ANTIGEN RECOGNITION AND ANTIBODY SPECIFICITY

Carrier Specificity and Genetic Control of Anti-Dinitrophenyl-Oligolysine Antibody*

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The specificity of antibody formed in response to an antigenic challenge is a function of both the nature of the antigenic determinants used and the genetic capacity of the animal to differentiate among and respond to those determinants. At present neither the mechanism of specific antigen recognition nor the nature of its genetic control is well understood.

It is widely held that the immunologically competent or committed cell is triggered by the interaction of antigen with a complementary and stereospecific receptor identical to the antibody subsequently produced (1–5). However, on thermodynamic grounds, the difference between the binding energies of anti-hapten antibody for those hapten-containing compounds which can trigger the cell and for those that cannot is small and does not readily account for the disparity between humoral antibody specificity and the specificity of cells involved in both cellular immunity and secondary anti-hapten antibody synthesis (6–13). To account for these differences, several investigators have shown that cell cooperation occurs in the secondary anti-hapten antibody response between hapten-specific and carrier-specific helper cells (14–17) and that the carrier does more than provide a complete antigenic determinant for the haptenic group. Their experiments indicate that an interaction between carrier and carrier-specific helper cell facilitates the triggering of the bone marrow-derived anti-hapten antibody-forming cell. Such a mechanism provides a basis for the carrier effect noted in triggering the secondary anti-hapten antibody response. But this mechanism implies that the haptenic group by itself constitutes an antigenic determinant capable of triggering the hapten-specific antibody-forming cell provided that the carrier-specific cell "delivers" the antigen to the bone marrow-derived antibody-forming cell, and that the anti-hapten antibody receptor on such a cell possesses sufficient affinity for the haptenic group to be triggered.

A carrier cell–hapten cell model for antigen recognition and gene action is best understood by presuming that the antibody-forming cell has only limited carrier

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specificity. The present studies, utilizing the technique of fluorescence quenching, were undertaken to explore the precise carrier specificity of anti-hapten antibody in responder and nonresponder guinea pigs. Animals were immunized with a series of singly substittued ϵ, dinitrophenyl (DNP)[1] and α, DNP–oligolysine peptides differing from one another only by the addition of several lysyl (Lys) residues to the end of the peptide chain furthest from the immunodominant DNP group. These added lysines, situated more than seven lysyl residues from the haptenic group, are clearly excluded from any previously proposed spatial limits of one antibody-binding site (6, 9, 18–20). Therefore, if the antibodies produced by immunization with DNP-oligolysines of slightly different chain lengths can recognize their homologous antigens, they presumably would be detecting conformational changes in the portion of the peptide chain adjacent to the haptenic group. More important, such a finding would suggest that each DNP-oligolysine compound requires either a specific carrier cell, a specific hapten-carrier antibody-forming cell, or both. Each alternative requires a much greater number of preexisting specific cells than previously estimated, thus diminishing the chance interaction between two or more such specific cells.

The ability to respond to polylysines or oligolysines is inherited in the guinea pig as an autosomal mendelian dominant trait (21–24) and the polylysine gene has been postulated to act at the level of the carrier-specific cell (24). This locus of gene action implies that responder and nonresponder guinea pigs possess the same group of antibody-forming cells. In support of this hypothesis was the demonstration that DNP-oliglysines in association with an immunogenic carrier evoke in nonresponder guinea pigs the formation of anti-DNP antibody no different from that obtained in responder animals under comparable conditions (25).

In the present studies it will be shown that antibody formed in responder animals to defined DNP-oligolysines is extraordinarily specific and capable of recognizing the precise chain length of the DNP-oligolysines used to induce the immune response. The antibody-forming cell is then also capable of a fine degree of carrier recognition. On the other hand, the anti-DNP antibody generated in nonresponder animals by immunization with DNP-oligolysines in association with a molecule recognized as immunogenic lacks this capacity to distingush between different DNP-oligolysines, suggesting that the polylysine gene may act at the level of the antibody-forming cell.

Materials and Methods

Preparation of Hapten-Substituted Compounds.—Poly, ϵ, *N*-benzyloxycarbonyl-L-lysine with an average chain length of 11 (n = 11) was prepared as previously described (26). After the blocked polymer was dissolved in dimethylformamide and triethylamine, 1-fluoro-2,4-dinitrobenzene (Eastman Organic Chemicals, Rochester, N. Y.) was added. The DNP-oligolysines were deblocked and the water-soluble peptides were resolved on carboxymethyl (CM) cellulose, yielding a series of α, DNP-oligolysines ranging in size from α, DNP-Lys_2 to α, DNP-

[1] *Abbreviations used in this paper:* BAC, bromoacetyl-cellulose; CM, carboxymethyl; DNP, dinitrophenyl; EACA, ϵ-aminocaproic acid; HSA, human serum albumin; PBS, phosphate-buffered saline, pH 7.4.

Lys_{15}. In addition three mixtures, α,DNP-Lys_{7-10}, α,DNP-Lys_{16-30} and α,DNP-Lys_{30-100} were pooled. α,DNP-Lys_{30-100} represents a mixture of large peptides with an upper limit in peptide size estimated to be approximately 100.

An homologous series of oligolysine peptides containing a single dinitrophenyl substitution on the ϵ-amino group of the C-terminal lysine was prepared by Yeda, Rehovot, Israel as previously described (27). The blocked polymer was deblocked and the peptides separated on a CM-cellulose column into relatively pure populations of oligopeptides ranging in size from 2,ϵ,DNP-Lys_{2} to 19,ϵ,DNP-Lys_{19}. Although fractionation was accomplished with good resolution, it is likely that any single peptide was contaminated with as much as 5% of those peptides containing one lysyl residue more or one less (26).

Human serum albumin (HSA) (Pentex Biochemicals, Kankakee, Ill.) was dinitrophenylated with dinitrobenzene sulfonic acid (Eastman Kodak) by standard methods (28). The DNP-HSA to be conjugated with the immunoabsorbant contained approximately 16 DNP groups per mole ($DNP_{\overline{16}}HSA$), whereas DNP-HSA to be used for quantitative precipitin titers contained approximately 25 groups per mole ($DNP_{\overline{25}}HSA$). 2,4,dinitrophenol was purchased from Eastman Kodak and recrystallized twice before use. ^{3}H-DNP-EACA was made by reacting ^{3}H-1-fluoro-2,4-dinitrobenzene (Nuclear Chicago, Des Plaines, Ill.) with ϵ-aminocaproic acid in dimethyl formamide and purified by preparative thin-layer chromatography using Chrom AR sheet-500 (Mallinckrodt Chemical Works, St. Louis, Mo.) and a solvent system containing toluene 60, pyridine 10, dioxane 10, and 2-chloroethanol 10. The final product had a specific activity of 8 Ci/mM.

Preparation of Immunoabsorbants.—$DNP_{\overline{16}}HSA$ was conjugated to bromoacetyl-cellulose (BAC) (Miles Laboratory, Elkhart, Ind.) by the method of Robbins et al. (29).

Immunization.—For antibody-specificity studies guinea pigs of the National Institutes of Health Hartley strain weighing 500–600 g were divided into five groups containing six to nine animals each. Five antigens were used for immunization: α,DNP-Lys_{7-10}; α,DNP-Lys_{16-30}; 8,ϵ,DNP-Lys_{8}; 10,ϵ,DNP-Lys_{10}; and 15,ϵ,DNP-Lys_{15}. The antigens were dissolved in saline and emulsified in a Virtis homogenizer (Virtis Co., Gardiner, N. Y.) with an equal volume of Freund's incomplete adjuvant (Difco Laboratories, Inc., Detroit, Mich.) to which had been added 2 mg/ml *Mycobacterium tuberculosis* H37Ra (Difco). Each animal received approximately 90 nmole of antigen (as determined by DNP content) in 0.8 ml of the homogenate distributed among the four footpads. They were boosted in the neck on the 7th day with 30 nmole of the same antigen dissolved in 0.25 ml of saline and emulsified with an equal volume of Freund's incomplete adjuvant. On days 14 and 21 they were skin tested intradermally with 2.4 nmole of the same antigen in 0.1 ml of saline. Animals with both positive Arthus and delayed-type skin tests were bled three times from day 25 to 32 and the serum from each group pooled. The responder rate was about 70%.

A sixth group of 12 NIH Hartley guinea pigs was immunized with an adjuvant containing 10 mg/ml *M. tuberculosis* H37Rv (a gift of Dr. B. Benacerraf). Each animal received a total of 90 nmole of 14,ϵ,DNP-Lys_{14} dissolved in 0.3 ml saline and emulsified with an equal volume of Freund's incomplete adjuvant containing 10 mg/ml H37Rv. The emulsion was distributed in all four footpads. A seventh group of 12 Camm-Hartley guinea pigs (Camm Research Institute, Inc., Wayne, N.J.) was similarly immunized except that 90 nmole of α,DNP-Lys_{16-30} was used as the antigen. The responder rate in the Camm-Hartley guinea pigs is reported to be between 20 and 30% (30). These animals were skin tested at 13 days with their respective immunizing compounds as described above, and only animals with definitely positive or negative tests were used for the antibody-specificity studies. Those animals with positive skin tests were considered responder animals and those with negative skin tests nonresponders (30). Between 33 and 43 days after immunization the animals were bled by cardiac puncture three times and the individual sera pooled.

Spectrophotometry.—A Gilford spectrophotometer (Gilford Instrument Co., Oberlin, Ohio)

with silica cells of 1 cm light path was used for all spectrophotometric determinations. The α,DNP-lysine content of these peptides was determined in 0.01 M sodium phosphate saline buffer, pH 7.0, at 360 nm (E_{360nm} = 16,800). The ϵ-DNP-Lysine content was similarly determined (E_{360nm} = 17,400). The concentration of purified anti-DNP antibody was obtained at 280 nm $E_{1cm}^{1\%}$ = 13.0.

Quantitative Precipitin Studies.—For the quantitative precipitin assay 0.5 ml of antiserum was added to a series of 3 ml centrifuge tubes containing known quantities of $DNP_{25}HSA$. The volume was adjusted to 1 ml with saline and the tubes mixed, incubated for 1 hr at 37°C, and allowed to stand for 2 days at 4°C with occasional stirring. The precipitates were washed twice with 0.7 ml of cold saline and dissolved in 0.75 ml of 0.02 M sodium lauryl sulfate. The absorbances of the resulting solutions were then read in a spectrophotometer at 360 and 280 nm. The estimated contribution of the $DNP_{25}HSA$ to the absorbance at 280 nm was then subtracted from the observed absorbance at 280 nm and the total concentration of anti-DNP antibody calculated (31).

Purification of Antibody.—The purification of antibody was carried out according to the method of Robbins et al. (29). $DNP_{16}HSA$ cellulose was added to the serum and mixed on a 6 rpm rotator for 2 hr at 4°C. The cellulose was centrifuged at 5000 *g* and washed with chilled saline until the supernatant contained no material absorbing at 280 nm. The packed cellulose was suspended in 0.1 M dinitrophenol–0.01 M phosphate-buffered saline, pH 7.4 (PBS), incubated at 37°C for 1 hr, and centrifuged at 5000 *g*. The yellow supernantant was passed through a small column of Bio-Rad Anion Exchange Resin AG 1 × 8 (200–400 mesh) (Calbiochem, Los Angeles, Calif.). Ammonium sulfate 0.3 g/ml was added to the colorless antibody-containing effluent and allowed to stand at 4°C for 18 hr. The purified antibody precipitate was redissolved in PBS and dialyzed exhaustively against PBS for 24 hr. The solution was then centrifuged to remove precipitated protein and the concentration of antibody determined spectrophotometrically. Purified antibody was run in immunoelectrophoresis against rabbit anti-whole guinea pig serum and rabbit anti-guinea pig γ_1 and γ_2 globulins (gift of Dr. Kurt Bloch).

Fluorescence Quenching.—Fluorescence quenching was carried out according to the method of Velick et al. and Eisen (32, 33). Purified antibody was diluted to a concentration of 50 μg/ml in PBS. 2 ml of the solution was pipetted into a 3 ml cuvette and varying amounts of DNP-Lysines added in 0.01 ml portions. The antibody solution was excited at 280 nm, and the emission spectrum recorded at 345 nm on a Hitachi-Perkin Elmer spectrophotofluorimeter (Hitachi America Ltd., Indianapolis, Ind.–Perkin-Elmer Corp., Norwalk, Conn.). All experiments were carried out at 25°C.

Of approximately 17 points obtained with each fluorescence quenching titration, 8–10 were used for calculating an equilibrium constant (K_0). As has been reported previously, initial titration points in this technique have a disproportionate amount of quenching, which results in negative values for free concentration of hapten, and must be discarded (33). The value of Q_{max} was taken as 100% yielding a K_0 which did not differ by more than 5% whed compared to one obtained with equilibrium dialysis with the same antibody using tritiated DNP-EACA or α,DNP-oligolysines. These results are consistent with our previous demonstration of the agreement between fluorescence quenching and equilibrium dialysis (6). The Q_{max} obtained empirically for each antibody ranged between 75 and 80%.

With the use of a time-shared PDP-1 computer the 8–10 titration values were converted to points on a Sips plot, $\ln r/n\text{-}r = a\ln K_0 + a \ln C$ (34, 35), where K_0 is the equilibrium constant; a, the index of heterogeneity; and C, the concentration of free hapten. The value of K_0 was obtained and converted to $\Delta F°$, the standard free energy, by the formula $\Delta F° = -RT \ln K_0$. Values for $\Delta F°$ had confidence limits at the 0.05 level between $\pm$150 cal/M, and duplicate titration for the same antibody and the same hapten done on different days did not differ by more than 100 cal. $\Delta F°$ values differing by more than 200 cal/M were considered signifi-

cant. The slopes (a) of the Sips plot (36) for each group of DNP-oligolysines used to examine a particular purified antibody did not differ significantly and were essentially parallel.

Equilibrium Dialysis.—Equilibrium dialysis was performed with microdialysis cells (Future Plastics, Cambridge, Mass.) having 0.1 ml chambers. 0.08 ml of antibody (300 μg/ml) in buffer was placed on one side of the membrane and 0.08 ml of labeled oligopeptide solution on the other. The cells were rotated at 6 rpm at 37°C for 1 hr and for 18 hr at 25°C. Similar tubes containing labeled peptides without antibody were prepared to insure that equilibrium was achieved. Portions of 0.05 ml were obtained from both sides of the membrane by syringe (Hamilton Co. Inc., Whittier, Cal.) and dissolved in 0.5 ml of NCS (Nuclear Chicago). To each sample, 10 ml of Spectrofluor (New England Nuclear Corp., Boston, Mass.) was added and the samples counted in a Packard Tri-Carb liquid scintillation counter (Packard Instrument Co., Downer's Grove, Ill.) (counting efficiency 55%).

RESULTS

Specificity of Anti–DNP-oligolysine Antibody in Responder Guinea pigs.—The total precipitable antibody of guinea pig antiserum to α,DNP-Lys_{7-10}, α,DNP-Lys_{16-30}, 8,ϵ,DNP-Lys_8, 10,ϵ,DNP-Lys_{10}, and 15,ϵ,DNP-Lys_{15} from responder animals immunized with Freund's adjuvant containing 2 mg/ml H37Ra ranged between 0.174 and 0.423 mg antibody per milliliter (Table I). Individual serum pools were purified and the yield of antibody was between 66 and 85% of the total precipitable antibody.

TABLE I

Titers of Serum Anti-DNP Antibody and Per Cent of Antibody Yield after Purification

Immunizing antigen	Serum antibody titer	Yield of purified antibody
	(mg/ml)	(%)
α,DNP-Lys_{7-10}	0.174	85
α,DNP-Lys_{16-30}	0.273	66
8,ϵ,DNP-Lys_8	0.179	80
10,ϵ,DNP-Lys_{10}	0.262	74
15,ϵ,DNP-Lys_{15}	0.423	85

The binding energies ($-\Delta F^\circ$) of α,DNP-Lys_n peptides, n,ϵ,DNP-Lys_n peptides, DNP-OH, and ϵ,DNP-EACA to purified guinea pig anti-α,DNP-Lys_{7-10} antibody were obtained by fluorescence quenching (Fig. 1). As is shown, there is a progressive increase in $-\Delta F^\circ$ with increasing chain length from α,DNP-Lys_3 to α,DNP-Lys_9 having a maximum of 10,030 cal/M. Further increase in oligiolysine chain length to α,DNP-Lys_{12}, α,DNP-Lys_{15}, or α,DNP-Lys_{16-30} resulted in a decrease of at least 1400 cal/M compared with α,DNP-Lys_{7-9}. It should be emphasized that these $-\Delta F^\circ$ have confidence limits at ± 150 cal/M at the 0.05 level. Two additional points are of interest. First, as previously shown (6, 37), the haptenic group contributed approximately 70% of the total binding energy of α,DNP-Lys_9 as measured by binding of DNP-OH or DNP-EACA, whereas the carrier portion of the DNP-oligolysine peptide can be esti-

mated to contribute the remaining 30%. Second, 2,ε,DNP-Lys_2, 7,ε,DNP-Lys_7, and 10,ε,DNP-Lys_{10} bind significantly better with anti-α,DNP-Lys_{7-10} antibody than would have been anticipated from the behavior of the haptenic group alone. It is not clear whether the additional contribution in binding energy of, for example, 2,ε,DNP-Lys_2 over DNP-EACA in its interaction with anti-α,DNP-Lys_{7-10} antibody results from populations of antibody with a higher affinity for 2,ε,DNP-Lys_2 or the capacity of ε,DNP oligolysines to conform to the α,DNP-Lys_{7-10} binding site. Although these cross-reactions do occur, it is apparent that anti-α,DNP-Lys_{7-10} antibody contains a measurable proportion of antibody molecules with specificity for the homologous immunizing antigen.

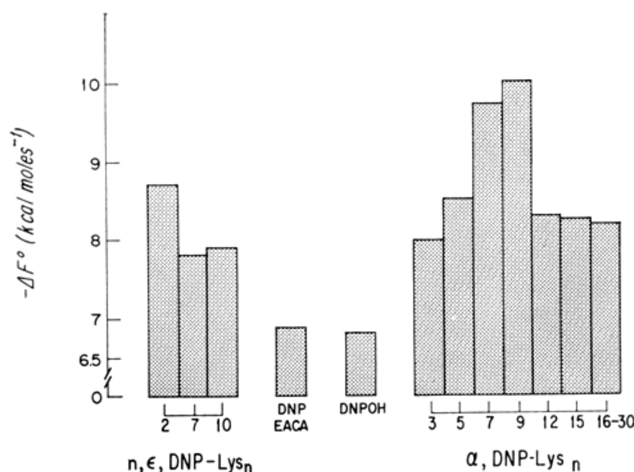

Fig. 1. Binding energies ($-\Delta F°$) of α,DNP-Lys_n peptides, n,ε,DNP-Lys_n peptides, DNP-EACA, and DNP-OH obtained with purified anti-α,DNP-Lys_{7-10} antibody in fluorescence quenching studies.

Antibody raised to α,DNP-Lys_{16-30}, in contrast, shows no progressive increase in binding energy from α,DNP-Lys_3 to α,DNP-Lys_9 (Fig. 2). The $-\Delta F°$ for those compounds ranged between 8309 and 8632 cal/M. More important, on increasing the chain length beyond the nonamer there is a progressive increase in $-\Delta F°$ reaching a maximum at α,DNP-Lys_{16-30} (9706 cal/M). Once again, the maximal $-\Delta F°$ obtained is achieved with those peptides used to initiate the immune response. Dinitrophenol,ε,DNP-oligolysines, and DNP-EACA, on the other hand, behaved almost identically with both anti-α,DNP-Lys_{16-30} and α,DNP-Lys_{7-10} antibody.

The binding characteristics of antibody made to 8,ε,DNP-Lys_8, 10,ε, DNP-Lys_{10}, and 15,ε,DNP-Lys_{15} are represented in Figs. 3, 4, and 5 respec-

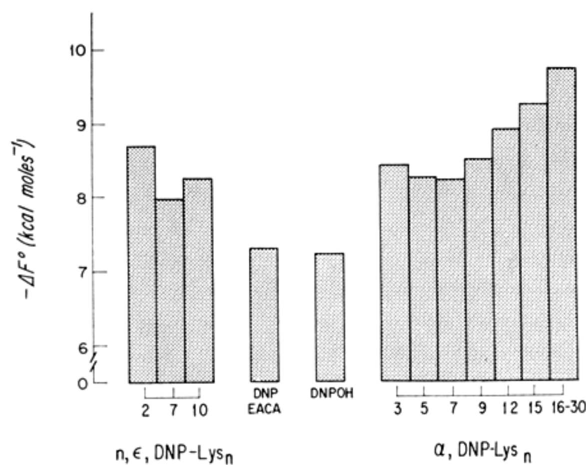

Fig. 2. Binding energies ($-\Delta F^\circ$) of α,DNP-Lys_n peptides, n,ϵ,DNP-Lys_n peptides, DNP-EACA, and DNP-OH obtained with purified anti-α,DNP-Lys_{16-30} antibody in fluorescence quenching studies.

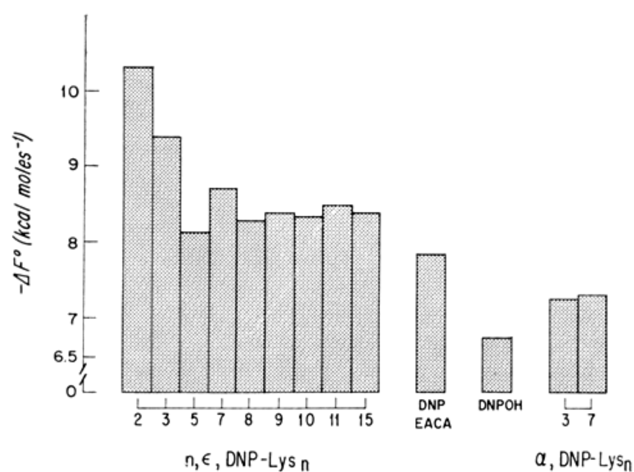

Fig. 3. Binding energies ($-\Delta F^\circ$) of α,DNP-Lys_n peptides, n,ϵ,DNP-Lys_n peptides, DNP-EACA, and DNP-OH obtained with purified anti-8, ϵ,DNP-Lys_8 antibody in fluorescence quenching studies.

tively. Anti-8,ϵ,DNP-Lys_8 antibody showed maximal binding with 2,ϵ,DNP-Lys_2. The $-\Delta F^\circ$ for this interaction was 10,369 cal/M. Lengthening the chain to 3,ϵ,DNP-Lys_3 and 5,ϵ,DNP-Lys_5 resulted in a decrease in $-\Delta F^\circ$. It was intriguing to find that the $-\Delta F^\circ$ for 7,ϵ,DNP-Lys_7 (8734 cal/M) was 431–587

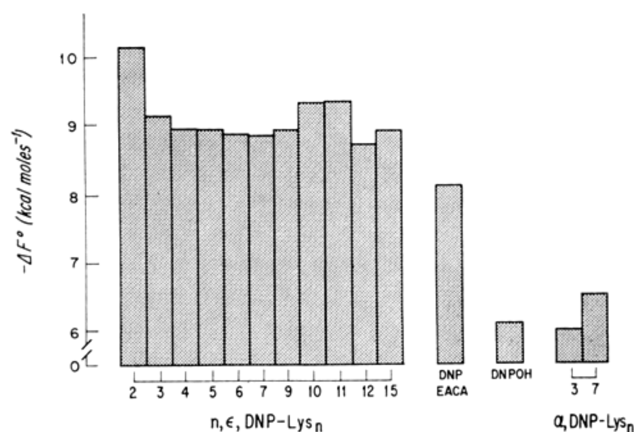

Fig. 4. Binding energies ($-\Delta F°$) of α, DNP-Lys$_n$ peptides, n,ϵ,DNP-Lys$_n$ peptides, DNP-EACA, and DNP-OH obtained with purified anti-10, ϵ,DNP-Lys$_{10}$ antibody in fluorescence quenching studies.

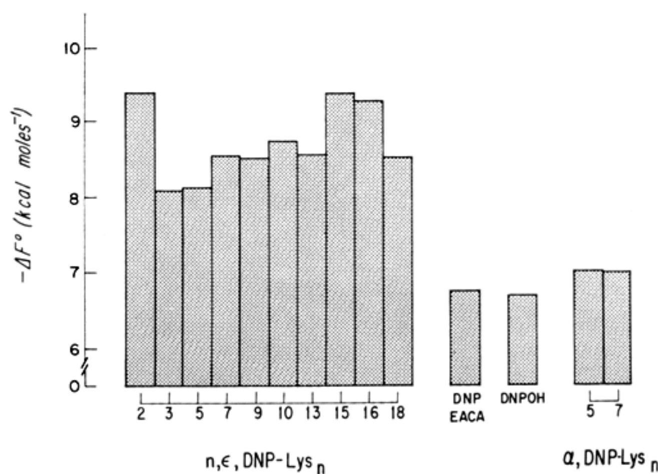

Fig. 5. Binding energies ($-\Delta F°$) of α,DNP-Lys$_n$ peptides, n,ϵ,DNP-Lys$_n$ peptides, DNP-EACA, and DNP-OH obtained with purified anti-15, ϵ,DNP-Lys$_{15}$ antibody in fluorescence quenching studies.

cal/M greater than that found with 5,ϵ,DNP-Lys$_5$ or 8,ϵ,DNP-Lys$_8$. Quenching with larger ϵ,DNP-oligolysines resulted in no significant increase in binding energy. For example, 9,ϵ,DNP-Lys$_9$ to 15,ϵ,DNP-Lys$_{15}$ had $-\Delta F°$ ranging between 8366 and 8482 cal/M, whereas the heptamer had a $-\Delta F°$ of 8734 cal/M. Anti-8,ϵ,DNP-Lys$_8$ antibody cross-reacts extensively with α,DNP-oligolysines. These cross-reactions, however, depend almost entirely on the

affinity for the DNP group since DNP-OH contributes 93% of the binding energy of α,DNP-oligolysines. Since it is possible to have contamination of 8,ϵ,DNP-Lys_8 with 7,ϵ,DNP-Lys_7, the presence of antibody with 7,ϵ, DNP-Lys_7 specificity in these animals is not unexpected.

The significance of the second peak in binding, i.e. 7,ϵ,DNP-Lys_7 with anti-8,ϵ,DNP-Lys_8 antibody, becomes clearer when the binding charactersitics of anti-10,ϵ,DNP-Lys_{10} and 15,ϵ,DNP-Lys_{15} antibody are measured. As occurred with anti-8,ϵ,DNP-Lys_8 antibody, antibody raised to 10,ϵ,DNP-Lys_{10} binds 2,ϵ,DNP-Lys_2 maximally. Moreover, the $-\Delta F°$ ranged between 8925 and 8989 cal/M in progressing from 4,ϵ,DNP-Lys_4 to 9,ϵ,DNP-Lys_9. No second peak is noted with 7,ϵ,DNP-Lys_7. At 10,ϵ,DNP-Lys_{10} (9380 cal/M) and 11,ϵ,DNP-Lys_{11} (9385 cal/M), however, there is a second peak in binding which corresponds to the size peptide used for immunization. This pattern of specificity is even more striking with antibody raised to 15,ϵ,DNP-Lys_{15}. Again, 2,ϵ,DNP-Lys_2 had a high binding energy of 9354 cal/M and as the ϵ,DNP-oligolysine chain length is increased from 3,ϵ,DNP-Lys_3 to 12,ϵ, DNP-Lys_{12}, the binding energies are less and range between 8110 and 8750 cal/M. A second peak of binding energy is noted with 15,ϵ,DNP-Lys_{15} (9411 cal/M) and to a lesser extent with 16,ϵ,DNP-Lys_{16} (9252 cal/M). Further increase in peptide chain length to 18,ϵ,DNP-Lys_{18} (8535 cal/M) resulted in a lower $-\Delta F°$ similar to that found for 7–13,ϵ,DNP-$Lys_{7\text{-}13}$. As was noted with antibody to 8,ϵ,DNP-Lys_8 and 10,ϵ,DNP-Lys_{10}, all ϵ,DNP-oligolysines tested had higher affinity for anti-15,ϵ,DNP-Lys_{15} antibody than did α,DNP-oligolysines, DNP-OH, or DNP-EACA.

Each of the purified antibody preparations tested above showed the capacity to discriminate α,DNP-oligolysines from ϵ,DNP-oligolysines and all could distinguish within $\pm$ one lysyl residue the precise oligolysine chain length used to initiate the immune response. Despite the use of these relatively homogeneous antigens for immunization, the index of heterogeneity with the homologous immunizing antigen calculated from the Sips plot ranged between 0.16 and 0.35.

Skin Reactivity and Serum Antibody Titers of Responder and Nonresponder Animals Immunized with DNP-Oligolysines.—9 out of 12 NIH-Hartley strain guinea pigs immunized with 14,ϵ,DNP-Lys_{14} in an adjuvant containing *M. tuberculosis* H37Rv responded in 2 wk with strongly positive delayed skin reactions; the remaining 3 animals were completely negative. The observed responder rate of 75% as determined by positive delayed skin reactions was similar to that obtained in this strain of guinea pigs with DNP-oligolysines in other adjuvants. Sera from three responders and three nonresponders to 14,ϵ, DNP-Lys_{14} were used for subsequent antibody studies and the skin test results for these animals are shown in Table II. Camm-Hartley guinea pigs were immunized in a similar fashion to the above but with α,DNP-$Lys_{16\text{-}30}$.

As expected, 4 of 12 gave a positive delayed skin reaction to α,DNP-Lys_{16-30} at 2 wk; 8 had negative skin reactions. The observed responder rate of 33% did not differ significantly from that expected. Two responder and two nonresponder animals were used in subsequent antibody studies and their skin reactions are shown in Table II.

Anti-DNP antibody titers from nonresponders ranged between 0.124 and 0.211 mg/ml whereas the titers for responders ranged between 0.146 and 0.247 mg/ml (Table II). These results are similar to those obtained by Green et al. (30) and indicate that irrespective of responder status all guinea pigs immunized with DNP-polylysines in H37Rv (10 mg/ml) produce significant levels of circulating anti-DNP antibody.

TABLE II

Titers, Purified Antibody Yield, and Skin Tests of Responder and Nonresponder Animals Immunized with an Adjuvant Containing M. tuberculosis (H37Rv)

Animal No.	Strain*	Antigen	Titer	Yield	Skin test‡	Responder status§
			(mg/ml)	*(%)*	*(mm)*	
L3	H	14,ϵ,DNP-Lys_{14}	0.140	70	0	N
L2	H	14,ϵ,DNP-Lys_{14}	0.127	65	0	N
L5	H	14,ϵ,DNP-Lys_{14}	0.124	68	0	N
R3	H	14,ϵ,DNP-Lys_{14}	0.149	60	22/20	R
R2	H	14,ϵ,DNP-Lys_{14}	0.150	60	20/20	R
R6	H	14,ϵ,DNP-Lys_{14}	0.170	65	17/16	R
B2	C	α,DNP-Lys_{16-30}	0.211	72	±	N
A5	C	α,DNP-Lys_{16-30}	0.155	99	±	N
A6	C	α,DNP-Lys_{16-30}	0.146	62	25/30	R
B3	C	α,DNP-Lys_{16-30}	0.247	82	30/30	R

* Camm-Hartley Strain, C; NIH Hartley, H.

† Diameter of induration in millimeters, two readings 90° from each other at 24 hr.

§ Responder, R; nonresponder, N.

Anti-hapten antibody from each individual animal was purified for the haptenic group with the insoluble absorbant DNP-HSA–cellulose and the yield ranged between 60 and 99% of the total precipitable antibody (Table II). Similar yields were obtained from both responder and nonresponder animals. Each purified antibody was analyzed by immunoelectrophoresis against rabbit anti-whole guinea pig serum and rabbit anti-guinea pig γ_1 and γ_2 antibody. The purified antibodies were all γ-globulin and consisted of a mixture of γ_1 and γ_2 globulins. No qualitative differences could be observed between the antibodies elaborated by responder and nonresponder animals to 14,ϵ,DNP-Lys_{14} and α,DNP-Lys_{16-30} .

Specificity of Anti–DNP-Oligolysine Antibody Made in Responder and Non-

responder Guinea Pigs.—Antibody from responder animals R2 and R6 (Figs. 6 *A* and *B* respectively) had maximum binding energy with $14,\epsilon$,DNP-Lys_{14}, the homologous immunizing antigen. The $-\Delta F°$ for this interaction were 10,055 and 10,326 cal/M for R2 and R6 respectively. Lengthening or shortening of the oligolysine chain length from $14,\epsilon$,DNP-Lys_{14} was also accompanied by a significant decrease in binding energy. The high $-\Delta F°$ for $2,\epsilon$,DNP-Lys_2 is again noted. Antibody raised in responder animal R3 (Fig. 6 *C*) to $14,\epsilon$,DNP-Lys_{14} showed a slightly different pattern of binding. In this animal, both $2,\epsilon$,DNP-Lys_2 and $5,\epsilon$,DNP-Lys_5 had $-\Delta F°$ equal to or greater than that obtained with the homologous immunizing antigen. An additional peak in binding energy is also seen with $14,\epsilon$,DNP-Lys_{14}, with a $-\Delta F°$ at least 550 cal/M greater than that obtained with either $13,\epsilon$,DNP-Lys_{13} or $15,\epsilon$,DNP-Lys_{15}. Thus, despite the use of an adjuvant containing H37Rv, responder guinea pigs are able to elaborate at least one population of anti-DNP antibody which is specifically reactive with the precise chain length of the peptide used to induce the immune response.

The binding characteristics of antibody raised to $14,\epsilon$,DNP-Lys_{14} in nonresponder animals are shown in Figs. 6 *D*, *E*, and *F*. Nonresponder antibody showed maximum binding energy with $2,\epsilon$,DNP-Lys_2 and the $-\Delta F°$ for this interaction ranged between 9329 and 10,107 cal/M. With antibody from nonresponder L3 and L2 (Figs. 6 *D* and *E*) there was no peak in binding energy with $14,\epsilon$,DNP-Lys_{14}, the homologous immunizing antigen. Similarly, antibody from nonresponder L5 (Fig. 6 *F*) showed no specific boost in binding energy with $14,\epsilon$,DNP-Lys_{14} but a slight increase in $-\Delta F°$ for $10\text{–}16,\epsilon$, DNP-$Lys_{10\text{-}16}$ peptides was noted. Of interest, and as noted with antibody raised in responders, dinitrophenol contributed approximately 70% of the total binding energy, the remainder being contributed by the "oligolysine carrier."

The specificity of anti-α,DNP-$Lys_{16\text{-}30}$ antibody raised in responder Camm-Hartley guinea pigs A6 and B3 is shown in Figs. 7 *A* and *B* respectively. With both purified antibody preparations, the highest $-\Delta F°$ is obtained with α,DNP-$Lys_{16\text{-}30}$, the homologous immunizing antigen. The $-\Delta F°$ for this interaction ranged between 7860 and 9131 cal/M and was 350–600 cal/M greater than any other interaction measured for each antibody. Of interest, the $-\Delta F°$ for α,DNP-Lys_3 was 732–988 cal/M less than that obtained with α,DNP-$Lys_{16\text{-}30}$. This pattern of binding is slightly different than that obtained with NIH-Hartley responder animals immunized with α,DNP-$Lys_{16\text{-}30}$ in an adjuvant containing 2 mg/ml *M. tuberculosis* (H37Ra) and may reflect either genetic differences in the animals or the use of an adjuvant containing 10 mg/ml *M. tuberculosis* (H37Rv) (Fig. 2). Both sets of experiments, however, show maximum $-\Delta F°$ with α,DNP-$Lys_{16\text{-}30}$, the homologous immunizing antigen.

Anti-α,DNP-$Lys_{16\text{-}30}$ antibody raised in nonresponder Camm-Hartley

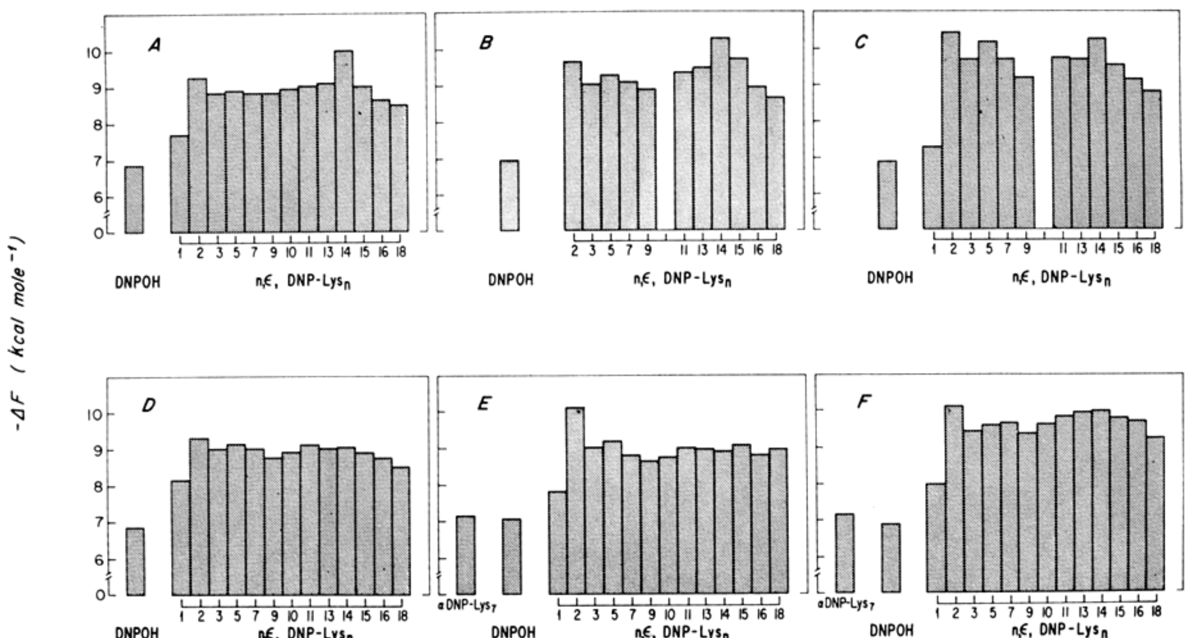

Fig. 6. The energy of binding ($-\Delta F^{\circ}$) of n, ϵ, DNP-Lys_n peptides, α,DNP-Lys_7, and dinitrophenol to anti-14,ϵ, DNP-Lys_{14} antibody rasised in responder and nonresponder Hartley strain guinea pigs. *A*, *B*, and *C* are responder animal R2, R6, and R3 respectively. *D*, *E*, and *F* are nonresponder animals L3, L2, anP L5 respectively.

guinea pigs with negative skin tests (Figs. 7 *C* and *D*) differed significantly from that obtained in responders (Figs. 7 *A* and *B*). Firstly, α,DNP-Lys$_3$ exhibited, of all the peptides tested, the highest binding energy (9336–9961 cal/M). Second, both nonresponder sera tested exhibited a lower binding energy for the homologous immunizing antigen, i.e. α, DNP-Lys$_{16-30}$, than for α, DNP-Lys$_3$. Finally, there is no peak in binding energy for α, DNP-Lys$_{16-30}$, i.e., nonresponder antibody could not discriminate α, DNP-Lys$_{16-30}$ from α, DNP-Lys$_{15}$ or α, DNP-Lys$_{30-100}$.

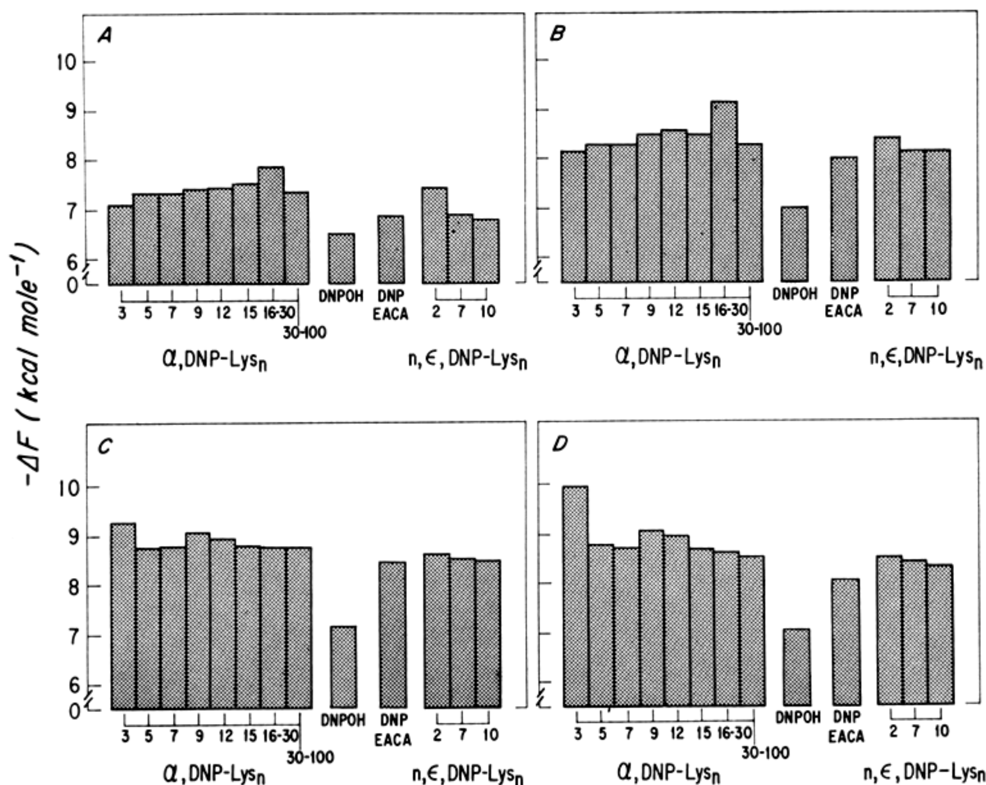

Fig. 7. The energy of binding ($-\Delta F^\circ$) of α, DNP-Lys$_n$ peptides, n, ϵ, DNP-Lys$_n$ peptides, DNP-EACA, and dinitrophenol to anti-α, DNP-Lys$_{16-30}$ antibody raised in responder and nonresponder Hartley strain guinea pigs. *A* and *B* are responder animals A6 and B3 respectively. *C* and D are nonresponder animals B2 and A5 respectively.

DISCUSSION

Present studies indicate that the antibody-forming cell has the capacity to elaborate antibody molecules which can specifically recognize the haptenic

group, the position of the haptenic group, and the number of lysyl residues on the oligolysine chain used to induce the immune response. The data presented demonstrate that antibody raised to α,DNP-Lys_{7-10} in responder animals can be readily distinguished from that produced to α,DNP-Lys_{16-30}, and similarly antibodies formed to 8,ϵ,DNP-Lys_8, 10,ϵ,DNP-Lys_{10}, 14,ϵ,DNP-Lys_{14}, and 15,ϵ,DNP-Lys_{15} are readily discriminated.

Although an upper limit for the size of an antibody-binding site has been estimated in several systems (6, 9, 18–20, 38), much less is known about the conformation of the antigenic determinant and its influence on the dimensions and specificity of the binding site. If we accept the premise that the antibody-binding site has a limited size or volume of about 7 residues or less, the addition of lysines to α,DNP-Lys_{7-10} or 8,ϵ,DNP-Lys_8 must result in compounds which are distinguished by antibody because of changes in the conformation of the lysines adjacent to the DNP group. Thus, we conclude that each immunogenic peptide possesses a preferred conformation or set of conformations that are recognized by the immunologically competent cell and that such cells have the capacity to elaborate a population of antibody molecules that can repeatedly distinguish the homologous immunizing antigen $\pm$ one lysyl residue. Previous estimates of the size of the antibody-combining site have been based on data supplied by a series of antibody–hapten interactions (quantitative inhibition of precipitation, equilibrium dialysis, and fluorescence quenching). It is now apparent that the lack of rigidity of the peptides or hapten–peptide conjugates places certain limitations on their use as measuring sticks. Just as it is unreasonable to suggest that anti-α,DNP-Lys_{16-30} antibody has a binding site 16–30 residues long, it is equally difficult to decide on an upper limit in site size of 7 residues without precise knowledge of the conformation of the antigenic determinant used for immunization and the fragment used to define the site.

The present data do not, however, permit us to estimate what percentage of the antibody is made to these "preferred sets of conformations." Moreover, if the antibody-forming apparatus can detect a preferred set of conformations, it is likely to respond to minor conformations of antigen in association with host protein or with the mycobacteria present in the adjuvant. The latter interactions may account for the heterogeneity of antibody as measured by the index of heterogeneity (a) in the Sips plot. Similarly, others have shown that single determinant haptenic antigens do not necessarily evoke homogeneous antibody responses (39–41).

The present studies confirm the observations of Green et al. demonstrating that responder guinea pigs developed delayed hypersensitivity and anti-DNP antibody when immunized with DNP-polylysines in an adjuvant containing *M. tuberculosis* H37Rv (30). Under these experimental conditions, nonresponder animals, lacking the PLL gene, elaborate anti-DNP antibody but do not de-

velop delayed skin reactivity or its in vitro correlates. Detailed analysis of the specificity of purified antibodies to α,DNP-Lys$_{16\text{-}30}$ and 14,ϵ,DNP-Lys$_{14}$ from both responder and nonresponder animals shows that the antibodies produced are different. The antibodies of all responder animals distinguish the precise DNP-oligolysine chain length used to induce the immune response $\pm$ one lysyl residue as reflected by a greater $-\Delta F^{\circ}$ for the interaction of antibody with the homologous immunizing antigen than for its interactions with others. Antibody made by nonresponder animals to the identical antigens, in contrast, shows no greater $-\Delta F^{\circ}$ with the homologous immunizing antigen and cannot distinguish the precise DNP oligolysine chain length used to induce the immune response. Although these differences permit distinction between responder and nonresponder antibody, it is still not known what fraction of the total antibody produced in the responder is directed at the precise DNP-oligolysine chain length used to induce the immune response. In the nonresponder, we presume, however, that interaction of DNP-polylysines with the adjuvant generates the antigenic determinant and that anti-hapten antibody-forming cells in the nonresponder recognize and respond not only to the DNP-polylysines but to portions of the adjuvant as well. More important, providing an immunogenic carrier for DNP-polylysines in the form of large amounts of H37Rv does not circumvent the genetically deficient recognition step in the nonresponder. If it did, the antibody formed to DNP polylysines in both groups of animals would have been identical. Evidently the polylysine gene also controls directly an antibody-forming system which can recognize the total antigenic determinant used to induce the immune response rather than only a carrier-specific cell which functions early in the immune response to present a non-immunogenic haptenic portion of a molecule to an anti-hapten antibody-forming cell. These observations are in keeping with a local environment hypothesis for antigen recognition by the antibody-forming cell (42). An alternative but less likely site of action of the PLL gene is at the carrier-specific cell, early in the immune response, controlling the manner in which antigen is presented to the anti-hapten antibody-forming cell (24). Under such circumstances DNP-oligolysine with H37Rv would affect the selection of the antibody-producing cell precursors in such a way that the antibody produced in responders and nonresponders would be different, despite the fact that both responders and nonresponders had an identical complement of anti–DNP-polylysine antibody-forming cells. It would be difficult to test for the latter possibility.

It is implicitly assumed by many immunologists that anti-hapten antibody is directed at the haptenic group alone and that the heterogeneity of antibody results from the multiplicity of responses to the haptenic group alone by the antibody-forming apparatus. In the present study we have shown that anti-hapten antibody purified with cross-reactive DNP-HSA–cellulose has carrier

specificity which is a function of the local chemical environment surrounding the haptenic group. The extent of the carrier specificity observed with anti–DNP-oligolysine antibody raises certain questions about the mechanism by which antibody is generated. In this regard, it has been shown that cellular cooperation occurs during the induction of the immune response (43–47) and that individual cells separately recognize carrier and haptenic portions of the antigen (14–17). The present studies indicate that the antibody-forming cell has an extraordinary degree of carrier specificity as measured by its capacity to elaborate a highly specific antibody molecule. Does the carrier-specific cell have an equivalent degree of carrier specificity and, if so, how can two such highly specific cells seek one another out to cooperate in antigen recognition? Alternatively, is the carrier-specific helper cell merely "oligolysine-specific" and capable of focussing DNP-polylysines on a wide variety of hapten-specific antibody-forming cells which recognize both the hapten and the adjacent carrier? While the evidence for cellular cooperation in the generation of antibody is impressive, the mechanism by which two cells cooperate in the recognition of an antigen by either of the above or still other schemes is obscure.

Finally, it is widely held that antibody synthesis is initiated by selection by the antigen of a population of cells with complementary and stereospecific receptors identical to the antibody molecule subsequently produced. In the present studies each DNP-oligolysine used induces at least one population of antibody molecules unique unto itself. Conservatively one could estimate that there preexist 20 separate antibody populations for the n, ϵ, DNP-Lys_n peptides and a similar number for the α, DNP-Lys_n series. Moreover, it was shown that anti-5, ϵ, DNP-Lys_9 antibody was specific (48), and a further extrapolation of this observation suggests that an additional 20 antibody populations may be generated by DNP groups on different ϵ, amino positions of the lysine chain. Thus, using a simple hapten on an oligolysine backbone of variable size, one could generate a minimum of 60 different antibody populations: 100–1000 may not be unrealistic. Probably, the oligolysine backbone in conjunction with another haptenic group such as penicilloyl would induce additional sets of antibody molecules (49). Given the hundreds of such haptens available, the minimal number of antibodies generated by the simplest synthetic immunogen–hapten complex could easily reach 10^4–10^5 separate molecules. Very likely, the degree of antibody specificity described in the present paper would occur with other synthetic polypeptide antigens as well as with naturally occurring antigens. The quantity of preexisting information required to account for the specificity of antibody is difficult to understand in terms of a germ line theory (50) and may be more compatible with theories depending on somatic mutation, recombination, or episomal insertion (51–56). The high specificity of antibody observed in these experiments demands an immune system capable of recognizing subtle changes in antigen and probably one more complex than is currently being postulated.

SUMMARY

The exact specifiicity of anti-DNP antibody produced by Hartley guinea pigs immunized with a series of defined α,DNP and ϵ,DNP-oligolysines was studied by fluorescence quenching. All responder animals made anti-DNP antibody which recognized the precise chain length, $\pm$ 1 lysyl residue, of the DNP-oligolysines used to induce the immune response as measured by an increase in binding energy ($-\Delta F^\circ$) for that antigen. The ability of the immune system to detect the smallest possible change in oligolysine chain length suggests that the anti-hapten antibody-forming cell possesses a highly specific recognition system for carrier conformation. When DNP-oligolysines are incorporated in an adjuvant containing *M. tuberculosis* H37Rv, both responder and nonresponder produce anti-DNP antibody, but only the responder develops delayed skin sensitivity. In addition to their failure to develop delayed hypersensitivity, nonresponders produced anti-DNP oligolysine antibody which did not show the increase in $-\Delta F^\circ$ for the immunizing antigen characteristic of responder antibody. These observations support a local environment hypothesis for antigen recognition at the level of the anti-hapten antibody-forming cell and suggest that the polylysine gene exerts its control at the same cell.

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