions of two purified antibodies of different association constants so that results might be contrasted in the same animal. As seen in Table I, the minimum eliciting dose for a PCA response varied directly with the antibody-hapten association constant. Antibody preparations with highest affinity gave positive reactions at concentrations of 7.8 to 31.3 μg/ml, whereas preparations of low affinity required 500 μg/ml or more.

Effect of affinity on the efficiency of complement fixation. Efficiency of complete fixation is taken in this study to represent the minimum concentration of antibody (reacting with the minimum amount of antigen) needed to fix a given amount of complement. This was determined for each antibody preparation. As seen in Table II, the efficiency of complement fixation varied directly with the association constants of the antibodies. Both fraction A and fraction B required a minimum concentration of 0.027 μg/ml of antigen and 2.1 μg/ml of antibody to fix 2 of 3 CH_{50} units. Optimal fixation by fraction C required an antigen concentration of 0.081 μg/ml and an antibody concentration of 8.3 μg/ml. Since the antibody in question is IgG, altering the incubation conditions of the hapten, antibody and complement mixture (37°C for 1 hr, instead of 4°C for 18 hr) leads to decreased complement fixation (19, 20), and therefore to an increase in the minimum concentration of antigen and antibody necessary to fix a given amount of complement. Table III shows that the decreased complement fixation associated with incubation

TABLE III

Two dimensional complement fixation test with purified rabbit anti-DNP and dinitrophenylated bovine γ globulin under altered conditions of incubation

DNP_{22}-BGG (μg/ml)	Antibody Dilution (μg/ml)											
	133	66.5	33.3	16.6	8.3	4.2	2.1	1.0	0.5	0.3	0.1	0
					Fraction A							
0.243	0[a]	0	0	1	2	4	4	4	4	4	4	4
0.081	0	0	0	1	2	3	3.5	4	4	4	4	4
0.027	1	2	2	2	3	3.5	4	4	4	4	4	4
0.009	3.5	3.5	3.5	3.5	3.5	3.5	4	4	4	4	4	4
0.003	4	4	3.5	4	4	3.5	4	4	4	4	4	4
0.001	3.5	4	4	4	4	4	4	4	4	4	4	4
0.000	3.5	4	4	4	4	4	4	4	4	4	4	4
					Fraction B							
0.243	0	0	0	1	3	4	4	4	4	4	4	4
0.081	0	0	0	1	2	3.5	4	4	4	4	4	4
0.027	1.5	2	2	2.5	3	3.5	4	4	4	4	4	4
0.009	4	4	4	4	4	4	4	4	4	4	4	4
0.003	4	4	4	4	4	4	4	4	4	4	4	4
0.001	4	4	4	4	4	4	4	4	4	4	4	4
0.000	4	4	4	4	4	4	4	4	4	4	4	4
					Fraction C							
0.243	2	3	4	4	4	4	4	4	4	4	4	4
0.081	2.5	2.5	4	4	4	4	4	4	4	4	4	4
0.027	3.5	4	4	4	4	4	4	4	4	4	4	4
0.009	4	4	4	4	4	4	4	4	4	4	4	4
0.003	4	4	4	4	4	4	4	4	4	4	4	4
0.001	4	4	4	4	4	4	4	4	4	4	4	4
0.000	3.5	3.5	4	4	4	4	4	4	4	4	4	4

[a] 0, no lysis; 4, complete lysis.

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at 37°C was noted to about the same degree in all three preparations.

DISCUSSION

Recent studies correlating the avidity of antibody for its antigenic determinant with its role in biologic reactions (1, 8, 9) raise the possibility that antibody affinity is directly related to the complement-fixing efficiency of the immune complex. The work of Siskind and Eisen (1) and others (8) indicates that high affinity antibody is more efficient in eliciting allergic skin responses. Our findings with PCA confirm these data. Levine (9) has found that increasing affinities of dinitrophenyl specific antibodies parallel the increasing sensitivity of a hemagglutination assay method for the antibody. However, it has been suggested (21) that low affinity antibody may wander from site to site on an antigenic surface activating complement components at each site and thus increasing the damaging effects of the antibody. It has recently been shown (22) that "Forssman shock" induced by injecting rabbit anti-Forssman antibody intravenously into guinea pigs is produced inefficiently, if at all, by IgM antibody prepared by a method which yields high affinity antibody, but is regularly produced by IgG anti-Forssman antibody with known low affinity.

We have attempted to evaluate systematically the effect of variation of antibody affinity for its antigenic determinant upon its efficiency of complement fixation. Our studies indicate that when the same antigen is allowed to complex with antibodies of varying affinity under the same conditions, low affinity antibody does not fix complement as efficiently as does high affinity antibody. Moreover, the previously described phenomenon (19, 20) of greater fixation at 4°C as compared with fixation at 37°C by IgG antibody is qualitatively independent of the affinity of the antibody. These studies give further support to the concept of differing mechanisms of complement fixation between IgM and IgG antibodies as the cause of their different biologic effectiveness.

Complement fixation by an IgG antibody-antigen system requires the presence of complexes with lattice formation (23). The smallest complex that will fix complement has been shown to be Ag_2Ab_3 (23). In order for complement to react with immune complexes, aggregation of antibody appears necessary (24), and it has further been shown (24) that the initiation of complement fixation in the fluid phase requires two IgG molecules side by side (doublet formation). In our experiments, the same antigen (DNP-BGG) with a known DNP/BGG molar ratio of 32 was used throughout. In such a system in which all other features are constant, the frequency of doublet formation must be a function of antibody affinity. It seems reasonable to suggest that a smaller proportion of low affinity antibody is complexed with the soluble antigen during the period of fixation and, therefore, less complement is fixed. Whether similar results can be obtained with antibodies directed at cell surface antigens has yet to be established.

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