The Relationship between Antigenic Complexity and Heterogeneity in the Antibody Response*

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ABSTRACT: Rabbit antibodies to the defined-sequence polymer [(D-Ala-L-ala)$_5$-ε-dinitrophenyl-L-Lys$_1$]$_{10.2av.}$ have been prepared. Unlike antibodies to dinitrophenyl-substituted proteins, these have a high energy of interaction with [^{3}H]ε-dinitrophenyllysine at 2.5 weeks after immunization with average intrinsic association constants of the order of 1–5 $\times$ 10^{-9} M^{-1}. No evidence of a rise in these association constants could be demonstrated later in the immune response. Gel radioimmunoelectrophoresis, separation on DEAE-Sephadex columns, and partial immunoadsorption experiments indicated that the antibody population showed some restriction in heterogeneity as compared with antibodies to dinitrophenylated bovine γ-globulin. Antibodies to an *N*-carboxyanhydride polymer having approximately the same composition but a less rigidly ordered environment of the dinitrophenylaminoalkyl residue gave rise to antibodies having intermediate maturation properties and some differences in charge distribution.

A major barrier to the investigation of the relationship between the structure and function of antibodies has been the heterogeneity of most natural antibodies. A number of experiments have demonstrated clearly that when a single hapten is attached to a known residue of a pure protein, or to a polypeptide backbone (Kantor *et al.*, 1963; Eisen *et al.*, 1964) not single antibodies but whole families of antibodies are produced in response. Such heterogeneity is in part due to the presence of the different antibody classes such as γG, γM, and γA and their respective subclasses as well as to genetic differences. Further, even when a single class of antibodies is isolated from a single animal immunized with defined hapten conjugates, heterogeneous antibody fractions reacting with hapten are produced (Haber *et al.*, 1967). It has been suggested previously that it is perhaps an oversimplification to regard a hapten as an immunogen in isolation when it is attached to a macromolecular carrier and that it may be more appropriate to consider the hapten together with its environment as constituting the immunogen (Haber *et al.*, 1967). The dimensions of the antibody combining site are considerably larger than most of the small organic molecules used as haptens (Kabat, 1960, 1966; Sage *et al.*, 1964) and therefore the sites may encompass not only the hapten but also the hapten plus many different sections of the protein. In an attempt to construct an antigen in which some of the complexities of substituted proteins are reduced, a defined-sequence amino acid copolymer has been synthesized, having a molecular weight of about 10,000 and bearing the 2,4-DNP hapten at regular intervals of about 30 Å along the backbone when the polymer is in its extended form. This polymer has the structure [ε-DNP-L-Lys-(D-Ala-L-Ala)$_5$]$_n$, where n = 10.2 (av). This polymer has a relatively homogeneous and nonantigenic environment around the hapten, and in the extended form, the haptens are separated by distances greater than the largest dimension of the antibody combining site. The polymer in aqueous solution at pH 7.0 is a random coil; there is no evidence of any β conformation (Richards *et al.*, 1967). The alternate D- and L-amino acid sequence prevents an α-helical structure.

This report describes studies performed with rabbit antisera produced in response to immunization with the DNP hapten placed in three environments of increasing homogeneity. The antigens used were: (1) randomly dinitrophenylated bovine γ-globulin; (2) a "statistical" *N*-carboxyanhydride polymer of residue ratio DL-alanine to DNP-L-lysine (10.6:1), having the same amino acid composition as the defined-sequence polymer; and (3) the defined-sequence polymer. The results obtained suggest that a reduction in the complexity of the antigen leads to a reduction in heterogeneity of the antibodies produced.

Materials and Methods. IMMUNIZATION. Male New Zealand white rabbits were immunized by emulsifying the antigen in complete Freund's adjuvant containing killed *Mycobacterium tuberculosis* (5 mg/ml) and injecting this preparation into all four footpads.

Preparation of γ-Globulin Fractions. The γ-globulin fraction of normal rabbit sera and specific antisera were prepared by the DEAE-cellulose chromatography (Campbell *et al.*, 1964). Such fractions contained 80–85% of the hapten binding activity of whole anti-

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TABLE I: Representative Rabbit Antibody Titers to the Defined-Sequence Polymer [(D-Ala-L-Ala)$_5$ε-DNP-L-Lys$_{10.2}$ (av) as a Function of Time after Immunization.[a]

Weeks after Immunization	mg of Antibody Protein/ml of Serum					
	Rabbit A	Rabbit B	Rabbit C	Rabbit D	Rabbit E	Rabbit F
2.5	800	300	330	24	2	2
9	805	635	400	37	9	10
14	1000	585	1000	25	33	120
21	830	15	60	27	40	240
27	80	15	90	14	11	60

[a] Rabbits A, B, and C are representative of the group of animals producing relatively high antibody titers (about 10–15% of all immunized rabbits). Rabbits D, E, and F represent the group of rabbits producing lower titers of antibody. Antibody titers were estimated by the method of Haber *et al.* (1965).

serum (as determined by equilibrium dialysis and by the method of Haber *et al.* (1965)) γ-globulin fractions of normal serum had 1–5% of the hapten binding activity of antisera used in the present study.

Radioactive Hapten and Equilibrium Dialysis. [^{3}H]-DNP-L-lysine of specific radioactivity (9000 Ci/mole) was prepared by methods described previously (Richards and Haber, 1967). Equilibrium dialysis was carried out as described by Eisen (1964) using solid Lucite 1-ml cells. Each cell compartment was emptied with a Hamilton syringe and the volume was estimated by weight.

Immuno- and Radioimmunoelectrophoresis. Rabbit antiserum was subjected to electrophoresis in agar gel; precipitin lines were developed with sheep antiserum to rabbit γ-globulin. After washing, dinitrophenylated [^{125}I]bovine γ-globulin solution was added to the troughs and the plates were stored at room temperature for 24 hr. Thereafter the plates were washed, dried, wrapped in Saran, and overlayed with X-ray film (Bloch *et al.*, 1968).

Synthesis of Polymers. The synthesis of the defined-sequence polymer [ε-DNP-Lys-(D-Ala-L-Ala)$_5$]$_{10.2}$ has been described previously (Richards *et al.*, 1967). The "statistical" *N*-carboxyanhydride polymer of residue ratio DNP-L-Lys to DL-Ala (1:10.6) was synthesized from ε-Z-L-Lys-*N*-carboxyanhydride and DL-alanine-*N*-carboxyanhydride (Pilot Chemicals, Watertown, Mass.) at an initial molar ratio of 1:16,[1] using sodium ethoxide (0.004 M) as initiator. Removal of carbobenzoxy-blocking groups was carried out with gaseous HBr for 1 hr at 20° in benzene and the complete removal of these groups was inferred by the absence in the infrared spectrum of the ester band at 1700–1730 cm^{-1}. A lysine to alanine ratio of 1:10.6 was found on amino acid analysis. The ε-amino groups of the lysine residues as well as the *N*-terminal amino groups were dinitrophenylated using a threefold molar excess of 1-fluoro-2,4-dinitrobenzene with respect to the lysine residues. This procedure was carried out twice on the same polymer. Amino acid analysis of the product revealed that less than 1% of the lysine residues had free ε-amino groups. The approximate molecular weight of the polymer was determined by the method of Whitaker (1963) using ribonuclease and trypsin as molecular weight standards. The average molecular weight was approximately 20,000.

Dinitrophenylated bovine γ-globulin was prepared according to Eisen *et al.* (1953). It contained 38 moles of DNP radical/mole of globulin.

Fractional Absorption Experiment. This method is based on the removal of fractions of the antibody population from antiserum and estimation of the average K_A of the population before and after fractionation. An immunoabsorbent was prepared by the method of Axen *et al.* (1967). For each absorption experiment an aliquot of the product was suspended with stirring in the serum for 1 hr at 37° and removed by centrifugation. Equilibrium dialysis was carried out on the supernatant remaining after each fractionation step.

DEAE-Sephadex Column Method for Determining Charge Heterogeneity. The methods of Sela and Mozes (1966) were followed exactly.

Results

Representative titers of anti-DNP antibody in sera from rabbits immunized with the defined-sequence polymer are presented in Table I. About 15% of the rabbits gave relatively high titers (0.5–0.1 mg of antibody/ml of serum). The remainder gave lower titers. Figure 1 shows the temporal pattern of the average binding energies observed in rabbits immunized with the three different antigens; 16 days after immunization, antibodies to dinitrophenylated bovine γ-globulin exhibited an average K_A of 1–9 × 10^5 M^{-1} (Eisen and Siskind, 1964). This value increased with time. In sharp contrast to this, antibodies directed against the defined-sequence polymer showed an initial average K_A of 2–5 × 10^9 M^{-1}. An increase in K_A with time was *not* detected in this case, the average K_A remaining constant for periods in excess of 6 months. The pattern observed with the random copolymer was intermediate. The earliest average K_A was 1 × 10^8 M^{-1}. There was a rise in K_A over several weeks but this was only about one order of magnitude.

[1] The exact ratio vary with each lot of *N*-carboxyanhydrides employed depending upon the content of undecomposed *N*-carboxyanhydride.

TABLE II: The Average K_A of Antibody Populations in Rabbits Immunized with Varying Doses of the Defined-Sequence Polymer [ε-DNP-L-Lys-(D-Ala-L-Ala)$_5$]$_{10.2\ av.}$

Animal	Dose (μg)	Weeks	$K_A \times 10^9$	Weeks	$K_A \times 10^9$
24	50	2.5	0.12	10	0.14
25	50	2.5	6.2	9	1.9
26	50	2.5	4.0	10	6.7
	50	2.5	0.5	10	0.66
28	100	2.5	3.9	10	1.0
29	100		3.0		4.4
Av of 10 expt	1000	2.50	2.6	20.0	5.6
31	5000	2	7.0	10	
32	5000	2	8.2, 6.9	10	6.9
33	5000	2	2.2	10	3.7

TABLE III: Association Constants and Heterogeneity "*a*" Values on Early and Late Sera of Ten Rabbits Immunized with the Defined-Sequence Polymer.

Animal	Weeks after Immunization	$K_A \times 10^9$	"*a*"	Weeks after Immunization	$K_A \times 10^9$	"*a*"
1	3.0	9.0	0.99	22	8.3	0.80
2	3.0	4.7	0.83	29	6.9	1.14
3	2.5	5.5	0.90	19	6.2	0.81
4	2.5	10.8	0.88	13	8.6	0.90
5	2.5	6.6	0.96	28	7.7	0.89
6	2.5	0.12	0.66	24	6.6	0.75
7	2.5	4.0	0.85	28	4.7	0.87
8	2.5	0.44	1.00	10	0.12	1.02
9	2.5	3.8	0.83	10	5.7	
10	2.5	0.12	1.10	15	0.98	0.87

Most of the equilibrium dialysis experiments were performed with antibodies obtained from rabbits which were immunized with 1.0 mg of defined-sequence polymer. This dose is equivalent to 60% of the dose of dinitrophenylated bovine γ-globulin (based on hapten content) used by Eisen and Siskind (1964). These authors produced a maximal rise in average association constants with this dose. Therefore, to test the possibility that the reduction in temporal heterogeneity was due to the particular dose of polymer used, immunizing doses ranging from 50 μg to 5 mg were employed. Doses below 50 μg gave no antibody response and a sufficient quantity of the polymer was not available to test doses greater than 5 mg. Table II shows that within these dose limits, the average K_A was initially high and remained high thereafter. As a test for the presence of antibodies with lower association constants, a number of equilibrium dialyses were performed using higher concentrations of hapten. No antibodies with low affinity for hapten were detected.

Because of the marked restriction in temporal heterogeneity of binding observed with antibodies to the defined-sequence polymer, the binding data obtained from ten rabbits immunized with this antigen were analyzed by the Sips equation (Sips, 1948) and the heterogeneity index ("*a*" value) was thereby obtained. Table III lists the "*a*" values obtained; it should be noted that these values do not show a consistent pattern, some values being 1.0 or close to 1.0, while others range from 0.66 upward. It seems unlikely that such diverse values are due to a single antibody population.

Antisera obtained at comparable intervals following injection of individual rabbits with one of the three antigens emulsified in complete Freund's adjuvant were subjected to immunoelectrophoresis. Representative results are shown in Figure 2. Nearly identical γG precipitin arcs were obtained. However, following the addition of ^{125}I-labeled dinitrophenylated [^{125}I]bovine γ-globulin, there was a considerable difference in the pattern of antigen binding observed. Antisera obtained early in the course of immunization with dinitrophenylated bovine γ-globulin showed antigen binding to almost the entire γG precipitin arc; antisera obtained later in the course showed more intense binding

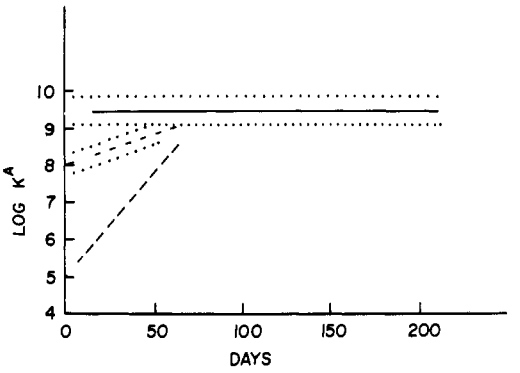

FIGURE 1: Plot of log K_A *vs.* time in days following immunization. Values for 19 rabbits injected with 1.0 mg of defined-sequence polymer, 42 data points from 17 to 196 days are represented by ————; values for 7 rabbits injected with 1.0 mg of "random" *N*-carboxyanhydride polymer, 14 data points 17–93 days are represented by -----; and values for rabbits injected with 5.0 mg of dinitrophenylated bovine γ-globulin, 9 data points (recalculated from Eisen and Siskind, 1964) are represented by — — —. All K_A values were determined by equilibrium dialysis against [^{3}H]DNP-lysine of specific radioactivity 9000 Ci/mole or in suitable dilution. All data lines, one standard deviation from the mean, are calculated on the PDP9 or IBM 360 computer using a FORTRAN LV/CYTOS system, to determine best fit by least-squares analysis.

TABLE IV: Absorption Studies.[a]

Absorption Step	% of Total Antibody Left in Soln	Av K_A of Antibody Population in Soln
Antibodies to Dinitrophenylated Bovine γ-Globulin		
Before absorption	100	2.7×10^8
1	54	2.0×10^8
2	21	1.2×10^8
3	19	1.5×10^7
Antibodies to the Defined-Sequence Copolymer		
Before absorption	100	6.8×10^8
1	43	6.1×10^8
2	28	4.2×10^8
3	6	2.9×10^8

[a] Partial absorption experiment: a comparison between the partial absorption patterns of antibodies to dinitrophenylated bovine γ-globulin and the defined-sequence of copolymer. At each step a fraction of the antibody population present was absorbed and removed. On an aliquot of the supernatant solution, the concentration of antibody, the average K_A and the heterogeneity index were estimated by equilibrium dialysis.

of antigen to the entire γG precipitin arc. Antisera obtained early in the course of immunization with the defined-sequence polymer showed antigen binding to the cathodal end of the γG precipitin arc; and although the intensity of binding increased with antisera obtained later in immunization, the distribution of binding tended to remain confined to the cathodal region. Similar results were obtained with early and "late" antisera from rabbits injected with the "random" *N*-carboxyanhydride polymer and with a few antisera obtained early (less than 3 weeks) in the course of immunization with dinitrophenylated bovine γ-globulin.

In preparation for the fractional absorption experiments, two rabbit antisera, an antidinitrophenylated bovine γ-globulin antiserum and an antiserum to the defined-sequence polymer, were sought which had similar binding affinity for ε-DNP-lysine. The anti-dinitrophenylated bovine γ-globulin antiserum used was obtained 5 weeks after immunization, and the other antiserum was obtained 12 weeks after immunization. The association constant of the anti-defined sequence polymer antiserum was lower than most such antisera tested. Sequential absorption of the anti-dinitrophenylated bovine γ-globulin antiserum (Table IV) revealed subpopulations of antibodies with average K_A values ranging from 1.5×10^7 to 2.7×10^8 l. M^{-1}. Antiserum to the defined-sequence polymer exhibited subpopulations with average K_A values ranging from 2.9×10^8 to 6.8×10^8 l. M^{-1}. These results suggest that on the basis of binding energies, there is somewhat less heterogeneity in the antibodies to the defined-sequence polymer.

Sela and Mozes (1966) demonstrated that specific antibodies to DNP varied in their net charge, depending upon the charge of the carrier of this determinant. Positively charged amino acid copolymers resulted in negatively charged antibodies, as well as the converse. Fractionation into two arbitrary categories by DEAE-Sephadex was applied to antibodies directed against the defined-sequence polymer, to the random polymer as well as to dinitrophenylated bovine γ-globulin. Table V details these results and clearly indicates that antibodies to the defined-sequence polymer are confined solely to one of these arbitrary classes, while antibodies to the "random" polymer and to dinitrophenylated bovine γ-globulin are distributed between these classes.

Discussion

There have been a number of studies indicating that restriction of antibody heterogeneity has been achieved either fortuitously or by the use of special antigens (for review, see Haber, 1968). Demonstration that a single protein species is present has proved difficult. In other studies, as well as in ours, inferences on homogeneity have been drawn from properties such as binding curves (Nisonoff *et al.*, 1967), charge distribution (Sela and Mozes, 1966), and light-chain patterns (Kantor *et al.*, 1963; Brenneman and Singer, 1968). There can be no doubt that a variety of different antigens such as pneumococcal polysaccharides (Miller *et al.*, 1967), some monosubstituted proteins (Brenneman and Singer, 1968), and certain peptides (Haber *et al.*, 1967) can produce modification in the antibody response, although at present no simple theory ties

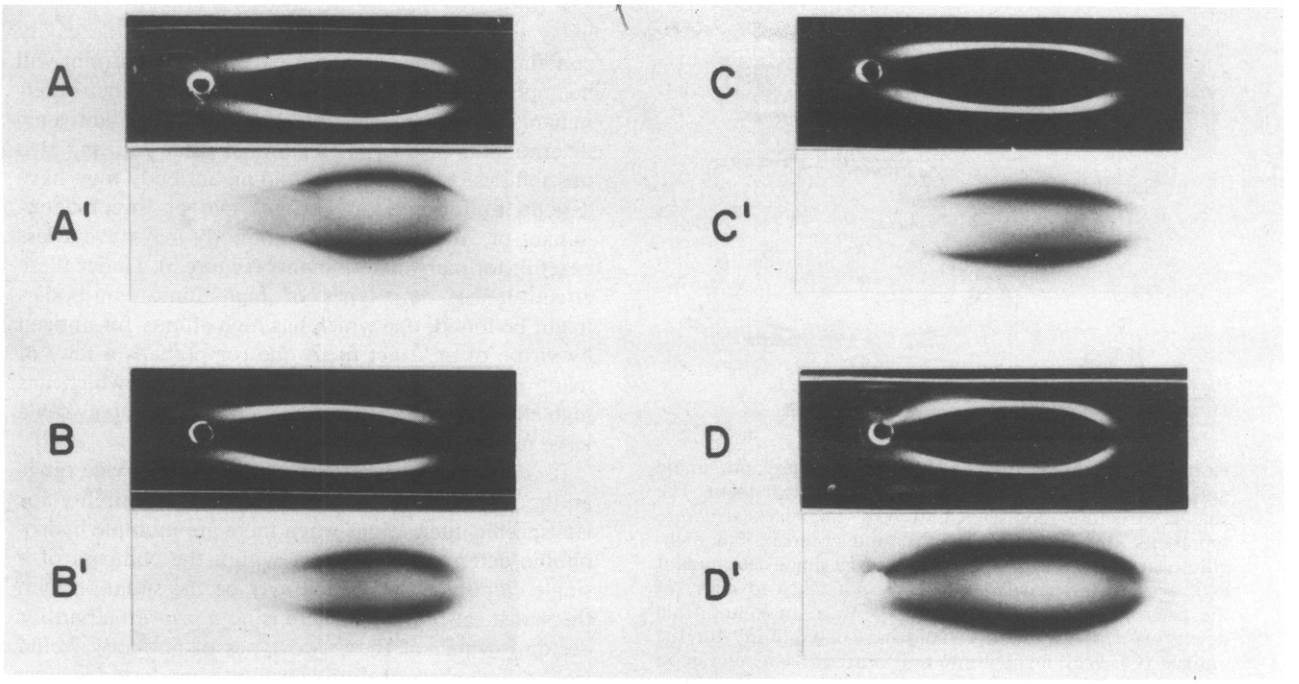

FIGURE 2: Paired immuno- (upper) and radioimmunoelectrophoretic (lower) patterns of rabbit antidefined-sequence polymer [ϵ-DNP-L-Lys(D-Ala-L-Ala)$_5$]$_{10.2}$ and antidinitrophenylated bovine γ-globulin antiserums. Antiserum to defined-sequence polymer obtained 2.5 (A) and 12 (B) weeks after start of immunization was placed in wells as shown; antiserum to dinitrophenylated bovine γ-globulin obtained at the same times was placed in wells C and D. Precipitin patterns were developed with sheep antiserum to rabbit γ-globulin placed in adjoining troughs. Anode to left of figure.

together their mechanisms of action. The studies presented here are a more detailed examination of the antibody response to one such agent, a defined-sequence DNP-amino acid polymer and to its less ordered structural analog.

This defined-sequence polymer [(D-Ala-L-Ala)$_5$-ϵ-DNP-L-Lys]$_{10.2\,av}$ was designed to test the hypothesis that one important factor in the heterogeneity of the antibody response is the complexity of the antigenic structure. Previous studies have demonstrated that when antigens such as monosubstituted DNP ribonuclease and polylysine which has been randomly substituted with DNP are used as antigens, the antibody response is still complex, although Brenneman and Singer (1968) have produced antibodies with a single light-chain type using a mono-DNP-papain antigen. The defined-sequence polymer used in the present study is still not a simple antigen, but introduces a relatively uniform environment for single haptens which previous antigens have not possessed. The "statistical" *N*-carboxyanhydride copolymer is a useful control because only the order, but not the composition of the molecule is changed. The dinitrophenylated bovine γ-globulin antigen however represents the same hapten in a much more polymorphic environment. Studies of the immune response of rabbits injected with these antigens indicate that as increasing degrees of "order" are imposed on the haptenic environment, the antibody response becomes less complex. These conclusions are based on experiments involving the dispersion of molecular charge distribution and on the variations in magnitude of noncovalent interactions between the hapten and the antibody populations produced. According to these criteria, antibodies to the defined-sequence polymer are less heterogeneous than the population of antibodies produced in response to a DNP-protein conjugate, the antibodies to the "statistical" polymer occupy an intermediate position. The methods used do not indicate whether the reduction in functional heterogeneity is accompanied by a reduction in structural heterogeneity.

It was previously considered that an increase in average intrinsic association constant, K_A, with time after immunization was a constant feature of all anti-hapten antibody responses. A surprising finding in the present study was the lack of temporal heterogeneity of average K_A's of the anti-defined sequence polymer antibody population, *i.e.*, a high binding constant was obtained in experiments with early antisera, and this value did not change significantly when determined with antisera obtained later in the course of the immune response. Similarly, in sequential studies performed with antisera to the "statistical" polymer, there was only a relatively small rise in average K_A extending over one order of magnitude. It is possible that the immune response to the defined-sequence polymer "matures" very rapidly. According to this view, antibodies to the defined-sequence polymer might have lower binding affinities for the hapten prior to the time (2.5 weeks after the start of immunization) when the methods used permitted an accurate measurement of binding energies. This possibility cannot be further explored with present methods.

Paul *et al.* (1967) have suggested that maturation of the immune response depends upon a cellular selection process based on the ability of sensitive cells to bind

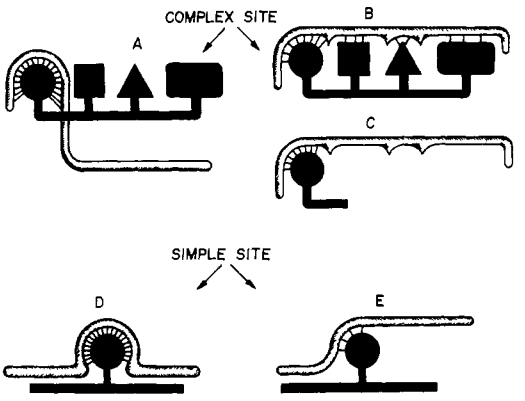

FIGURE 3: Schematic diagram relating complex and simple antigenic sites to the antibodies formed against them. The shaded structure represents antibody; the black structure represents the antigenic site. With a complex site high-affinity antibody may be directed against a single determinant (A), or with lesser degrees of correspondence to many of the determinants (B). When tested with a single antigenic determinant, the antibody would have low affinity for the antigen (C). With a single site only antibody having a good conformational correspondence (D) will form high-affinity antibodies. Antibodies having other conformations are likely to have very low affinity for the determinant (E).

antigen to their surface. Cells producing high-affinity antibodies are postulated to bind antigen with high-affinity and low-affinity antibody-producing cells bind antigen with low affinity. Binding of antigen to the cell is postulated to stimulate cell division and an increase in antibody production. Early in the immune response, when the concentration of antigen is high, cells capable of forming both high- and low-affinity antibodies are stimulated. Later, when the concentration of antigen is low, only cells capable of producing high-affinity antibodies are stimulated. As a result, the average binding energy of the late antibody population rises.

Despite the wide dose range of defined-sequence polymer administered in the present experiments, it is possible that the dose of effective immunogen attained at the surface of sensitive cells was nevertheless sufficient only for the stimulation of cells capable of producing high-affinity antibodies. The defined-sequence polymer consists in part of D- and L-amino acid residues. Such polymers are known to be degraded more slowly than polymers consisting of L-amino acids. Hence the concentration of effective immunogen may have been limited by the rate at which the immunized animal can process the antigen. Other unique, unidentified properties of the defined-sequence polymer may also be involved in determining the response of cells capable of producing high-affinity antibodies.

The influence of the hapten and its environment on the generation of binding energy will be considered next. The size of an antibody combining site is such that it may be complementary to a small hapten plus a number of surrounding determinants (Kabat, 1966; Richards *et al.*, 1967). Since the site has a definite location on the antibody molecule (Feinstein and Rowe, 1965; Valentine and Green, 1967), there is likely to be a limitation to the volume of the cavity containing the site. As a consequence, a constraint will be imposed on the specificity of antibodies. When determinants are close together, or when many antigenic determinants crowd a region of the antigen, two possibilities exist. A corresponding antibody may have a good fit for one (or perhaps two or three) determinant or, alternatively, the antibody may have a less exact fit for many determinants (Figure 3). Under these circumstances two types of high-affinity antibodies might be found, one which has high affinity for antigen by virtue of an exact fit to one (or perhaps a few) of many determinants and a second antibody which has high affinity as a result of additive interaction between a large number of antigenic determinants.

It would perhaps be expected that such wide-range antibodies would present an increased possibility for less specific interactions when there are multiple hydrophobic determinants present within the compass of a single combining site. Such may be the situation with the statistical polymer. There is no *a priori* method for deciding which of these two types of antibody would yield a higher association constant when tested against the complete antigen. It is however assumed that the above description would apply to the hypothetical antibody-like receptors on sensitive cells.

Parker *et al.* (1966) have studied the antibodies produced against DNP-peptide–protein complexes. It was shown that when the DNP determinant is placed in a relatively ordered but still complex environment containing many different haptenic amino acid residues different antibodies are formed which have specificity either for the DNP-aminoalkyl group or for the peptide plus the DNP-aminoalkyl group. The maturation phenomenon is not dependent upon replacement of peptide-directed antibodies by DNP-directed antibodies or *vice versa*. Fluorometric titrations of antibodies evoked by a DNP-tetrapeptide–protein complex were carried out as a function of time after immunization. The ratio of $-\Delta F^\circ$ for the peptide over $1/-\Delta F^\circ$ for DNP-lysine showed no consistent change with time. This indicates that there is no gross shift in the relative proportions of single determinant antibodies and anti-peptide antibodies in the system studied. These data also show that both types of antibody contribute to the maturation effect.

These authors further observed that following the administration of 2.0 mg of DNP-peptide–protein complex, the antibodies produced showed an increase in binding affinity for ε-DNP-L-lysine with time after immunization. The response was small, being less than one order of magnitude over an 8-week period. This rise in affinity was considerably smaller than that observed by Eisen and Siskind (1964) using 5.0 mg of dinitrophenylated bovine γ-globulin as antigen. It is possible that the more ordered environment of the DNP-peptide–protein complex contributed less binding energy and hence led to the activation of fewer cells capable of producing high-affinity antibodies than did the randomly oriented environment of the DNP-protein conjugate.

It is possible that the defined-sequence polymer may

TABLE V[a]

Antigen	Sample	Total Globulin in Peak (mg)	Total Anti-DNP Antibody in Peak (mg of Antibody Protein)	% of Total Anti-DNP Antibody	K_A Av	Total Globulin in Peak (mg)	Total Anti-DNP Antibody in Peak (mg of Antibody Protein)	% of Total Anti-DNP Antibody	K_A Av
Dinitrophenylated Bovine γ-globulin	1	31.6	0.0580	83.2	2.8×10^9	102.8	0.0098	16.8	1.4×10^9
	2	6.5	0.0117	61.5	3.0×10^9	19.4	0.0045	38.5	4.9×10^9
	Mean			72.3				27.7	
Random copolymer	3	11.8	0.0126	87.3	4.0×10^9	27.4	0.0016	12.7	2.9×10^9
Defined-sequence polymer	4	22.3	0.0105	100	5.1×10^9	29.6	*b*	*b*	*b*
	5	16.2	0.0201	100	6.3×10^9	26.4	*b*	*b*	*b*
	Mean			100					

[a] Rabbit globulins containing antibodies to dinitrophenylated bovine γ-globulin, the "random" *N*-carboxyanhydride polymer (ε-DNP-Lys:DL-Ala, 1:10) and the defined-sequence polymer $[\varepsilon\text{-DNP-L-Lys-(D-Ala-L-Ala)}_5]_{10\cdot 2\ av.}$ obtained 12 weeks after immunization have each been separated on DEAE-Sephadex G-50 into two populations (peak I and peak II) on the basis of charge differences according to Sela and Mozes (1966). Each peak has been assayed for γ-globulin content spectrophotometrically. The amount of antibody to the DNP group and its binding energy have been determined by equilibrium dialysis. [b] No binding activity detected.

give rise to the same phenomenon in a more extreme form. Poly-DL-alanine is known to be only weakly antigenic and consequently it may contribute little binding energy to stimulate a sensitive cell. Consequently in animals immunized with the defined-sequence polymer, only cells specific for the DNP determinant and capable of binding this determinant with sufficient energy to stimulate the cell will be involved in the immune response. The range of binding energy in this group of cells may be restricted and consequently a rise in affinity of the antibodies produced by such cells over a period of time, may be not appearant as measured by the methods employed. In other words, if the antigenic site is composed of relatively few strongly interacting units, steric considerations will rule out all but a few high-energy interactions with wide-range antibodies and these are statistically more likely to have a smaller range of association constants. The "statistical" polymer and the defined-sequence polymer are identical in composition, but the former may allow two or more DNP groups to be adjacent to one another. Consequently, in animals immunized with the "statistical" polymer cells specific for a single DNP determinant, as well as cells specific for varying numbers of DNP groups may be stimulated. It is likely that the binding energies of these varying groups of cells would be different and consequently maturation of the immune response with a rise in binding energy of the antibodies produced might be expected and indeed, a rise in K_A of one order of magnitude was observed.

These results suggest that the degree of order in the haptenic environment is important and that it may be profitable to map the combining region of anti-DNP antibodies by studying the interaction energies of peptides which have hydrophobic residues introduced at varying distances from the DNP-aminoalkyl group. This is analogous to the mapping of the active site of papain in the studies of Schechter and Berger (1968).

While the results of these studies do not indicate the mechanism by which restriction in complexity is effected, it is clear that increasing order in the antigen results in decreasing heterogeneity of the antibody response.

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