THE BEHAVIOR OF HAPTEN–POLY-L-LYSINE CONJUGATES AS COMPLETE ANTIGENS IN GENETIC RESPONDER AND AS HAPTENS IN NONRESPONDER GUINEA PIGS*

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About 30 to 40% of random bred Hartley strain (1) and all strain 2 guinea pigs (2) recognize hapten-poly-L-lysine conjugates (H-PLL) as antigens. Strain 13 guinea pigs (2), mice, rats, and rabbits (3) do not make an immune response to these compounds. The immune response of guinea pigs to H-PLL conjugates has been shown to be controlled by a dominant autosomal gene (2, 4). Contrasting with the strong antigenicity of H-PLL conjugates in "genetic responders," previous attempts to demonstrate an immune response to PLL itself in these animals have failed (5, 6), confirming the widely held belief (3) that homopolymers of L-amino acids are not antigenic.

The capacity to form an immune response to H-PLL conjugates was initially believed to depend on the ability of "genetic responders" to metabolize the PLL carrier in a way required to induce an immune response, but it was established that neither the enzymatic digestion of the PLL molecule (7) nor its uptake by lymph node macrophages (8) was impaired in unresponsive guinea pigs. It was postulated that if the gene governing the antigenicity of H-PLL controls a specific metabolic step on the PLL carrier prior to the recognition of specificity, this step must involve the formation of an unidentified immunogenic inducer (1, 7). However, the nature of the immunological specificity of the response to H-PLL conjugates led us to entertain the alternative possibility that this gene may control the recognition of the PLL specificity as an antigen. The immunological specificity of specifically isolated anti-2,4-dinitrophenyl-poly-L-lysine antibodies (DNP-PLL) was indeed shown to extend to the PLL carrier

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(9), illustrating that in this system as in hapten-protein systems the antihapten antibodies induced by the same hapten on different carriers have different immunological specificities (10). The marked carrier specificity involving the PLL molecule had already been observed in delayed hypersensitivity reactions to hapten-PLL conjugates (1). Studies on the immunological specificity of hapten systems emphasize the concept that antigenic determinants in these systems must necessarily involve both hapten and carrier. These considerations led us to a reevaluation of the hapten-carrier relationship in the hapten-PLL system and to consider the possibility that the single gene control of the response to hapten-PLL conjugates may involve the recognition of the partial specificity attributable to the PLL carrier. The antigenicity of unconjugated PLL was therefore reinvestigated and this homopolymer was found to provoke a very weak but definitely recognizable delayed hypersensitivity reaction in guinea pigs capable of responding to hapten-PLL conjugates.

A related study, which also gives some insight into the nature of the genetic control of the immune response to H-PLL, was performed. In these investigations we determined whether hapten-PLL conjugates could be shown to behave as a complex hapten in nonresponder guinea pigs if mixed with an immunogenic carrier. The term hapten was originally proposed by Landsteiner (11) in a paper which characterized the immunological behavior of alcoholic extract of horse kidney which although capable of combining with Forssman antibody, was itself nonantigenic. It was found later that the nonantigenic Forssman antigen could be made antigenic for rabbits by simply mixing it with pig serum (12). Other materials such as Wasserman antigens and other lipid antigens were also shown to display similar behavior (13, 14). Although the mechanism of this phenomenon was and is not clearly understood, the term "Schlepper Funktion" or conveyor was used by these early investigators to describe the immunogenicity enhancing action of the association of antigenic proteins with haptens. More recently, Plescia et al. (15) made use of this method to induce the formation of antibodies in rabbits against nonantigenic or poorly antigenic macromolecular substances. DNA, and pneumococcal polysaccharide behaved as haptens when allowed to form salt bridges with the positively charged antigen, methylated bovine serum albumin. We have taken advantage of the strong positive charge of hapten PLL molecules at neutral pH to allow them to react similarly with negatively charged foreign and guinea pig serum albumins, in order to investigate their behavior as macromolecular haptens in "nonresponder" guinea pigs and to study the immunological specificity of the antibodies produced.

To obtain background data, immunizations with hapten-polyamino acid conjugates without the use of adjuvants, and with adjuvants containing *Mycobacteria tuberculosis* substituted for *Mycobacteria butyricum*, were also performed.

Materials and Methods

Polypeptides and Proteins.—Two poly-L-lysine (PLL) hydrobromide preparations of average molecular weight of 50,000 and 90,000 respectively and poly-D-lysine (PDL) hydrobromide of an average molecular weight of 23,000 were obtained from Pilot Chemical Co., Watertown, Massachusetts.

Bovine serum albumin (BSA), and bovine fibrinogen (BF) were obtained from Armour Pharmaceutical Co., Chicago. Twice recrystallized hen ovalbumin (Ova) was obtained from Pentex, Inc., Kankakee, Illinois; human serum albumin (HSA) was purchased from Merck, Sharpe, and Dohme, Philadelphia. Guinea pig serum albumin (GPA) was prepared by starch block electrophoresis (16). Acetylated BSA was the gift of Dr. Paul Maurer, New Jersey College of Medicine and Dentistry.

Polyanions.—Carboxymethylcellulose and polystyrene sulfonate were the gift of Dr. Max Shubert, New York University School of Medicine. Hyaluronic acid and dextran sulfate (molecular weight: 37,000 to 43,000) were obtained from Nutritional Biochemicals Corp., Cleveland. Highly polymerized deoxyribonucleic acid (DNA) was purchased from Mann Research Laboratories, New York, and heparin sodium (10 mg/ml) from Vitarine Co., Inc., New York.

Other Reagents.—Dried, killed *Mycobacteria tuberculosis hominis* was the gift of Dr. Byron Waksman, Yale University School of Medicine. Incomplete and complete (containing *M. butyricum*) Freund's adjuvants were obtained from Difco Laboratories, Detroit. Kaolin was purchased from Mallinckrodt Chemical Works, St. Louis.

1-fluoro-2,4-dinitrobenzene (DNFB) was obtained from Eastman Organic Chemicals, Rochester, New York. DNFB-^{3}H (156 mc/mmole) was obtained from Nuclear-Chicago Corp., Chicago; 1-chloro-2,4-dinitrobenzene-^{3}H (DNCB-^{3}H) (19.5 mc/mmole) was obtained from New England Nuclear Corporation, Boston. *N*, ε-2,4-dinitrophenyl-L-lysine (ε-DNP-L-lysine) was purchased from Cyclo Chemicals, Los Angeles. 2,4-dinitrophenol (DNP-OH, Fisher Scientific Co., New York) was recrystallized once from hot water. ε-Aminocaproic acid (EACA) and carbobenzoxy (CBZ) chloride were purchased from Mann Research Laboratories.

Preparation of Conjugates.—DNP-PLL was prepared by reacting PLL with DNFB under alkaline conditions as described previously (1). The synthesis of DNP-PDL by the reaction of PDL and DNFB was carried out at pH 7.6 in order to minimize the possibility of racemization of D-lysine residues. DNP-PDL was freed of any unreacted DNFB by exhaustive dialysis, lyophilization, and acetone washing. DNP-GPA was prepared under alkaline conditions utilizing DNFB as previously described (17). Polypeptide or protein concentrations were determined from micro-Kjeldahl nitrogen measurements; the degree of DNP substitution was calculated from absorbancy at 360 mμ on the basis of the molar extinction coefficient of free ε-DNP-L-lysine [ε 360 mμ = 17,400 (18)]. The following DNP conjugates were prepared: $DNP_{0.6}$-PLL_{240} (used only for fluorescence studies), DNP_{11}-PLL_{240}, DNP_{14}-PLL_{240}, $DNP_{2.5}$-PDL_{109}, DNP_{41}-GPA. Subscripts refer to the average number of groups per molecule of polypeptide or protein.

Arsanilic acid azo-GPA (ASGPA) was prepared according to methods previously described (19). Acetylated GPA was prepared by reacting GPA with an excess of acetic anhydride at pH 10.

Carbobenzoxy-PLL was prepared by reacting carbobenzoxy chloride with PLL at pH 10; any unreacted material was removed by dialysis.

DNP-^{3}H-EACA was prepared by the reaction of EACA in 0.01 N NaOH with DNFB-^{3}H (dissolved in benzene) or DNCB-^{3}H (dissolved in dioxane). Reactants were mixed in the ratio 1 mole DNP:8 moles EACA. The reaction was allowed to proceed overnight at room temperature with constant stirring and the product was acid precipitated four times. DNP-^{3}H-EACA prepared with DNCB-^{3}H was recrystallized from slightly acidified water.

Preparation of DNP-PLL·Conveyor Complexes.—Equimolar amounts of BSA, HSA, GPA, and Ova were added to DNP-PLL in 0.075 M NaCl. 0.1 N NaOH was then added dropwise until a precipitate formed (20).

Acetylated BSA and acetylated GPA were added to DNP-PLL or to DNP-PDL in equal weight amounts; no alkali was added.

Polyanions were dissolved in saline and added to the DNP-PLL in the amounts shown in Table VI.

Immunization.—Male and female Hartley strain guinea pigs weighing 300 to 400 g were purchased from Camm Research Inc., Wayne, New Jersey. Strain 13 guinea pigs were obtained from the Laboratory of Immunology, National Institute of Allergy and Infectious Diseases, Bethesda, Maryland.

The basic immunization procedure consisted of the injection of 0.4 ml of an emulsion of equal parts DNP-polylysine (mol wt 50,000) in 0.15 M saline and complete Freund's adjuvant (*M. butyricum*) into the four foot-pads. The total dose was 100 μg. Guinea pigs were tested twice with 10 μg DNP-PLL injected intradermally in 0.1 ml during the 2 wk after initial immunization. They were bled between days 21 and 25.

In order to determine if adjuvants were required to obtain an immune response to DNP-PLL, guinea pigs received intraperitoneal injections of 2 mg of DNP-PLL in 0.15 M NaCl and front foot-pad injections of 500 μg of DNP-PLL in saline. The animals received 100 μg injections of DNP-PLL in saline in each of 4 skin sites at 7 and at 14 days and were bled at 18 to 23 days. They were then injected with 100 μg DNP-PLL in complete adjuvant (*M. butyricum*) as described in the basic schedule.

The effect of substitution of *M. tuberculosis* for *M. butyricum* in adjuvant was studied by immunizing a group of guinea pigs according to the basic schedule with the exception that *M. tuberculosis* in incomplete Freund's adjuvant (2 mg/ml) was used instead of the usual complete Freund's adjuvant (*M. butyricum*).

Finally, immunization with DNP-PLL bound to various conveyor molecules (both protein and polyanion) was carried out according to the basic schedule except that booster doses were 60 to 100 μg DNP-PLL·conveyor in addition to the 10 μg of DNP-PLL usually employed.

Immunization with Unconjugated PLL.—The determination of an immune response to PLL has proven to be a difficult problem because of the large positive charge of this molecule at neutral pH. Thus nonspecific precipitation with many serum proteins (3, 20) often occurs and anaphylactic reactions may be affected by interaction of PLL with tissue components. However, intradermal injections of 10 μg or less cause only slight nonspecific inflammatory skin reactions. Thus, immunization of 29 guinea pigs with 100 μg PLL emulsified in incomplete adjuvant with *M. tuberculosis* (2 mg/ml) was performed and skin reactivity tested at 9 and 16 days with 10 μg PLL injected intradermally. The guinea pigs were then immunized intradermally and intraperitoneally with 100 μg DNP-PLL emulsified in complete adjuvant (*M. butyricum*) and skin tested with 10 μg DNP-PLL 1 and 2 wk subsequently. All animals were then bled.

Assay of the Immune Response to DNP-PLL Conjugates.—The assay of the immune response of guinea pigs to H-PLL in previous studies (1, 2, 4, 7) has relied on precipitin reactions where they could be demonstrated or on various types of hypersensitivity reactions: delayed hypersensitivity, systemic anaphylaxis, and passive cutaneous anaphylaxis (PCA). Delayed hypersensitivity reactions have generally been found to be the most reliable test of an immune response to H-PLL in adjuvant immunized animals. Considering that these various methods may measure only certain classes of antibody, (systemic anaphylaxis and PCA only γ_1-antibodies (21)), a comparison of various antibody assays was made in the DNP-PLL system. It was observed that certain sera in which anti-DNP antibodies were demonstrable by gel diffusion against DNP-GPA failed to sensitize for PCA reactions with DNP-GPA. Similarly some

animals whose sera gave DNP-specific precipitin lines in gel developed only mild, or no systemic anaphylaxis when challenged with 250 μg DNP-GPA intravenously. The failure of anaphylactic reactions to measure antibody demonstrable by precipitin methods could be explained by the expected low levels of γ_1-, and high levels of γ_2-antibodies produced by guinea pigs in the early stages of immunization with antigen in complete adjuvant (22).

Some anti–DNP-PLL sera which produced specific precipitin lines with DNP-GPA failed to agglutinate DNP-GPA coated tanned sheep erythrocytes; anti–DNP-BGG sera in high dilution caused satisfactory agglutination of the same cell preparations. Such findings emphasize that various assays of serum antibody which involve reactions subsequent to simple binding of antibody to antigen may be unreliable when one is attempting to demonstrate the ability or inability to make an immune response to a given antigen. In order to avoid possible ambiguities of this sort, simple measurements of antigen binding by antibody were employed in the current study. Equilibrium dialysis was chosen as an unequivocal method for making these measurements. Three criteria were therefore used for evidence of an immune response to DNP-PLL in guinea pigs injected with the antigen in complete adjuvants: delayed reactions to DNP-PLL, double diffusion in agar gel with DNP-GPA, and equilibrium dialysis.

Delayed hypersensitivity of guinea pigs was determined by skin reaction 24 hr after intradermal injection of 10 μg of hapten-polylysine. Double diffusion in agar gel was performed utilizing commercially prepared agar plates (Immunoplates, pattern c, Hyland Laboratories Los Angeles). DNP_{41}-GPA, 250 μg/ml, was placed in central wells and antisera in peripheral wells. PCA (23), systemic anaphylaxis (24), and hemagglutination of DNP-GPA sensitized, tanned sheep erythrocytes (25) were performed according to the techniques cited.

Equilibrium Dialysis.—(26) of globulin fractions of antisera against DNP-^{3}H-EACA was performed utilizing washed Visking dialysis tubing and glass test tubes. Globulin fractions were precipitated 2X from 33% saturated $(NH_4)_2SO_4$ and dissolved in 0.15 M NaCl, 0.01 M PO_4, pH 7.2 (PBS) in a volume 5 times the original serum volume. They were then extensively dialyzed against PBS in the cold. 1 ml of globulin preparation was placed inside the dialysis sac and 0.9 ml of DNP-^{3}H·EACA in PBS outside of the sac. Tubes were sealed and rotated for 18 hr at room temperature. Volume changes within the sac were determined by difference in weights of the dialysis bags before and after dialysis. 0.5 ml samples from inside and outside the sac were placed in 15 ml of Bray's solution (27) and counted in a Tricarb liquid scintillation counter (Packard Instrument Co., La Grange, Illinois). Bound hapten was calculated from the expression:

$$\frac{\text{Bound hapten}}{\text{mM/ml}} = \frac{5\ (\text{counts inside sac} - \text{counts outside sac}) \times (\text{volume inside sac})/0.5\ \text{ml}}{\text{Specific activity (counts/mM)}}$$

Where small amounts of antibody were expected, DNP-^{3}H-EACA at 2×10^{-7} M was used; when large amounts of antibody were anticipated, a hapten concentration of 5×10^{-6} M was used. The values obtained measure total antibody when small amounts of antibody were present; however, when large amounts of antibody were present, there was insufficient hapten to occupy all sites and thus a minimum estimate of antibody content is obtained in such cases. In each group of equilibrium dialyses, the globulin fraction from the serum of a normal guinea pig and a buffer control were run. The globulin fraction from each experimental animal was analyzed separately.

Globulin fractions from the sera of 41 guinea pigs immunized with complete adjuvant a one, unconjugated PLL, or BSA in complete adjuvants were subjected to equilibrium dialysis to obtain values for nonspecific binding. The mean was 240×10^{-10} mM/ml serum with a standard deviation of 205×10^{-10}. Values which were in excess of 650×10^{-10} mM/ml (+ 2 SD) were considered to demonstrate specific binding by antibody (Table II). Considering the large

amount of data, only mean values for each group have been tabulated. In each instance in which only some animals in a group showed serum antibody by equilibrium dialysis the number of such animals is indicated.

Purification of Anti-DNP Antibody.—Anti-DNP antibodies were specifically purified according to the methods of Farah et al. (28). Antibody was precipitated from sera, in the presence of 0.01 M disodiumethylenediaminetetraacetate, by equivalence amounts of DNP-BF. It was specifically eluted with 0.1 M DNPOH; DNP-BF was removed by precipitation with streptomycin sulfate (35 mg/ml). DNPOH was removed by dialysis against PBS followed by chromatography with Dowex 1-X8 (200 to 400 mesh). Purified antibody preparations were essentially free of DNPOH and were shown by immunoelectrophoresis against rabbit anti-whole guinea pig serum to contain only γ_1- and γ_2-globulins.

Immunological Specificity of Anti-DNP-PLL Antibodies.—To evaluate the specificity of anti-DNP antibodies produced in these experiments, titrations of the quenching of the native fluorescence of antibody by hapten were performed according to the technique of Velick et al. (29). Fluorescence at approximately 350 mμ of antibody excited at 280 mμ was measured in a thermostated Aminco-Bowman spectrophotofluorometer at 26°C. Duplicate 2.0 ml samples of specifically purified anti-DNP antibody at a concentration of 30 μg/ml in PBS, pH 7.6, were titrated with ε-DNP-L-lysine and with $DNP_{0.6}$-PLL_{240} as described previously (9, 10). This DNP-PLL conjugate was shown in previous studies to be suitable for this type of measurement (9). The degree of quenching of antibody fluorescence by these two ligands was compared in terms of the amount of DNP groups added.

Average intrinsic association constants (K_0) were calculated from fluorescence quenching data according to the Sips equation (30, 31): $\log r/(n - r) = a \log c + a \log K_0$, where r is antibody sites bound/antibody molecules, c is concentration of free hapten, and a is Sips heterogeneity index. K_0's determined from fluorescence quenching titrations of guinea pig anti-DNP antibodies have been demonstrated to be accurate as shown by comparison with equilibrium dialysis. Similar results were obtained with the two methods when a value for maximum possible quenching of antibody fluorescence of 100% was used (10).

RESULTS

Immune Response of Guinea Pigs to DNP-PLL with and without Adjuvants.—Immunization of guinea pigs with DNP-PLL in saline gave the following results: Of a total of 34 animals, 10 produced circulating anti-DNP antibodies as judged by the results of double diffusion analysis in agar and PCA studies. The sera of the rest of the animals were entirely negative. The animals were then reimmunized with DNP-PLL with complete adjuvant containing *M. butyricum*. With one exception, the same animals that produced antibody now gave delayed reactions to the immunizing antigen. One animal that had initially produced antibody failed to give a delayed reaction (Table I).

In the past all guinea pigs not developing delayed sensitivity to DNP-PLL had not produced any anti-DNP antibody as judged by the techniques utilized. In order to more firmly establish that non-skin reactor guinea pigs did not produce any anti-DNP antibody, 13 guinea pigs were immunized with DNP-PLL in the usual fashion with complete adjuvant (*M. butyricum*). Six of these 13 animals responded by developing delayed sensitivity and by producing circulating antibody. The other 7 animals failed to develop a delayed skin reaction

and did not produce any anti-DNP antibody as judged by equilibrium dialysis (Table II), thus confirming the results of previous studies.

Next, the effect of substitution of *Mycobacterium tuberculosis* for *Mycobacterium butyricum* in the adjuvant was tested. Twenty guinea pigs were immunized with DNP-PLL and incomplete Difco adjuvant containing 2 mg/ml of dried *M. tuberculosis*. Fourteen of these animals made an immune response as demonstrated by the development of delayed sensitivity and the production of circulating antibody (Table II). Because the percentage of positive animals was higher than usually observed in random bred guinea pigs, the following experiments were performed: (*a*) Animals were immunized in the usual fashion with DNP-PLL in complete adjuvant (*M. butyricum*). Ten nonresponding animals

TABLE I

Antibody Production and Delayed Hypersensitivity Reactions of Guinea Pigs Immunized First with DNP-PLL in Saline and Then with DNP-PLL in Adjuvant

First immunizing antigen	No. animals	Gel diffusion*	PCA‡	Second immunizing antigen	Delayed reactions to 10 μg DNP-PLL
		No. pos./ total	*No. pos./ total*		
3 mg DNP-PLL in saline	34	10§/34	10§/34	100 μg DNP-PLL in adjuvant containing *M. butyricum*	9§/34

* 250 μg/ml DNP_{41}-GPA.

‡ Positive in titers of 1:40 to 1:160 with 250 μg DNP_{41}-GPA.

§ These are the same animals; one of these animals that produced antibodies later failed to show a delayed response to DNP-PLL.

were selected and were subsequently reimmunized with 100 μg DNP-PLL in adjuvant in which *M. tuberculosis* 2 mg/ml was substituted for the *M. butyricum*. These previously negative animals were then retested. They did not become positive. (*b*) Another group of 10 guinea pigs was immunized with DNP-PLL without adjuvant according to the schedule previously described. Two of these animals made circulating antibodies as detected by gel diffusion and PCA. The other 8 animals did not produce antibodies. All the animals were then immunized with 100 μg DNP-PLL in adjuvant containing 2 mg/ml *M. tuberculosis*. The animals were subsequently skin tested with 10 μg DNP-PLL; only the 2 animals that had previously responded showed delayed reactions to DNP-PLL. The remainder of the animals were negative. The substitution in the adjuvant used for immunization of *M. tuberculosis* for *M. butyricum* does not convert previously DNP-PLL nonresponding animals to responding animals. The somewhat higher percentage of positive animals obtained in the initial experiment using DNP-PLL in adjuvant containing *M. tuberculosis* was

apparently due to the fortuitous presence of a larger number of reactors in this group.

Animals immunized with DNP-PDL and complete adjuvant containing *M. butyricum* did not develop delayed sensitivity or produce circulating antibodies,

TABLE II

Delayed Sensitivity to DNP-PLL and Serum Level of Anti-DNP Antibodies in Animals Immunized with DNP-PLL or DNP-PDL with Adjuvants Containing M. Butyricum or M. Tuberculosis

Immunizing antigen	No. animals	No. of responder animals as judged by delayed reactions to DNP-PLL 10 μg	Gel Diffusion*	Equilibrium dialysis c DNP-EACA (2×10^{-7} M)	Equilibrium dialysis c DNP-EACA (5×10^{-6} M)
				mM Hapten bound/cc of serum $\times 10^{-10}$‡	
			No. pos./ total		
100 μg DNP-PLL in adjuvant containing *M. butyricum*	13	Responders 6 Nonresponders 7	6/6 0/7	6508§ 370	36,691 (5)§
100 μg DNP-PLL in adjuvant containing *M. tuberculosis*	20	Responders 14 Nonresponders 6	14/14 0/6	6942 450‖	
100 μg DNP-PDL in adjuvant containing *M. butyricum*	10		0/10	195	
100 μg DNP-PDL in adjuvant containing *M. tuberculosis*	9		0/9	366	
Control Group				*Average for 41 Animals*	
100 μg PLL in adjuvant	25			240	
Adjuvant in saline	6			+1 Standard deviation = 445	
10 μg BSA in adjuvant	*10*			+2 Standard deviation = 650	
Total.....	41				

* 250 μg/ml DNP_{41}-GPA.

‡ 7.5 μg of antibody can bind 1000×10^{-10} mM of hapten when both sites are occupied considering 150,000 mol wt for guinea pig antibody.

§ This figure represents an average value. The number in parentheses is the number of individual animals tested when all animals were not tested.

‖ One individual value fell outside of 2 standard deviations from the mean of the control animals.

confirming the lack of antigenicity of hapten-PDL conjugates (32, 33) (Table II).

Antigenicity of PLL.—Fourteen out of 29 guinea pigs immunized with PLL in complete adjuvant with *M. tuberculosis* showed delayed hypersensitivity reactions to 10 μg of PLL, when tested at 2 and 3 wk after immunization. The reactions were mildly indurated and ranged from about 7 to 12 mm in diameter (Table III). To control for the presence of an unknown impurity in the PLL

preparation used for immunization the animals were also tested with another PLL with different average molecular weight (90,000) with identical results. Similar reactions were also obtained with a PLL_{10-30} preparation synthesized by Dr. Stuart Schlossman, Harvard Medical School, the polymerization of which had been initiated with NAOH. A haptenic material possibly associated with PLL which might account for these results is the carbobenzoxy (CBZ) group. PLL is synthesized by the manufacturer according to the method of Katchalski (34) which involves blocking the ϵ-amino group of the N-carboxy anhydride of lysine with carbobenzoxy chloride prior to polymerization. The CBZ groups are split off after polymerization and the preparation is believed to

TABLE III

Delayed Sensitivity to PLL and DNP-PLL, and Serum Levels of Anti-DNP Antibodies in Animals Immunized First with PLL and Then with DNP-PLL

First immunizing antigen	No. animals	Delayed reaction to PLL 10 μg	Second immunizing antigen	No. of responder animals as judged by delayed reactions to DNP-PLL 10 μg	Gel Diffusion*	Equilibrium dialysis ‡ DNP-EACA mM Hapten bound/cc of serum × 10^{-10}
		No. pos./total			*No. pos./total*	(2×10^{-7} *M*)
100 μg PLL in adjuvant containing *M. tuberculosis*	29	14/29	100 μg DNP-PLL in adjuvant containing *M. butyricum*	Responders 14§ Nonresponders 15§	14/14 0/15	139

* 250 μg/ml DNP_{41}-GPA.

‡ 7.5 μg of antibody can bind 1000 × 10^{-10} mM of hapten when both sites are occupied considering 150,000 mol wt for guinea pig antibody.

§ These are the same animals that showed a delayed reaction or failed to show a delayed reaction to PLL alone

be free of conjugated CBZ. The possibility had to be considered that the PLL preparation used for immunization contained traces of CBZ-PLL and that the delayed reactions observed to PLL could in fact be the result of immunization and testing with CBZ-PLL. In order to test this, CBZ-PLL were prepared by the reaction of CBZ chloride with PLL hydrobromide. PLL-sensitized guinea pigs were tested with 10 μg intradermal injections of PLL and of CBZ-PLL. The reactions to both products were identical in size and intensity. No delayed reactivity attributable to CBZ could be demonstrated as the relatively highly conjugated CBZ-PLL would have been expected to give a considerably more intense delayed reaction than PLL possibly containing traces of CBZ. Rather, the results indicate that the immune response was to PLL. No antibodies specific for PLL or for CBZ-GPA could be detected by active cutaneous anaphylaxis in the PLL responder guinea pigs.

Considering the relative mildness of the delayed reactions to PLL and the known irritating properties of PLL, it was felt that the evidence of an immune response to PLL would be strengthened if the guinea pigs showing delayed re-

action to this homopolymer were the same animals genetically capable of producing antibodies to DNP-PLL. Indeed when all the PLL immunized guinea pigs were reimmunized with DNP-PLL in adjuvants the same 14 positive guinea pigs produced anti-DNP antibodies. The 15 animals which had not shown delayed reactions to PLL failed to show delayed reactions to DNP-PLL or to produce anti-DNP antibodies detectable by equilibrium dialysis and were considered nonresponders (Table III).

The Effect of Interaction with Foreign and Homologous Serum Albumin on the Immune Response to DNP-PLL and DNP-PDL.—When foreign serum albumins (BSA, acetylated BSA, HSA, or ovalbumin) were allowed to interact with DNP-PLL to form electrostatic complexes and the animals were immunized with such preparations in complete adjuvant, a new pattern of immune response to DNP-PLL was observed. The usual percentage of Hartley strain guinea pigs (30 to 40%) became delayed hypersensitive to DNP-PLL but all the animals, including the ones without delayed hypersensitivity to DNP-PLL, produced a considerable amount of antibody against the dinitrophenyl hapten, ranging from about 170 to 830 μg of antibody/ml as estimated by equilibrium dialysis measurements (Table IV). No significant differences could be noted between the amounts of anti-DNP antibody produced by the guinea pigs with and without delayed reactions to DNP-PLL. Four strain 13 ("genetic nonresponders") guinea pigs immunized with DNP-PLL·BSA in complete adjuvants also made significant amounts of anti-DNP antibodies as measured by equilibrium dialysis with DNP-^{3}H-EACA and showed absence of delayed hypersensitivity to DNP-PLL. All guinea pigs also showed strong delayed reactions to the foreign albumins used for immunization. These findings are in distinct contrast with all previous results of immunization with DNP-PLL in complete adjuvants without foreign albumins (1, 2) (Tables II, VI). In the previous experiments there was always a complete correlation between the development of delayed hypersensitivity to H-PLL conjugates and the production of antihapten antibodies in responder guinea pigs. Thus, immunization with DNP-PLL bound to foreign albumins by electrostatic forces can induce nonresponder guinea pigs to produce anti-DNP antibodies but cannot render them "complete responders," since they cannot show delayed reactions to the H-PLL conjugates. These experiments demonstrate that DNP-PLL which is a complete antigen in "responders" can behave as a hapten in nonresponder guinea pigs.

A similar pattern of immune response is observed in guinea pigs immunized with DNP-PLL mixed with guinea pig albumin, or with guinea pig albumin conjugated with acetyl or benzarsonate groups, but the level of anti-DNP antibodies produced was very much lower, especially in animals immunized with DNP-PLL·GPA (Table IV), and could only be detected by equilibrium dialysis. Furthermore not all animals immunized with DNP-PLL bound to guinea pig albumin produced anti-DNP antibodies. The sera of 4 out of 12

TABLE IV

The Effect of Various Albumins Combined with DNP-PLL on the Production of Anti-DNP Antibodies by Responder and Nonresponder Guinea Pigs

Immunizing antigen*	No. animals	No. of responder animals as judged by delayed reactions to DNP-PLL 10 μg		Gel diffusion‡	Equilibrium dialysis c̄ DNP-EACA (2×10^{-7} M)	Equilibrium dialysis c̄ DNP-EACA (5×10^{-6} M)
					mM Hapten Bound/cc of Serum $\times 10^{-10}$§	
				No. pos./ total		
100 μg DNP-PLL combined c̄ 100 μg acet. BSA	32	Responders	10	10/10		88,389 (4)‖
		Nonresponders	22	22/22		111,508 (5)
100 μg DNP-PLL combined c̄ 120 μg BSA	39	Responders	10	10/10		97,890 (4)
		Nonresponders	29	29/29		67,173 (9)
100 μg DNP-PLL combined c̄ 120 μg HSA	9	Responders	3	3/3		52,228
		Nonresponders	6	6/6		23,041
100 μg DNP-PLL combined c̄ 80 μg ovalbumin	10	Responders	6	6/6		76,135
		Nonresponders	4	4/4		54,124
100 μg DNP-PLL combined c̄ 120 μg GPA	16	Responders	4	4/4	7041‖	
		Nonresponders	12	0/12	978¶	
100 μg DNP-PLL combined c̄ 100 μg arsanilic-GPA	16	Responders	6	6/6	8179	
		Nonresponders	10	1/10	1182**	
100 μg DNP-PLL combined c̄ 100 μg acet. GPA	12	Responders	2	2/2	7150	
		Nonresponders	10	2/10	2308‡‡	

* Adjuvant containing *M. butyricum* used with these antigens.

‡ 250 μg/ml DNP_{41}-GPA.

§ 7.5 μg of antibody can bind 1000×10^{-10} mM of hapten when both sites are occupied considering 150,000 mol wt for guinea pig antibody.

‖ This figure represents an average value. The number in parenthesis is the number of individual animals tested when all animals were not tested.

¶ Four individual values outside of 2 standard deviations from mean of the control animals (see Table I).

** Eight individual values under 2 standard deviations from mean of the control animals (see Table I).

‡‡ 2 individual values under 2 standard deviations from mean of the control animals.

nonresponder animals immunized with DNP-PLL·GPA, 2 out of 10 immunized with DNP-PLL·AS-GPA and 8 out of 10 immunized with DNP-PLL·acetyl-GPA showed hapten-binding values outside of 2 standard deviations from the mean of control animals (Table IV).

Immunization with DNP-PDL associated with acetylated BSA also induced the formation of anti-DNP antibodies in guinea pigs; no delayed hypersensitivity was observed to DNP-PDL. However, the level of anti-DNP antibodies in the sera of these animals was just at the limit that could be considered significant, by equilibrium dialysis measurements (see Table V). Guinea pigs immunized with DNP-PDL mixed with BSA did not produce significant amounts of anti-DNP antibodies (Table V).

TABLE V

The Effect of BSA and Acetylated BSA Combined with DNP-PDL on the Production of Anti-DNP Antibodies

Immunizing antigen	No. animals	Delayed reaction to DNP-PDL 10 μg	Gel diffusion*	Equilibrium dialysis c̄ DNP-EACA (2×10^{-7} M)
		No. pos./ total	*No. pos./ total*	*mM Hapten bound/cc of serum* $\times 10^{-10}$‡
100 μg DNP-PDL combined c̄ 120 μg BSA	18	0/18	0/18	484§
100 μg DNP-PDL combined c̄ 100 μg acet. BSA	6	0/6	0/6	678‖

* 250 μg/ml DNP_{41}-GPA.

‡ 7.5 μg of antibody can bind 1000×10^{-10} mM of hapten when both sites are occupied considering 150,000 mol wt for guinea pig antibody.

§ These figures represent average values of the number of animals shown in each group.

‖ The values from 3 sera fell above 2 standard deviations from the mean of the control (see Table I).

Considering that albumins used as conveyor molecules for DNP-PLL in these experiments reacted with DNP-PLL to form insoluble complexes, control experiments were carried out investigating the result of immunization with DNP-PLL precipitated by various nonantigenic macromolecular polyanionic polymers: DNA, dextran sulfate, heparin, carboxymethylcellulose, polystyrene, and hyaluronic acid. The results of these experiments are presented in Table VI. Irrespective of the polymer used only the usual 30 to 40% of guinea pigs gave an immune response to DNP-PLL and there was again a complete correlation between delayed hypersensitivity to DNP-PLL and the production of anti-DNP antibodies by responder guinea pigs. The sera of nonresponder guinea pigs showed hapten-binding values comparable to those observed in non-immunized control animals, demonstrating the absence of anti-DNP antibodies in these animals.

PLL Specificity of Anti-DNP Antibodies Produced by Nonresponder Guinea Pigs Immunized with DNP-PLL Complexed with Foreign Albumins as Conveyors. —Studies of the immunological specificity of anti-DNP-PLL antibodies pro-

TABLE VI

The Effect of Various Polyanions Combined with DNP-PLL on the Production of Anti-DNP Antibodies by Responder and Nonresponder Guinea Pigs

Immunizing antigen*	No. animals	No. of responder animals as judged by delayed reactions to DNP-PLL 10 μg		Gel Diffusion‡	Equilibrium dialysis c̄ DNP-EACA (2×10^{-7} M)
				No. pos./ total	*mM Hapten bound/cc of serum* $\times 10^{-10}$§
100 μg DNP-PLL combined c̄ 200 μg dextran sulfate	5	Responders	2	2/2	3440‖
		Nonresponders	3	0/3	122
100 μg DNP-PLL combined c̄ 2 mg heparin	5	Responders	1	1/1	Not done
		Nonresponders	4	0/4	Not done
190 μg DNP-PLL combined c̄ 1 mg DNA	5	Responders	1	1/1	4380
		Nonresponders	4	0/4	182
100 μg DNP-PLL combined c̄ 100 μg polystyrene sulfonate	4	Responders	2	2/2	3428
		Nonresponders	2	0/2	107
100 μg DNP-PLL combined c̄ 100 μg carboxymethyl cellulose	5	Responders	2	2/2	6389
		Nonresponders	3	0/3	192
100 μg DNP-PLL combined c̄ 200 μg hyaluronic acid	5	Responders	2	2/2	3917
		Nonresponders	3	0/3	366
100 μg DNP-PLL combined c̄ 2 mg Kaolin	5	Responders	3	3/3	6041
		Nonresponders	2	0/2	122

* Adjuvant containing *M. butyricum* used with these antigens.

‡ 250 μg/ml DNP_{41}-GPA.

§ 7.5 μg of antibody can bind 1000×10^{-10} mM hapten when both sites are occupied considering 150,000 mol wt for guinea pig antibody.

‖ This figure represents an average value of the number of animals shown in the responder and nonresponder groups.

duced by responder guinea pigs were made (9) utilizing the technique of fluorescence quenching. Immunological specificity of anti-DNP-PLL antibodies for the PLL carrier was demonstrated by a higher K_0 of these antibodies with $DNP_{0.6}$-PLL_{240} than with ε-DNP-L-lysine. The free energy contribution of the PLL carrier to the interaction of anti-DNP-PLL antibodies with $DNP_{0.6}$-PLL_{240} was from −0.8 to −2.1 kcal/mole (9). This value should be viewed as a minimum estimate of energy of carrier specificity contributed by the PLL mole-

cule because $DNP_{0.6}$-PLL_{240} which was used for fluorescence quenching measurements must be considered as highly cross-reactive with and not identical to the more highly substituted antigens used for immunization. In contrast, anti-DNP antibodies produced by guinea pigs immunized with DNP-BSA and other DNP-proteins were quenched to a greater degree by ε-DNP-L-lysine than by $DNP_{0.6}$-PLL_{240} (9).

TABLE VII

Average Intrinsic Association Constants (K_0) *of Purified Anti–DNP Protein Antibodies and Anti–DNP-PLL Antibodies Prepared in Nonresponder Guinea Pigs Utilizing Conveyor Molecules*

Immunizing antigen	Identification No.	$K_0 \times 10^{-6}$ $DNP_{0.6}$-PLL_{240}	$K_0 \times 10^{-6}$ ε-DNP-L-lysine	$\frac{K_{0_{DNP_{0.6}\text{-}PLL_{240}}}}{K_{0_{\epsilon\text{-DNP-L-lysine}}}}$
		liters/mole	*liters/mole*	
DNP-PLL·Acet. BSA	Pool 4	28.	3.0	9.3
DNP-PLL·Ova	Pool 1A	>100.*	8.6	>11.6
DNP-PLL·HSA	Pool 7	3.2	0.71	4.5
DNP-PLL·BSA	123–5	>100.*	9.6	>10.4
DNP-PLL·BSA	123–7	35.	4.1	8.5
DNP-PLL·BSA	123–8	48.	1.5	32.0
DNP-PLL·BSA	123–9	27.	3.0	9.0
DNP-PLL‡	Pool 1	24.	3.2	7.5
DNP-BSA§	75	0.059	0.80	0.074
DNP-Ova§	66	1.6	8.2	0.20

* Fluorescence quenching titrations of guinea pig anti-DNP antibodies do not accurately measure K_0's in excess of 10^8 liters/mole.

‡ Anti–DNP-PLL antibodies were prepared in responder guinea pigs. Data is taken from reference 9.

§ Anti–DNP-BSA and anti–DNP-Ova antibodies were prepared in Hartley strain guinea pigs. Data from reference 9.

These observations on carrier specificity of anti–DNP-PLL and anti-DNP protein antibodies made it essential to study as part of these experiments the specificity of anti-DNP antibodies produced by nonresponder guinea pigs immunized with DNP-PLL reacted with foreign proteins to ascertain whether these antibodies also showed specificity for the PLL molecule or whether they differ in this respect from the anti–DNP-PLL antibodies produced by responder guinea pigs.

Pooled, specifically purified anti-DNP antibodies from animals immunized with DNP-PLL associated with acetylated BSA, HSA, and ovalbumin were examined; in addition, purified antibodies from 4 individual animals immunized with DNP-PLL·BSA were titrated. In all cases these antibodies were obtained

from guinea pigs without delayed hypersensitivity to DNP-PLL ("nonresponders"). The results of these experiments are presented in Table VII and Fig. 1. The fluorescence of each of these antibody preparations was quenched to a greater degree by $DNP_{0.6}$-PLL_{240} than by ε-DNP-L-lysine, demonstrating their specificity for the PLL molecule. No difference could be detected in this respect between these antibodies and anti–DNP-PLL antibodies produced by responder animals immunized with DNP-PLL without carrier proteins.

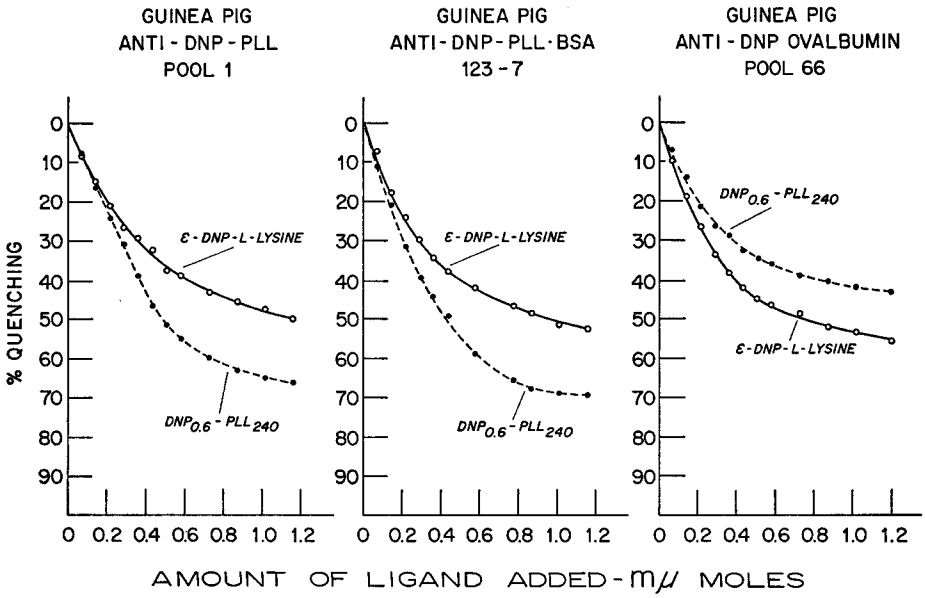

FIG. 1. Fluorescence quenching of anti-DNP antibodies by ε-DNP-L-lysine and by $DNP_{0.6}$ PLL_{240}. Measurements were carried out on 2 ml samples containing 30 μg purified antibody/ml in PBS at 26°C. Amounts of ligands added are expressed in terms of DNP groups.

DISCUSSION

DNP-PLL, a complete antigen in genetic responder guinea pigs, behaves as a hapten in nonresponder guinea pigs. Thus, guinea pigs genetically unable to recognize the antigenicity of DNP-PLL nevertheless form anti-DNP antibodies when immunized with electrostatic aggregates of this positively charged conjugate with several negatively charged albumins. In spite of making anti-DNP antibodies, such animals do not show delayed hypersensitivity to DNP-PLL which is normally exhibited by genetic responder guinea pigs immunized with this antigen. That the protein carrier molecules do not play a passive role in these immunogenic complexes, is shown by the lack of antigenicity of complexes of DNP-PLL and nonprotein polyanions for genetic nonresponder animals.

The use of covalently bound protein carriers to induce the formation of antibodies against haptenic determinants is a classical immunological technique introduced by the pioneer studies of Landsteiner (14). It has generally been assumed, however, that haptens are low molecular weight compounds which need to be bound to the protein carrier by covalent linkage. The production of anti-DNA antibodies by immunization with DNA complexed with methylated BSA (15), and the results of our experiments where positively charged DNP-PLL complexed with negatively charged foreign albumins stimulated the synthesis of antihapten antibodies demonstrate that haptens can be indeed of very large size and that the bonds between hapten and carrier molecule can be the result of electrostatic interaction. Similar results were reported by Maurer for an aggregate of poly-L-glutamic acid and methylated BSA (35). Since the reaction of a carboxyl ion with an ionized amino group involves about 10 kcal of energy (36), several such interactions can provide an energy of binding equal to that of a covalent bond.

Contrasting with the high level of anti-DNP antibodies produced by nonresponder guinea pigs immunized with DNP-PLL and foreign albumins, animals immunized with DNP-PDL and acetylated BSA produced barely detectable levels of DNP antibodies showing that DNP-PLL and DNP-PDL are not equally immunogenic haptens.

Landsteiner reported in his early studies with an alcoholic extract of the Forssman hapten that only antigenic proteins could act as effective carriers while homologous proteins could not. Similar results have been obtained with DNP-PLL in nonresponder guinea pigs; foreign albumins behave as much more efficient conveyors to induce the formation of anti-DNP antibodies than does guinea pig albumin or its benzarsonate or acetyl conjugates. However, haptens covalently linked to nonimmunogenic autologous proteins are known to induce a good immune response (17). More drastic alterations in the structure of nonimmunogenic autologous proteins resulting from covalent conjugation with the hapten may in part account for these differences.

The concept of hapten introduced by Landsteiner has been a great stimulant to immunological thought, but it has had the consequence of focusing the concern of investigators on the hapten and the carrier as distinct entities and distinct antigenic determinants. When antihapten antibodies were investigated for carrier specificity it became apparent that their immunological specificity extended clearly to areas of the carrier molecule adjacent to the site of attachment of the hapten (9, 10, 37–39). The antigenic determinants able to best react with the specific antibody and able also to induce and boost (40) the specific immune response in hapten-protein systems and in the H-PLL system includes both hapten and the covalently bound carrier molecule both of which can be considered as partial determinants supplying part of the required energy of interaction with the specific antibody.

Thus, it is not surprising that poly-L-lysine was found to be weakly antigenic in guinea pigs able to respond to haptenic conjugates of the polymer. The immune response to PLL was only demonstrable by mild delayed hypersensitivity reactions; in guinea pigs immunized with weak antigens in complete adjuvants this is the most sensitive test as was shown in studies of antigenicity of denatured autologous gamma globulin (41). The gene controlling the antigenicity of H-PLL conjugates in responder guinea pigs can be considered therefore to be the same gene necessary to form an immune response to poly-L-lysine although this does not allow as yet a definition of what is governed by this gene. The two alternative possibilities previously considered must be discussed: (*a*) a metabolic reaction on the PLL, prior to the recognition of specificity, possibly in the macrophages, to form the specific inducer (this will be referred to as the "metabolic gene hypothesis"), and (*b*) the specific immunological recognition of PLL as a partial antigenic determinant because of the previous synthesis of a cellular antibody capable of reacting with it. This will be referred to as the "specificity gene hypothesis."

If this last possibility is true, one needs also to postulate that a single gene may control only part of the immunological specificity of an antibody to account for the data obtained in the H-PLL system, where it has been shown that the ability to synthesize antibodies against different hapten conjugates of PLL is under control of the same gene (42).

The "metabolic gene hypothesis" was initially proposed because PLL was believed not to be antigenic and it was observed that any hapten on PLL, as well as the copolymer of L-glutamic acid and L-lysine would stimulate antibody formation in genetic responder animals (1). The results of these experiments however are more favorable to the "specificity gene" hypothesis because (*a*) PLL alone was shown to be capable of inducing an immune response in genetic responders, and (*b*) the formation of anti-DNP antibodies by nonresponder animals immunized with H-PLL complexes with negatively charged albumins is stimulated much more effectively with foreign albumin than with guinea pig albumin. This last observation suggests that it is not the presence of any individual L-amino acids in the carrier molecule but the presence of recognizable foreign protein determinants which are necessary for immunogenicity. This interpretation of the nature of the genetic control of the immune response against H-PLL antigens is analogous to that proposed for similar genetic data in mice with other synthetic antigens, copolymers of L-amino acids, whose antigenicity depends also upon the presence of single dominant autosomal genes (43, 44), or to that proposed to explain the recognition by strain 2 and strain 13 guinea pigs of different antigenic determinants on the beef insulin molecule (45).

A most intriguing observation in our experiments was that nonresponder guinea pigs immunized with DNP-PLL complexed with foreign albumins form

anti–DNP-PLL antibodies but do not show delayed hypersensitivity reactions to DNP-PLL. It would appear from these observations that the same properties of the antigen are required to induce an immune response and to elicit delayed reactions in sensitized animals, and that an operation controlled by the same gene is involved at some stage in both reactions.

A similar conclusion was reached by Schlossman et al. (46) in the course of their studies of the minimum size of α,N-DNP-oligo-L-lysine required to be antigenic and to elicit delayed hypersensitivity reactions in genetic responder guinea pigs. Immunogenicity was observed with α,N-DNP-hepta, octa, and nona-L-lysine, smaller α,N-DNP-poly-L-lysines not being antigenic; delayed hypersensitivity reactions could be elicited only with α,N-DNP-PLL containing the same minimum number of lysines. Both observations can probably best be explained by the marked immunological specificity of delayed hypersensitivity reactions, of which carrier specificity is an example. Delayed hypersensitivity reactions, being the result of the interaction of sensitized cells with antigen, have the same strict immunological specificity as the anamnestic response (40) and the proliferative response of sensitized cells to antigen in vitro (47).

The reaction of sensitized cells with antigen requires a considerable energy of interaction which can be provided only by the immunizing antigen at the concentration generally used. These considerations imply that the nonresponder guinea pigs which were induced to make DNP-PLL antibodies by the injection of the polymer complexed with foreign albumins, in fact never made an immune response to the DNP-PLL determinant alone (of which they are genetically incapable) but made it rather to some determinant involving both a portion of the DNP-PLL and a portion of the foreign albumin used as carrier. The fact that the anti-DNP antibodies produced by these animals showed specificity for the PLL molecule by the technique of fluorescence quenching is in apparent contradiction with this interpretation. However, it must be pointed out that although this technique demonstrates clearly that these antibodies show specificity for PLL, it does not provide an exact measurement of the total specificity involved because of the nature of the technique (29) and because $DNP_{0.6}$-PLL_{240} which had to be used for these measurements must be considered an antigen cross-reactive with the more highly coupled DNP-PLL used for immunization.

In the foregoing discussion we have assumed that the "Schlepper" in DNP-PLL protein complexes induces the formation of anti-DNP antibodies by forming with the hapten a complete antigenic determinant, similar to what occurs with hapten covalently bound with a carrier protein. An alternative possibility has to be considered: that is, that the electrostatically combined antigenic Schlepper molecule initiates the formation of antibodies to the haptenic determinants without at all being involved in the specificity of these

antibodies, or being itself a part of the determinant; the recognition of antigenic protein being sufficient to induce (through mechanisms unknown) an immune response to haptens introduced with them into susceptible cells.

SUMMARY

30 to 40% of Hartley strain guinea pigs have previously been demonstrated to possess a dominant autosomal gene which enables them to recognize the antigenicity of hapten-poly-L-lysine conjugates as expressed by the development of both antihapten antibodies and delayed hypersensitivity to the immunizing antigen. In the present study, it was shown that PLL alone was weakly antigenic in such genetic responder animals. Immunization with DNP-PLL electrostatically combined with foreign albumins elicits the production of anti-DNP antibodies in all Hartley strain guinea pigs, although the percentage of animals demonstrating a delayed response to DNP-PLL and therefore considered genetic responders remains 30 to 40%. Immunization with nonantigenic polyanions combined with DNP-PLL does not produce such an effect. Some degree of PLL specificity of purified anti-DNP antibodies produced by genetic nonresponder animals by immunization with DNP-PLL combined with foreign albumins was demonstrated by means of fluorescence quenching.

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