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Structural Control of Immunogenicity

IV. RELATIVE SPECIFICITY OF ELICITATION OF CELLULAR IMMUNE RESPONSES AND OF LIGAND BINDING TO ANTI-HAPTEN ANTIBODY AFTER IMMUNIZATION WITH MONO-ε-DNP-*NONA*-L-LYSINE*

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Summary. An evaluation of the specificity of antigen binding receptors possessed by cells involved in cellular immune responses and by cells in the antibody synthesizing line was made in order to determine to what extent each cell type could distinguish between closely related mono-ε-DNP-*oligo*-L-lysines. The antigen binding receptors of cells involved in cellular immune responses were studied by the elicitation of delayed hypersensitivity reactions *in vivo* and by the stimulation of DNA synthesis by lymph node cells *in vitro* using 1-ε-DNP-*nona*-L-lysine and 9-ε-DNP-*nona*-L-lysine. These experiments demonstrated that closely related compounds could be distinguished by this cell population. On the other hand, serum antibody from animals immunized with 1-ε-DNP-*nona*-L-lysine did not regularly distinguish between various mono-ε-DNP-*oligo*-L-lysines of differing immunogenicity or between 1-ε-DNP-*nona*-L-lysine and 9-ε-DNP-*nona*-L-lysine. Serum antibody specificity is assumed to reflect the specificity of the receptors possessed by the precursors of antibody producing cells. Thus, it appears likely that the cells involved in cellular immune responses or functioning as 'helper' cells in the stimulation of antibody synthesis by other cells are capable of determining the immunogenicity of a compound. On the other hand, precursors of antibody producing cells, at least in primed animals and in the mono-ε-DNP-*oligo*-L-lysine response, do not appear to make distinctions between peptides of differing immunogenicity.

INTRODUCTION

In previous papers of this series (Stupp, Paul and Benacerraf, 1971a,b), we have characterized the cellular and humoral immune response of guinea-pigs to a series of mono-ε-DNP-*oligo*-L-lysines and have confirmed the findings of Schlossman and his associates (Schlossman, Yaron, Ben-Efraim and Sober, 1965; Schlossman, Ben-Efraim,

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* A preliminary account of some of the experiments has been presented (Paul, Stupp, Siskind and Benacerraf, 1970).

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Yaron and Sober, 1966) concerning the marked change in immunogenicity as the length of the oligopeptide chain is increased from 6 to 8. Further, we demonstrated that priming for anamnestic anti-hapten antibody responses to DNP-poly-L-lysines also requires the use of the larger peptides for detectable effects.

One possible mechanism by which distinctions are made between immunogenic and non-immunogenic (or marginally immunogenic) DNP-oligomers is through the specificity exhibited by antigen-binding receptors possessed by precursors of antibody forming cells or, alternatively, by the receptors present on cells participating in cellular immune responses or acting as 'helper' cells in humoral immune responses (Mitchison, Rajewsky and Taylor, 1970; Paul, Katz, Goidl and Benacerraf, 1970). In the previous paper (Stupp *et al.*, 1971b), it was shown that 'helper' cells very likely can make a distinction between *nona*-L-lysine and *tetra*-L-lysine either through their antigen-binding receptors or through another unspecified mechanism.

In the current paper, we have attempted to evaluate receptor specificity of cells participating in cellular immune responses on the basis of cross reactivity in the elicitation of delayed hypersensitivity reactions and in the *in vitro* stimulation of DNA synthesis by lymph node cells. Inferential data on receptor specificity of the precursors of antibody forming cells has been obtained by an analysis of the equilibrium binding properties of anti-DNP antibody produced by guinea-pigs immunized with 1-ε-DNP-*nona*-L-lysine. The latter is felt to be a reasonable measure of the specificity of the receptors present on precursors of antibody forming cells as it is most likely that such individual cells have a receptor with equivalent binding properties to the antibody which is secreted by the progeny of the stimulated cell (Mitchison, 1967; Siskind and Benacerraf, 1969). The data obtained are consistent with the notion that receptors possessed by cells involved in cellular immune responses distinguish between alternate mono-ε-DNP-derivatives of *nona*-L-lysine whereas, in primed animals, the receptors of the precursors of anti-hapten antibody forming cells are not as discriminative.

MATERIALS AND METHODS

Mono-ε-DNP-*oligo*-L-lysines were prepared as described in the first paper of this series (Paul and Kask, 1971). DNP-bovine fibrinogen (DNP-BF), DNP-guinea-pig albumin (DNP-GPA), a DNP derivative of a copolymer of L-glutamic acid and L-lysine (GL, 60 per cent glutamic acid, 40 per cent lysine; molecular weight = 115,000) and a DNP derivative of poly-L-lysine (PLL, 550 lysyl residues/mole) were prepared by methods analogous to those described by Benacerraf and Levine (1962). These compounds contained an average of 138, 40, 20 and 62 moles of DNP per mole of carrier, respectively.

Hartley and strain 2 guinea-pigs were immunized by injection into the foot pads of 100 μg of DNP conjugate, emulsified in Freund's complete adjuvant (CFA). The adjuvant contained 2 mg *M. tuberculosis* per ml of oil. Another group of Hartley guinea-pigs was immunized with a Freund's incomplete adjuvant emulsion of 1-ε-DNP-*nona*-L-lysine; 2 weeks later, an intradermal injection of 50 μg of 1-ε-DNP-*nona*-L-lysine was administered and the animals bled 4 weeks later.

Delayed hypersensitivity was evaluated by the intradermal injection of either 10 or 50 μg of antigen, dissolved in phosphate buffered saline (PBS; 0·15 M NaCl, 0·01 M potassium phosphate, pH 7·6). The skin site was observed at 2 hours and at 24 hours and evaluated in terms of the average diameter of erythema. In each experiment, the response

of immunized animals was compared with that of control animals immunized only with Freund's complete adjuvant.

Poly-L-lysine (PLL) gene status was evaluated, after the completion of an experiment, by active immunization with 100 μg of GL emulsified in Freund's complete adjuvant. Those animals manifesting delayed hypersensitivity to GL were classed as PLL+ (Kantor, Ojeda and Benacerraf, 1963).

Affinity of anti-DNP antibodies for mono-ε-DNP-*oligo*-L-lysines and for ε-DNP-L-lysine was measured by fluorescence quenching analysis (Velick, Parker and Eisen, 1960; Eisen and Siskind, 1964). Anti-DNP antibody was specifically purified from sera by the technique of Farah, Kern and Eisen (1960). One or 2 ml of purified anti-DNP antibody (10–30 μg/ml) in PBS was pipetted into a cuvette and fluorescence measured in an Aminco-Bowman spectrophotofluorometer at 20°. Wavelength of exciting light was 2800 Å and the intensity of the emitted light was determined at 3300 Å. Ten to 50 μl aliquots of DNP-ligand were added and fluorescence determined after each addition. The data were calculated according to the Sips equation (Sips, 1948; Karush, 1962), as previously described, utilizing a value of 100 per cent for maximum quenchable fluorescence (Siskind, Paul and Benacerraf, 1966).

Affinities of anti-DNP antibodies were also evaluated by determining the effect of dilution on antigen-binding capacity in a Farr ammonium sulphate precipitation assay (Farr, 1958; Green, Benacerraf and Stone, 1969). Association constants (K) can be calculated according to the following relation (Paul, 1971):

$$K = \frac{[B/F)_x \cdot R \cdot B_i] - [(B/F)_i \cdot B_x]}{(1\text{-}R)\ (B_i)\ (B_x)}$$

in which

B_i = bound ligand concentration at standard condition; B_x = bound ligand concentration at test condition; $(B/F)_i$ = ratio of bound to free ligand at standard condition; $(B/F)_x$ = ratio of bound to free ligand at test condition; R = ratio of antigen binding capacity at test condition to antigen binding capacity at standard condition; antigen binding capacity is serum dilution required for a given B/F multiplied by B under those conditions.

In effect, the relation gives the equilibrium constant of a homogeneous population of molecules which displays a given R at a given B_x and $(B/F)_x$. A plot of log K versus log R reveals, therefore, certain information concerning the degree of heterogeneity within the population. Of course, in heterogeneous collections of molecules, those low affinity antibodies which are not titrated are, in essence, ignored.

In practice, the technique requires the determination of antigen binding capacities of antiserum at a given set of ligand concentrations. The standard conditions utilized here were $(B/F)_i = 0{\cdot}5$ (33·3 per cent ligand bound) and $B_i = 0{\cdot}167 \times 10^{-6}$ M; the test conditions were $(B/F)_x = 0{\cdot}5$; $B_x = 0{\cdot}167 \times 10^{-7}$ M; $0{\cdot}167 \times 10^{-8}$ M; and $0{\cdot}167 \times 10^{-9}$ M.

The determination of antigen binding capacity involves addition of 10 μl of serum or of diluted serum (all dilutions being made in 1:5 normal guinea-pig serum) to 10 μl of [^{3}H]-ε-DNP-L-lysine (10 Ci/mM); after overnight incubation at 4°, 20 μl of saturated ammonium sulphate is added and the mixture centrifuged 30 minutes later. Radioactivity in an aliquot of supernatant is measured in a liquid scintillation spectrometer utilizing a scintillation solvent consisting of 25 parts toluene: 5 parts Bio-Solv 3 (Beckman Instrument Co., Fullerton, California): 1 part Liquiflor (New England Nuclear Corp.,

Boston, Mass.). Percent bound ligand is equal to

$$100\left(1-\frac{\text{Supernatant radioactivity with antisera}}{\text{Supernatant radioactivity with normal sera}}\right).$$

Dilution of serum required for 33·3 per cent bound ligand is determined from a plot of per cent binding versus log of dilution.

Specificity of anti-DNP antibody was evaluated by the relative ability of a series of DNP ligands to inhibit the binding of [^{3}H]-ε-DNP-L-lysine to anti-DNP antibody. Using an ammonium sulphate precipitation technique, percent inhibition was calculated as follows:

$$100\times\frac{\text{Supernatant radioactivity in presence of inhibitor}-\text{supernatant radioactivity in absence of inhibitor}}{\text{Total radioactivity}-\text{supernatant radioactivity in absence of inhibitor}}$$

Free concentration of inhibitor was equal to total inhibitor concentration minus the concentration of [^{3}H]-ε-DNP-L-lysine freed by the action of inhibitor (numerator of above expression). A plot of per cent inhibition versus log free ligand concentration enabled a determination of free ligand concentration required for 50 per cent inhibition. Difference in standard free energy of binding ($\triangle(\triangle F°)$) of a given ligand and of the index ligand (ε-DNP-L-lysine) is:

$$-\ln\frac{\text{Free concentration of index ligand required for 50 per cent inhibition}}{\text{Free concentration of given ligand required for 50 per cent inhibition}}\times R\times T$$

where R is the gas constant (1·987 cal/degree mole) and T, absolute temperature.

Stimulation of DNA synthesis in lymph node cells from immunized guinea-pigs by specific antigens was determined as previously described (Paul, Siskind and Benacerraf, 1968). Lymph node cell suspensions were prepared, sterilely, from guinea-pigs immunized 2–3 weeks previously with 100 μg of mono-ε-DNP-*oligo*-L-lysine in Freund's complete adjuvant. Five × 10^6 cells were suspended in 1·4 ml of tissue culture medium RPMI 1640 (Grand Island Biological Co., Inc.) supplemented with glutamine (2 μM/ml), penicillin (100 units/ml) and 10 per cent heated strain 13 guinea-pig serum. Antigen, in 0·1 ml of tissue culture medium, was then added; cells were incubated at 37° in a humid atmosphere of 5 per cent CO_2–95 per cent air for 24 hours. One μCi of [^{3}H]-thymidine (2 Ci/mM; New England Nuclear Corp., Boston, Mass.) was added and incubation continued for an additional 24 hours. Radioactivity incorporated into perchloric acid precipitable material was measured as described previously (Foerster, Lamelin, Green and Benacerraf, 1969).

RESULTS

1. SPECIFICITY OF CELLULAR IMMUNE RESPONSES

A series of Hartley and strain 2 guinea-pigs were immunized with 100 μg of 1-ε-DNP-*nona*-L-lysine, emulsified in Freund's complete adjuvant containing *M. tuberculosis* (2 mg/ml). Two to 4 weeks later the animals were skin tested with 50 μg of 1-ε-DNP-*nona*-L-lysine and of 9-ε-DNP-*nona*-L-lysine and with 10 μg of DNP-PLL and of PLL. The mean diameter of the responses at 24 hours in each of the PLL+ Hartley guinea-pigs and in each of the strain 2 guinea-pigs is presented in Table 1. In every case the response to the immunizing peptide, 1-ε-DNP-*nona*-L-lysine, was greater than that to 9-ε-DNP-*nona*-L-

TABLE 1

SPECIFICITY OF ELICITATION OF DELAYED HYPERSENSITIVITY IN GUINEA-PIGS IMMUNIZED WITH 1-ε-DNP-*nona*-L-LYSINE

Strain	Animal No.	Average diameter of delayed skin reaction (mm) Eliciting antigen: 1-ε-DNP-*nona*-L-lysine (50 μg)*	9-ε-DNP-*nona*-L-lysine (50 μg)*	DNP-PLL (10 μg)*	PLL (10 μg)*
Hartley	1	14	6	12	5
	2	17	7	15	4
	3	16	12	12	6
	4	8	6	9	10
	5	16	12	14	7
	6	14	11	15	9
	Mean	14·1	9·0	12·8	6·8
2	7	11	9	12	4
	8	17	9	19	5
	9	5	3	9	0
	10	8	6	10	3
	11	8	5	13	2
	12	13	10	17	5
	Mean	10·3	7·0	13·3	3·2

* Dose of antigen used for elicitation.

lysine. However, in almost every instance, a significant response to 9-ε-DNP-*nona*-L-lysine was obtained. In each of the strain 2 guinea-pigs and in two of the six Hartley guinea-pigs, the response to 10 μg of DNP-PLL exceeded, albeit by a small margin, the response to 50 μg of the immunizing peptide. The response to PLL was usually of small magnitude. Indeed, in strain two guinea-pigs, the response to PLL was usually insignificant.

Studies of the specificity of stimulation of DNA synthesis in lymph node cell cultures, performed 2–3 weeks after immunization, yielded generally similar results (Table 2).

TABLE 2

SPECIFICITY OF STIMULATION OF DNA SYNTHETIC RESPONSES IN LYMPH NODE CELL CULTURES FROM GUINEA-PIGS IMMUNIZED WITH EITHER 1-ε-DNP-*nona*-L-LYSINE OR 9-ε-DNP-*nona*-L-LYSINE

		Immunizing peptides: 1-ε-DNP-*nona*-L-lysine Hartley	1-ε-DNP-*nona*-L-lysine Strain 2	9-ε-DNP-*nona*-L-lysine Hartley
No. of cultures		8	7	3
Stimulant	Conc. (μg/ml)			
1-ε-DNP-*nona*-L-lysine	1	3·6*	3·6	1·2
	10	6·0	3·5	2·5
9-ε-DNP-*nona*-L-lysine	1	1·3	0·9	2·6
	10	3·0	1·4	3·7
DNP-PLL	1	2·5	2·8	1·5
	10	5·6		2·6
PLL	1	1·0	1·1	1·0
	10	1·3		0·9

*Ratio of incorporation of [^{3}H]thymidine in cultures incubated with indicated antigen to incorporation in cultures incubated without antigen. Result is the mean of the indicated number of independent experiments.

Guinea-pigs were immunized as for the delayed hypersensitivity experiments described above. The response of cultures obtained from both PLL+ Hartley and strain 2 guinea-pigs distinguished between the immunizing and the alternate mono-ε-DNP-*nona*-lysine. The response to 10 μg/ml of 9-ε-DNP-*nona*-L-lysine of cell cultures obtained from Hartley guinea-pigs immunized with 1-ε-DNP-*nona*-L-lysine was similar to that observed to 1 μg/ml of the immunizing peptide. Cultures from strain 2 guinea-pigs immunized to 1-ε-DNP-*nona*-L-lysine failed to display significant stimulatory responses to 9-ε-DNP-*nona*-L-lysine. Cultures from Hartley guinea-pigs immunized to 9-ε-DNP-*nona*-L-lysine were stimulated to a greater degree by the immunizing peptide than by the alternate mono-ε-DNP-*nona*-L-lysine, although the latter did give a definite response.

DNP-PLL stimulated cultures in each instance and, on the average, slightly less well than the immunizing peptide. However, in some cases DNP-PLL stimulated DNA synthesis to a greater degree than did the immunizing peptide. PLL alone failed to cause significant stimulatory responses.

Thus, the cellular immune responses, both delayed hypersensitivity and the stimulation of DNA synthesis by lymph node cells in culture, can distinguish between the alternate mono-ε-DNP-*nona*-L-lysines used in this study although some response is generally obtained with both DNP-*nona*-lysines.

2. SPECIFICITY OF ANTI-DNP ANTIBODY PRODUCED BY GUINEA-PIGS IMMUNIZED WITH 1-ε-DNP-*nona*-L-LYSINE

PLL+ Hartley guinea-pigs were immunized with 100 μg of 1-ε-DNP-*nona*-L-lysine as described above. Four weeks later, the animals were bled and quantitative precipitin analysis with DNP-BF performed on their sera. Anti-DNP antibodies were then purified as described in the Methods section and the affinity of the purified anti-DNP antibody for a series of DNP-ligands measured by fluorescence quenching analysis. Similar measurements were also made on anti-DNP antibodies purified from the serum of guinea-pigs immunized to DNP-GPA. A sample of purified guinea-pig anti-DNP-PLL antibody was the gift of Dr I. Green and of Dr V. Nussenzweig.

The results of these studies are presented in Table 3. The binding characteristics of anti-DNP antibodies produced by different individual guinea-pigs immunized with 1-ε-DNP-*nona*-L-lysine differ. Thus, one preparation (106–1) displays an increasing trend of binding activity as chain length of the ligand is increased. The two mono-ε-DNP-*nona*-L-lysines have the greatest binding activity. Several important points may be noted. There is no difference between the binding of 1-ε-DNP-*nona*-L-lysine and 9-ε-DNP-*nona*-L-lysine, although the cellular responses, as described in Section I, distinguish these compounds. Compounds with little or no capacity to induce cellular immune responses or to prepare for secondary response (such as 1-ε-DNP-*tetra*-L-lysine) bind this anti-DNP antibody only slightly less well than the clearly immunogenic mono-ε-DNP-*nona*-L-lysines.

The other two anti-1-ε-DNP-*nona*-L-lysine preparations bound 1-ε-DNP-*tetra*-L-lysine more energetically than they bound 1-ε-DNP-*nona*-L-lysine. Indeed, they bound ε-DNP-L-lysine as well or better than they bound 1-ε-DNP-*nona*-L-lysine. Thus, the analysis of binding characteristics of these sera provides no support for the thesis that the determination of immunogenicity and the specificity of cellular responses can be simply explained by the specificity of a cell associated anti-DNP antibody of a type similar to that in serum of immunized animals.

TABLE 3
RELATIVE AFFINITY OF ANTI-DNP-ANTIBODIES FOR VARIOUS DNP LIGANDS; IMMUNIZATION WITH MYCOBACTERIAL ADJUVANT

Ligand	Δ(ΔF°)* Kcal/mole				
	Anti-1-ε-DNP-*nona*-L-lysine			Anti-DNP-PLL	Anti-DNP-GPA
	106–1	105–1	105—2		
ε-DNP-L-lysine	O	O	O	O	O
DNP-EACA	> +1·4		+·64	> +1·3	−·25
1-ε-DNP-*di*-lysine	−·58			−·75	
1-ε-DNP-*tri*-lysine				−·62	
1-ε-DNP-*tetra*-lysine	−·63	−·11	−·40	−·74	+·54
4-ε-DNP-*tetra*-lysine	−·78				
1-ε-DNP-*hexa*-lysine	−·62			−·26	
6-ε-DNP-*hexa*-lysine	−·76				
6-ε-DNP-*octa*-lysine	−·67				
1-ε-DNP-*nona*-lysine	−·86	+·05	+·17	0	+1·00
9-ε-DNP-*nona*-lysine	−·87				
Standard free energy of binding (ΔF°) for ε-DNP-L-lysine (kcal/mole)	−8·10	−7·92	−11·05	−8·02	−9·48

* Δ(ΔF°) = ΔF° for binding indicated ligand − ΔF° for binding ε-DNP-L-lysine.
ΔF° = −RT ln Ko, where R is 1·987 cal degree^{-1} mole^{-1} and T, absolute temperature. Negative values for Δ(ΔF°) indicate that the given ligand is bound more energetically than is ε-DNP-L-lysine.

Anti-DNP-PLL antibodies bind 1-ε-DNP-*di*, *tri* and *tetra*-L-lysine to a greater degree than they bind ε-DNP-lysine. This increased binding, as compared with the binding of ε-DNP-L-lysine, is considerably diminished when 1-ε-DNP-*hexa*-L-lysine is tested and is entirely absent with 1-ε-DNP-*nona*-L-lysine. Anti-DNP-GPA binds ε-DNP-L-lysine more energetically than either 1-ε-DNP-*tetra*-L-lysine or 1-ε-DNP-*nona*-L-lysine.

One point of some interest is that the two highly positively charged immunogens studied here (1-ε-DNP-*nona*-L-lysine and DNP-PLL) gave rise to anti-DNP antibodies that bind neutral ε-DNP-L-lysine more avidly than they bind negatively charged DNP-ε-aminocaproic acid; on the other hand, immunization with DNP-GPA (a negatively charged substance) led to the production of an anti-DNP antibody population which binds negatively charged DNP-ε-aminocaproic acid more avidly than neutral ε-DNP-L-lysine and that, in turn, more avidly than the positively charged 1-ε-DNP-*tetra*-L-lysine and 1-ε-DNP-*nona*-L-lysine. This finding is consistent with the reports of Sela and Mozes (1966) of a reciprocal relation of the net charge of antigen and of antibody in rabbits.

In view of the demonstration in the second paper of this series (Stupp *et al.*, 1970a) that the production of anti-DNP antibodies in response to immunization with mono-ε-DNP-*oligo*-L-lysine was independent of PLL gene status when mycobacterial adjuvants were employed and that, under such conditions, the smaller mono-ε-DNP-*oligo*-L-lysines ($n = 2$ to $n = 6$) elicited the formation of significant amounts of antibody, it is possible that an evaluation of antibodies produced through immunization involving Freund's complete adjuvant is not completely relevant to the goal of this study.

It might be more appropriate to consider the antibodies produced under conditions of immunization in which only the larger peptides cause antibody synthesis and in which the response is under the control of the PLL gene. We, therefore, studied the binding characteristics of an anti-DNP antiserum produced by a PLL+ Hartley guinea-pig in

response to immunization with 1-ε-DNP-*nona*-L-lysine emulsified in Freund's incomplete adjuvant. The amount of anti-DNP antibody synthesized was too small to allow purification and subsequent fluorescence quenching analysis so that binding characteristics were studied by utilizing a series of non-radioactive DNP-ligands to inhibit the binding of [^{3}H]-ε-DNP-L-lysine by the globulin fraction prepared from the anti-1-ε-DNP-*nona*-L-lysine antiserum.

The results are presented in terms of the concentrations of the various ligands required to cause 50 per cent inhibition of the binding of [^{3}H]-ε-DNP-L-lysine by antibody. From these data, the difference in standard free energy of binding ($\triangle(\triangle F°)$) for any of the test ligands compared to the index ligand (ε-DNP-L-lysine) can be calculated (Table 4). The

TABLE 4

RELATIVE AFFINITY OF ANTI-1-ε-DNP-NONA-L-LYSINE FOR VARIOUS DNP-LIGANDS; IMMUNIZATION WITHOUT MYCOBACTERIAL ADJUVANT

Ligand	Concentration for 50 per cent inhibition* 10^{-8} M	$\Delta(\Delta F°)$† (kCal/mole)
ε-DNP-L-lysine	0·96	0·00
1-ε-DNP-*tetra*-L-lysine	0·96	0·00
6-ε-DNP-*octa*-L-lysine	1·11	+0·08
1-ε-DNP-*nona*-L-lysine	1·00	+0·02
9-ε-DNP-*nona*-L-lysine	1·30	+0·17

The capacity of a series of DNP-ligands to inhibit the binding of [^{3}H]-ε-DNP-L-lysine to the globulin fraction of an anti-1-ε-DNP-*nona*-L-lysine antiserum, raised by immunization of a PLL+ Hartley guinea-pig using Freund's incomplete adjuvant, was studied. Total concentration of radioactive ligand was $1·0 \times 10^{-8}$ M and, in the absence of inhibitor, 61 per cent of ligand was bound by the antibody.

* Concentration for 50 per cent inhibition was determined by plotting per cent inhibition against log free inhibition concentration. The regression line best fitting the data was calculated by the least squares method.

† $$\Delta(\Delta F°) = -RT \ln \left\{ \frac{\text{Concentration of } \varepsilon\text{-DNP-lysine for 50 per cent inhibition}}{\text{Concentration of test ligand for 50 per cent inhibition}} \right\}$$

Thus, negative values indicate a given ligand is bound more energetically than is ε-DNP-L-lysine.

$\triangle(\triangle F°)$ for each of the ligands tested is quite close to zero. Thus, 1-ε-DNP-*nona*-L-lysine has no binding advantage over 1-ε-DNP-*tetra*-L-lysine or even ε-DNP-L-lysine; further the antibody does not appear to discriminate, to a significant degree, between 9-ε-DNP-*nona*-L-lysine and 1-ε-DNP-*nona*-L-lysine. This antibody has binding characteristics very similar to those displayed by preparation 105–1 (See Table 3). Neither of these antibody populations distinguished the various DNP ligands tested, in contrast to 106–1 and 105–2 which bound 1-ε-DNP-*tetra*-L-lysine more energetically than ε-DNP-L-lysine. These data illustrate that antibodies produced by individual animals differ in their relative capacity to bind the immunizing peptide and shorter peptides, including ε-DNP-L-lysine. Even in the instance in which the binding of the immunogen (1-ε-DNP-*nona*-L-lysine) is more energetic than is the binding of shorter DNP-ligands, the energy difference is of small magnitude, particularly in relation to the total binding energy.

Finally, the use of a pure peptide as antigen allows a study of the relationship between structural homogeneity of antigen and thermodynamic homogeneity of the antibody

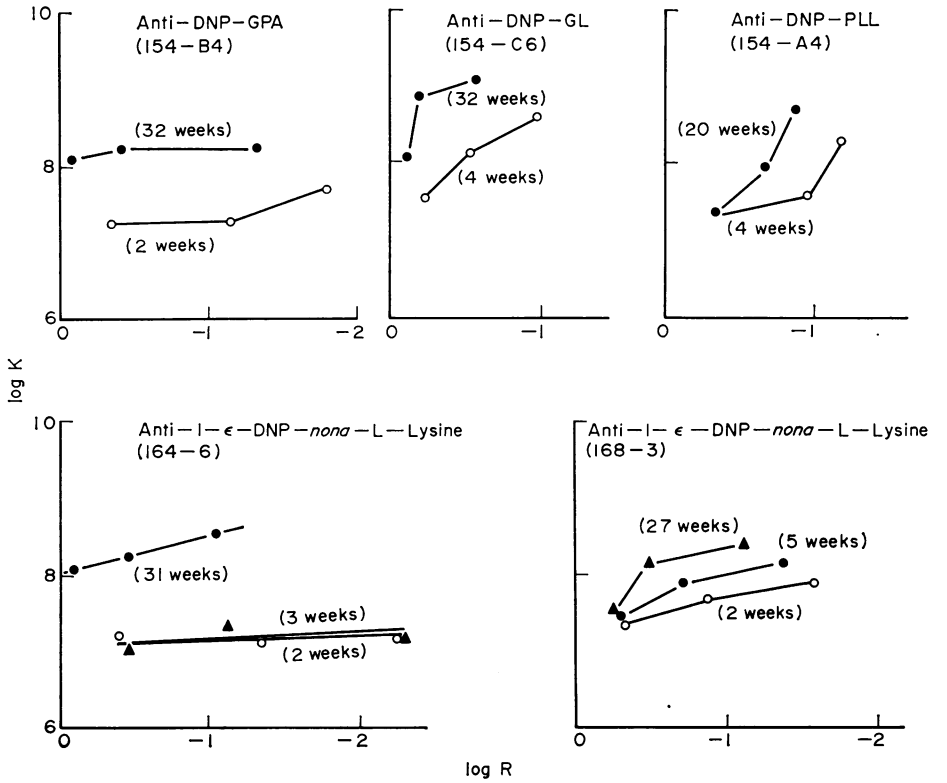

FIG. 1. Affinity of anti-DNP antibodies for [^{3}H]-ε-DNP-L-lysine at various times in the course of the immune response. K is association constant; R is antigen binding capacity at test condition/antigen binding capacity at index condition.

response. In order to study this question we asked whether at two different times in the course of the immune response to these peptides, the affinity of the serum antibody was similar and whether, at each time, there was evidence of thermodynamic heterogeneity. Hartley guinea-pigs were immunized with 1-ε-DNP-*nona*-L-lysine, emulsified in Freund's complete adjuvant containing 2 mg *M. tuberculosis* per ml of oil, and other animals were similarly immunized with DNP-GPA, DNP-GL or DNP-PLL. The animals were bled at various times in the course of the response. Because at the later bleedings (27–35 weeks), the amount of antibody obtained was often insufficient to study by fluorescence quenching analysis or equilibrium dialysis, we utilized the dilutional approach described in the Methods section. Fig. 1 displays the results of these studies. The anti-DNP antibodies present in the serum of two guinea-pigs immunized with 1-ε-DNP-*nona*-L-lysine increased in affinity for [^{3}H]-ε-DNP-L-lysine between 2 weeks after immunization and 27–31 weeks after immunization. The magnitude of this increase in animal 164–6 was equivalent to that noted in the response to DNP-GPA or to DNP-GL. Although antisera from both animals immunized with 1-ε-DNP-*nona*-L-lysine displayed relatively limited thermodynamic heterogeneity among the molecules measured in the affinity assay, the anti-DNP response to this peptide was quite similar in character to the response to the more complex antigen, DNP-GPA.

DISCUSSION

The current paper of this series presents studies aimed at determining what type of immune recognition system has the degree of specificity required to distinguish between closely related mono-ε-DNP-*oligo*-L-lysines and is, therefore, likely to be involved in the determination of the immunogenicity of the peptides under consideration. In order to achieve this aim, we compared the relative capacity of cellular immune responses and of serum antibody to discriminate between the mono-ε-DNP-*oligo*-L-lysine used for immunization and a similar peptide in which only the location of the DNP group on the peptide varied.

Guinea-pigs possessing the PLL gene (Hartley responder and strain 2) which had been immunized with 1-ε-DNP-*nona*-L-lysine displayed definite delayed hypersensitivity reactions to 1-ε-DNP-*nona*-L-lysine and significantly smaller reactions to 9-ε-DNP-*nona*-L-lysine. Poly-L-lysine elicited small or absent reactions. Similarly, lymph node cells from guinea-pigs immunized to 1-ε-DNP-*nona*-L-lysine were more effectively stimulated to synthesize DNA by the immunizing peptide than by 9-ε-DNP-*nona*-L-lysine. Again, poly-L-lysine had little effect. Further, cells from guinea-pigs immunized to 9-ε-DNP-*nona*-L-lysine were stimulated to a greater degree by 9-ε-DNP-*nona*-L-lysine than by 1-ε-DNP-*nona*-L-lysine. These findings demonstrate the high degree of specificity possessed by the recognition mechanism associated with the lymphoid cells which mediate cellular immune responses. They confirm findings of Schlossman and his colleagues concerning the DNA synthetic response of specific lymphoid cells to α-DNP-*oligo*-L-lysines and to mono-ε-DNP-*oligo*-L-lysines (Stulbarg and Schlossman, 1968; Schlossman, Herman and Yaron, 1969). In addition to the data reported here, extensive studies of the carrier specificity of cellular immune responses to hapten-carrier conjugates (see Paul, 1970) and the recent demonstration of a high degree of carrier specificity in the removal, by antigen-agarose beads, of lymph node cells which mediate specific DNA synthetic responses (Davie and Paul, 1970) all strongly support the concept that these cells possess receptors with a very high degree of specificity.

Serum antibody has also been evaluated for its capacity to differentiate between related mono-ε-DNP-*oligo*-L-lysines. The rationale for studying serum antibody is the assumption that precursors of antibody producing cells possess cell associated antibodies which function as receptors and which are identical, in their binding properties, to the antibody to be secreted by the progeny of that cell. Circulating antibody should thus reflect the receptors present on the precursors of antibody forming cells which are activated by the antigen used for immunization. Our studies of antibodies produced by PLL+ Hartley guinea-pigs in response to immunization with 1-ε-DNP-*nona*-L-lysine emulsified either in Freund's complete adjuvant or in Freund's incomplete adjuvant do not reveal any significant capacity of these circulating antibodies to distinguish between 1-ε-DNP-*nona*-L-lysine and 9-ε-DNP-*nona*-L-lysine nor do these antibody populations regularly distinguish between the clearly immunogenic mono-ε-DNP-*nona*-L-lysines and the mono-ε-DNP-*tetra*-L-lysines which are either not immunogenic or only marginally so.

These studies thus suggest that in immune responses which involve the interaction of two types of specific immunocompetent cells, the receptors possessed by the cells involved in cellular immune reactions and by the antigen sensitive 'helper' cells (Stupp *et al.*, 1971b) recognize very subtle differences in antigen structure which are not recognized by receptors on the precursors of antibody forming cells which proliferate and secrete

antibody in response to the same antigen. One reservation must be expressed, however. The characterization of the receptors on precursors of antibody forming cells, being based on an analysis of antibody molecules, is thus directly applicable to the precursors in a primed animal. As it is will be known (Eisen and Siskind, 1964; Siskind and Benacerraf, 1969) that considerable selective pressure has been involved in the generation of this cell population from precursors in the non-primed animal, it is conceivable that the precursors of antibody forming cells in non-primed animals have receptors with more discriminatory ability that is suggested by the analyses presented here. Nonetheless, if either of these two cell types is involved in determining the intrinsic immunogenicity of a compound, it would appear to be the antigen sensitive 'helper' cell (and the cell involved in cellular immune responses) rather than the precursor of the antibody forming cell.

Such a scheme can explain the ability of the shorter mono-ε-DNP-*oligo*-L-lysines ($n = 2$ to $n = 6$) to cause the synthesis of anti-DNP antibody when mycobacterial adjuvants are employed (Stupp *et al.*, 1971a). In this case, it is proposed that charge complexes form between the positively charged peptide and mycobacterial protein. The immune response which ensues involves the interaction of the DNP-peptide 'hapten'-mycobacterial 'carrier' complex with 'helper' cells specific for mycobacterial protein and with precursors of antibody forming cells capable of interacting with the DNP group. Thus, the antibody which is formed interacts very well with the free DNP-peptide but no cellular immune response or carrier effect is elicited by the peptide.

A final point in these studies is that the anti-DNP antibodies produced several months after immunization with 1-ε-DNP-*nona*-L-lysine had a higher binding affinity, as judged by the dilutional affinity assay used here, than did the antibody present shortly after immunization. This suggests that the relationship between antigenic complexity and homogeneity of immune responses is not so straightforward as had been proposed (Singer, 1964; Haber, Richards, Spragg, Austen, Vallotton and Page, 1967). In this regard, it would be more relevant to consider the recent finding that thymus deprived AKR mice make anti-DNP antibody in response to DNP-hemocyanin or DNP-bovine serum albumin immunization which is of lower affinity than is the antibody produced by such mice which have been reconstituted with thymus cells (Gershon and Paul, 1971). This suggests that antigens which depend upon the activity of thymus derived cells to stimulate strong immune responses should, indeed, elicit the maturation of antibody affinity with time as described by Eisen and Siskind (1964). On the other hand, antigens which elicit responses in which the role of thymus derived cells is of less importance may be expected to cause the production of relatively small amounts of antibody of limited heterogeneity. In such responses only a modest degree of affinity increase may be anticipated.

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