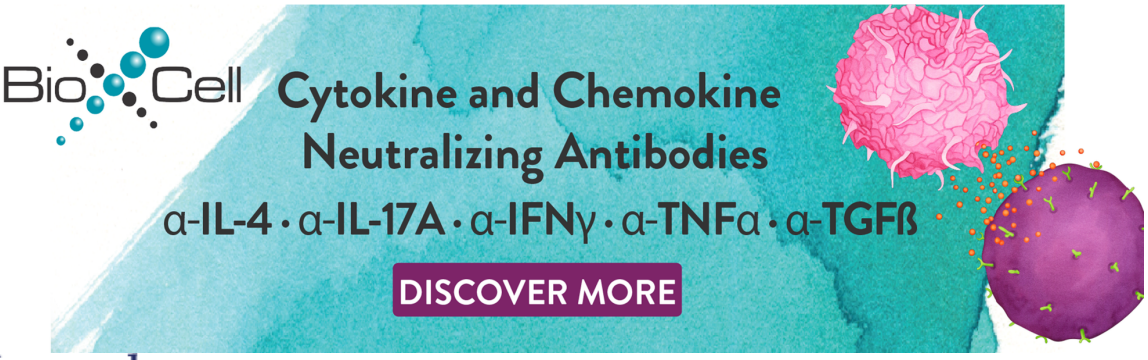

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EFFECT OF VARIATION IN ANTIBODY-HAPTEN ASSOCIATION CONSTANT UPON THE BIOLOGIC ACTIVITY OF THE ANTIBODY[1]

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It is well established by an extensive body of experimental results, obtained with a variety of independent methods, that the antibodies produced against even the simplest antigenic determinants are a heterogeneous population of molecules that vary widely with respect to affinity for the homologous determinant (e. g., 1–7). It has also been recognized that antisera produced against toxins and microorganisms often exhibit discrepancies when their activities are measured in different assays: e. g., between potency measured *in vitro* and biologic (protective) activity measured *in vivo* (8–11). Even within individual sera diverse fractions of antibody may differ in the ratio of activities as measured by various assays (8–10). These discrepancies are not easily interpreted since the number and variety of antigenic determinants in most antigens is so large as to leave unanswered the possibility that antibodies of different specificities are involved in different assays. Nevertheless, it has always seemed likely that differences in affinities of antibodies for any particular determinant might account for at least some of these discrepancies. Accordingly, the present work was carried out to determine how the affinities of different populations of antibody molecules influence their biologic activities. Antibodies specific for the 2,4-dinitrophenyl (DNP) group were used for this undertaking since their affinities can be readily measured (7, 12), and populations of anti-DNP molecules of varying affinity can be prepared (7). The biologic activity accessible to measurement in the case of the DNP group (or other simple haptenic groups) is the allergic response. That biologic activity of this kind can be influenced by affinity has been surmised from earlier observation with bis-DNP-L-lysine (13–15). This bivalent ligand elicited allergic skin responses in guinea pig and human skin sites passively sensitized with a preparation of rabbit anti-DNP antibodies of high affinity (for the DNP group) but not with an antibody preparation of low affinity (15). Further observations by Ovary (16) have also indicated a relationship between the affinity of anti-DNP antibody and its activity in passive cutaneous anaphylaxis (PCA). In addition, Cochrane (17) has reported that antiserum to bovine albumin (BSA) presumed from indirect measurements to be of low affinity, was not as effective in eliciting PCA reactions as was antibody of higher affinity. Direct measurements of association constants for anti-BSA antibodies could not, of course, be made. In the present paper the effect of variation in antibody-hapten association constant on passive cutaneous anaphylaxis has been studied with populations of purified anti-DNP antibodies that vary over a 1000-fold range in affinity for their homologous determinant (ε-DNP-L-lysine).

MATERIALS AND METHODS

Preparation of antibodies. Rabbits were immunized with varying doses (5 to 250 mg) of dinitrophenylated bovine γ-globulin (DNP_{47}-BγG),[3] prepared as previously described (18). The antigen was given as an emulsion in incomplete Freund's adjuvants (without mycobac-

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[3] When referring to dinitrophenylated proteins the subscript after DNP refers to the average number of DNP groups per molecule of protein, taking the molecular weight to be that of the unsubstituted protein.

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TABLE I

Association constants of anti-DNP preparations

Purified Antibody Preparation No.	K_0 [a]	Immunization Procedure	
		Antigen dose	Bleeding time
	$L/M \times 10^{-6}$	*mg*	*weeks*
1[b]	>1000	5	3
2[b]	~330	5	3
3[b]	8.1	5	3
4[b]	1.0	5	3
5[b]	0.23	5	3
6	1.6	5	5
7	32	5	5
8	5.9	5	5
9	20	5	8
10	0.60	5	2
11	0.32	5	2
12	0.14	250	2
13	0.13	250	5
14	0.36	250	2
15	0.23	250	5
16	0.38	250	8
17[c]	110	5	3
18[c]	0.28	250	2

[a] Association constants measured at 31(±1)°C in 0.1 M Tris-Cl, pH 7.5.

[b] Preparation Nos. 1 to 5 represent fractions prepared from a single bleeding of one rabbit (see "Methods").

[c] These preparations made from pooled rabbit serum. All other preparations made from single bleedings of individual rabbits.

teria) into the footpads. As reported in detail elsewhere (7), animals receiving high doses of antigen tend to produce antibodies of significantly lower binding affinity then animals immunized with low doses. In addition, antibody fractions of different affinities for the homologous determinant were prepared systematically by fractional precipitation (7). In brief, the procedure involved the successive addition of small aliquots of antigen, the precipitates forming after each addition being collected individually; antibodies were then isolated from each precipitate (see below). Antibody preparation Nos. 1 through 5 (Table I) were prepared by fractionation of sera from a single rabbit. Preparation Nos. 6 through 16 were prepared from specific precipitates formed with equivalent amounts of antigen from sera of individual rabbits. Preparations 17 and 18 were prepared from specific precipitates, formed in the equivalence zone, of pooled antisera of rabbits receiving either 5 or 250 mg of antigen, respectively.

Purification of antibodies. Purified antibody was prepared as previously described (20). Briefly, the purification involved precipitation of antibody with DNP-BγG, elution of the specific precipitate with 0.1 M dinitrophenol in the presence of streptomycin sulfate (35 mg/ml), passage of the eluent through a Dowex I column followed by ammonium sulfate precipitation and dialysis of the purified antibody against 0.15 M NaCl-0.01 M phosphate pH 7.5 (PBS) and then against 0.001 M potassium phosphate buffer, pH 7.5. The soluble (pseudo γ-globulin) fraction obtained after dialysis was used in all work reported here. The antibody was finally diluted in phosphate-buffered saline (PBS). The concentration of purified antibody was determined by absorbancy at 278 mμ in the Beckman-DU spectrophotometer ($E^{1\%}_{1\,cm}$ at 278 mμ was taken to be 13.6) (20).[4] The antibody preparations were found to be at least 70 to 90% precipitable with DNP-BγG. It must be emphasized that for two reasons the estimates of precipitability are minimal: *a*) precipitation was carried out at low concentrations of antibody, about 0.2 to 0.5 mg/ml; and *b*) only a single quantity of antigen was added to each sample of antibody; in order to conserve material complete precipitin curves were not obtained. It is clear from subsequent work in this laboratory that at antibody concentrations of the order of 5 mg/ml even the lowest affinity preparations are about 95% precipitable (21).

The electrophoretic mobilities of the different antibody preparations were determined in agar gel by immunoelectophoresis (22), using a goat antiserum specific for rabbit γ-globulin (2.0 mg of goat antibody/ml) to develop the precipitin patterns. Within the limits of resolution of the technique all samples of purified antibody showed only a single precipitin band and had the same electrophoretic mobility.

Measurement of antibody-hapten equilibrium constants. Association constants were measured by fluorescence quenching (7, 12). Antibody solutions, at a concentration of 100 μg/ml in 0.1 M Tris-Cl, pH 7.5, were titrated with a total of 3 mμ M ε-DNP-L-lysine added in small aliquots. Fluorescence was measured in an Aminco-

[4] Although 13.6 was used during this work for the extinction coefficient of rabbit antibody, more recent observations indicate that the value for rabbit anti-DNP antibody is 15.5 (7, 19).

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TABLE II

Effect of variations in antibody-hapten association constant on sensitivity of passive cutaneous anaphylaxis[a]

Antibody Preparation No.	K_0	Minimum Antibody Concentration Giving Positive Reaction[b]
	$L/M \times 10^{-6}$	μg/ml
1	>1000	6, 6, 6, 6, 3, 1.5
2	~330	6, 6, 3
3	8.1	25, 25, 12.5, 12.5, 12.5, 12.5
4	1.0	100, 50, 50, 100
5	0.23	170, 85, 85, >100
6	16	6, 6, 6, 3, 3, 3, 3, 3, 3, 1.5, 1.5, 1.5
7	32	3, 1.5, 1.5
8	5.9	3, 3, 3
9	20	6, 6, 3, 5
10	0.60	12.5, 6, 6, 6, 12.5
11	0.32	12.5, 6, 3, 3, 12.5
12	0.14	25, 25, 12.5, 12.5, 6, 6, 6
13	0.13	25, 12.5, 6, 25, 25
14	0.36	25, 12.5, 12.5
15	0.23	50, 50, 25, 12.5, 12.5, 12.5
16	0.38	50
17	110	7.5, 7.5, 7.5, 6, 3, 3, 1.5, 1.5
18	0.28	40, >40, >40, 12.5, 12.5, 12.5, 12.5

[a] Reaction elicited with 100 μg of DNP_{29}-HSA.

[b] Each figure represents a titration in an individual guinea pig. Each site received 0.1 ml of the appropriate antibody solution.

Bowman spectrophotofluorometer using incident exciting light of about 280 mμ and recording emission at 345 mμ. From the extent of fluorescence quenching observed with various amounts of added hapten the average intrinsic association constant (K_0) was calculated as described previously (7). The values for the binding of ε-DNP-L-lysine by the antibody preparations used in this work are listed in Table I.

Passive cutaneous anaphylaxis. Passive cutaneous anaphylaxis (PCA) was carried out according to Ovary (23). Guniea pigs weighing about 250 g (±50 g) received 0.1 ml of varying dilutions of purified antibody in PBS intradermally. Six to 10 hr later each animal received 0.5 ml of a 1% Evans blue solution in PBS containing 10, 100 or 1000 μg of dinitrophenylated human serum albumin (DNP_{29}-HSA) intravenously. Animals were sacrificed 20 to 30 min later, skinned, and reactions scored. A positive reaction was taken as a distinct area of bluing when observed from the flesh side of the skin.

Reverse passive cutaneous anaphylaxis reactions were carried out according to methods described by Ovary (24). An 0.1-ml amount of serial twofold dilutions of rabbit anti-DNP antibody was injected intradermally in guinea pigs. Six to 8 hr later the animals received 1.0 ml of goat antiserum specific for rabbit γ-globulin (2.0 mg of goat antibody/ml) mixed with 0.5 ml of 1% Evans blue in PBS intravenously. Animals were sacrificed 15 min later and the reactions scored as with the direct PCA reactions.

RESULTS

Effect of affinity on concentration of antibody required for PCA response. The minimum concentration of antibody that elicited a PCA response was determined for each preparation by injecting serial twofold dilutions of stock antibody solutions intradermally. The end point was recorded as the lowest concentration which gave a positive reaction (see "Materials and Methods") when the animal received 100 μg of antigen, with Evans blue, intravenously. Each preparation (except No. 16) was titrated in 3 to 12 individual guinea pigs. It should be mentioned that although the average affinities of the low affinity antibodies obtained after serial precipitation (preparation 5) and that obtained after injection of a large dose of antigen (e.g., preparation 15) may be very similar the distribution of affinities among the antibody molecules in these populations might be quite different.

As can be seen from Table II the minimum amount of antibody varied with the antibody-hapten association constant. Preparation Nos. 1 through 5, prepared by fractionation of the serum from a single rabbit, showed a progressive increase in the minimum concentration, paralleling the progressive decrease in binding constant. The preparations with highest affinity gave positive reactions at 1.5 to 6 μg/ml while the preparations with lowest affinity required 85 to 170 μg/ml.

Dependence of PCA sensitivity on dose of eliciting antigen. The effect of varying the dose of antigen was studied with regard to the variations in sensitivity of PCA described above. Pairs of antisera of high and low affinity were titrated on opposite sides of individual guinea pigs. PCA reactions were elicited with 10, 100 or 1000 μg of

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TABLE III

Effect of varying dose of antigen on sensitivity of PCA with high and low affinity antibodies

Antigen Dose	Ratio of Preparation Nos.[a]			
	#15: #6	#12: #6	#13: #	#3: #1
μg				
10	not done	6:<1.5 12.5:3 25:3 >25:6	12.5:1.5	not done
100	12.5:3	6:1.5	6:3	12.5:3 12.5:1.5
	12.5:1.5	6:1.5	12.5:6	25:6
	12.5:3	25:6 6:3 3:6		
1000	<3:1.5	6:6 3:3	3:3	6:6
	12.5:6	3:3 3:3 12.5:>6 <3:1.5 <3:1.5	6:6	6:6 6:6

[a] The ratio is the minimum concentration (μg/ml) of low affinity antibody required to the minimum concentration of high affinity required. For example with preparations 13 and 6, tested on opposite sides of a guinea pig injected intravenously with 10 μg of DNP-HSA, 12.5 μg/ml of #13 and 1.5 μg/ml of #6 were the respective minimum concentrations. Titrations were carried out on opposite sides of individual guinea pigs. Each site received 0.1 ml of appropriate antibody solution. Variations in minimum doses of antibody required for preparation 6 reflect differences in reactivity of individual guinea pigs. These variations do not influence the interpretation since each pair of sera were titrated simultaneously on opposite sides of the same animal. Preparation numbers refer to Tables I and II.

DNP_{29}-HSA. The minimum antibody concentration required for a positive reaction was determined in each case. The results are given in Table III.

It can be seen that for each combination tested, a four- to eightfold greater concentration of low affinity antibody than of high affinity antibody was required when 10 μg antigen were used to elicit the reaction. When 1000 μg antigen was used, these differences were eliminated in three out of the four combinations tested and reduced in the fourth. Thus, when the reactions were elicited with 1000 μg of antigen, high and low affinity antibody preparations behaved similarly, the minimum concentration for each being usually in the range of 1.5 to 6 μg of antibody/ml.

TABLE IV

Reverse passive cutaneous anaphylaxis using rabbit anti-DNP intradermally and goat antiserum to rabbit γ-globulin intravenously

Antibody Preparation No.	K_0	Minimum Concentration of Rabbit Anti-DNP Antibody Giving Positive Reactions[a]				
	$L/M \times 10^{-6}$	μg/ml				
17	112	0.75	0.75	0.4	0.4	0.75
18	0.28	0.75	1.5	0.4	0.4	0.75

[a] Each vertical column represents the results of titrations in an individual guinea pig.

Skin fixation of various antibody preparations. The possibility arises that the ineffectiveness of low affinity antibodies in PCA is due to poor fixation to skin sites rather than to low affinity for the ligand. In order to test this possibility, reverse passive cutaneous anaphylaxis (24) was carried out with representative samples of high and low affinity. Serial dilutions of representative samples of high and low affinity rabbit anti-DNP antibody were injected intradermally on opposite sides of the same guinea pigs. The animals were challenged 6 to 8 hr later with goat anti-rabbit γ-globulin and Evans blue intravenously (see "Materials and Methods"). As shown in Table IV, both high and low affinity antibody gave reverse PCA reactions at 0.4 to 1.5 μg/ml. Thus, differences in skin fixation did *not* account for the differences in behavior of the high and low affinity antibodies reported above.

DISCUSSION

If it is assumed that elicitation of a positive PCA reaction required a critical mass of antibody-antigen complexes at a localized skin site, then it is expected that PCA sensitivity would depend on the antibody's affinity for antigen. Further, to a first approximation, low affinity should be compensated for by increasing the

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concentration of either of the reactants. It is not generally possible to measure association constants for the determinant groups of a multivalent ligand with antibodies since multiple specificities are usually involved and insoluble complexes are usually formed. In the present study, the antibodies were formed against DNP-BγG and the test antigen was DNP-HSA; hence, the tendency of the antibodies to form stable complexes with DNP-HSA is expected to reflect the antibody's affinity for ε-DNP-L-lysine, the group common to both DNP proteins. This assumption has recently been validated (25).

From the foregoing, increasing the amount of antigen used to elicit the reaction would be expected to minimize the functional differences between antibodies of high and low affinity for ε-DNP-L-lysine. Our experimental results are consistent with this expectation.

High and low affinity purified anti-DNP antibodies obtained either from different rabbits or by fractionation of sera of individual rabbits behave differently with respect to their ability to sensitive guinea pig skin for PCA. When a relatively small dose of antigen is used to elicit the reaction, a higher concentration of low affinity antibody than of high affinity antibody is required for a positive reaction. Differences in antibody-hapten-binding constants are thus reflected in the *in vivo* reactivity of the antibodies in a manner consistent with what would be expected from considerations of the law of mass action.

It has been pointed out by Ovary (23) that increasing the dose of antigen used to elicit PCA reaction increases the sensitivity of the technique. Our results support and extend his observations: increasing the eliciting dose of DNP-HSA from 10 to 100 μg markedly increased the sensitivity with low affinity antibody, but had no demonstrable effect on sensitivity with the highest affinity antibody.

It has been reported by Kabat and Benacerraf (26) that nonprecipitating antibodies (prepared as the residual soluble antibody after precipitation of a rabbit antiserum with sequential small additions of antigen) is as effective as precipitating antibody in passive sensitization of guinea pigs for systemic anaphylaxis. Since their nonprecipitating antibodies were probably of lower affinity for antigen than the precipitated antibodies (7), their results may be ascribed to the fact that relatively large amounts of antigen (1 mg) were used to elicit anaphylaxis.

It is reasonable to expect that under natural conditions of infection and of exposure to naturally occurring antigens, the conditions approximate those in the present experiments in which relatively small quantities of antigen were used to elicit a biologic reaction. Under natural conditions one would expect therefore that the biologic activities of antibodies would be dependent on their affinities for the corresponding antigenic determinants. It is not surprising therefore that biologic activities of antibodies often do not correlate simply and consistently with the concentration in serum.

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SUMMARY AND CONCLUSIONS

Under appropriate conditions the passive cutaneous anaphylaxis response can be shown to depend on the affinity of antibody for its homologous antigenic determinant. The variation in response observed is consistent with what would be expected from considerations of the law of mass action.

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