STUDIES ON ARTIFICIAL ANTIGENS*

I. Antigenicity of DNP-Polylysine and DNP Copolymer of Lysine and Glutamic Acid in Guinea Pigs

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The importance of proteins as antigens has stimulated numerous studies on the immunogenicity of these substances (1). Three lines of investigation have been generally followed.

1. Attempts have been made to alter the shape and structure of protein molecules by denaturation, deamination, or acetylation to explore whether antigenicity and specificity depend upon free amino groups, free carboxyl groups or the complex native protein structure (2, 3).

2. The conjugation of proteins with reactive chemical groups (haptens) has been shown to confer additional antigenic properties to foreign proteins, with the immunological specificity of the haptens (4). Similarly, the conjugation of hapten groups with non-antigenic, homologous, or even autologous serum albumins render them antigenic with the specificity directed primarily towards the hapten (5). The 2,4-dinitrophenyl group (DNP), recognized as a strong antigenic hapten by several animal species, has been extensively used in such experiments. In studies along similar lines, Sela and Arnon have conjugated amino acids to gelatin which is itself a very weak antigen (6). The attachment of aromatic amino acids, cyclohexylalanine, or cysteine to gelatin resulted in a great increase in antigenicity, and, in some preparations, modified specificity (7, 8).

3. The use of synthetic poly-α-amino acids as antigens has been another original approach to the nature of antigenicity of proteins. In studies of Maurer and associates (9–13), polymers of single amino acids such as poly-L-lysine or poly-L-glutamic acid have been found to lack antigenicity. In contrast random copolymers of these two amino acids, or of three amino acids, have been shown to be capable of inducing antibody formation in guinea pigs and rabbits. It should be stressed, however, that all animals of a given species did not recognize these copolymers as antigens. Similar results have been reported by Gill and Doty (14).

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Our experiments are concerned with a combination of two of these approaches. The conjugation of an antigenic hapten with synthetic poly α-amino acids provides simple and well defined tools to investigate the nature of antigenicity. The immunogenicity of two poly α-amino acids conjugates, DNP poly-L-lysine, and DNP copolymer of lysine and glutamic acid (glu-lys) was studied in guinea pigs and compared with the immune response shown by the same animals to the unconjugated polyamino acids. Immediate and delayed skin sensitivities and the production of precipitating serum antibodies to these preparations were investigated. These two polyamino acids were selected because poly-L-lysine is not an antigen while the copolymer glu-lys is antigenic in some guinea pigs (11). Conjugation with DNP was introduced because this hapten which conjugates easily with the free ϵ-NH_2 group of lysine is recognized by all random bred guinea pigs as an antigenic determinant when bound to non-antigenic guinea pig serum albumin (5).

DNP conjugates of poly-L-lysine or of polyglu-lys have been found to induce skin reactivity and precipitating antibodies with DNP specificity in approximately 30 to 40 per cent of guinea pigs. Furthermore, only those animals capable of responding to DNP polylysine were also capable of forming antibodies to the immunologically distinct polyglutamyl-lysine copolymer.

Materials and Methods

Reagents.—Poly-L-lysine hydrochloride, having an average molecular weight of 182,000 and containing a mean of 1420 lysyl residues per molecule, was obtained from Mann Research Laboratories, New York. Poly-L-lysine hydrobromide, average molecular weight 66,000 (about 316 lysyl residues per molecule), was obtained from Pilot Chemical Company, Watertown, Massachusetts. A third poly-L-lysine preparation, averaging 20 lysyl residues per molecule, was a gift of the Kremers-Urban Co., Milwaukee. Copolymer, average molecular weight 65,000, with a ratio of 6:4 of glutamyl/lysyl residues, was kindly provided for these experiments by Dr. Paul Maurer, Seton Hall College of Medicine and Dentistry, Jersey City. The value for all molecular weights are based on manufacturer's assays.

1-Fluoro-2,4-dinitrobenzene (DNFB) was obtained from Eastman Organic Chemicals, Rochester, New York. Other chemicals were of reagent grade.

Preparation of Conjugates.—The same method was used in all cases. Poly-α-amino acid preparations were dissolved in distilled water and the pH adjusted to 9.8 with 5 per cent sodium carbonate. Varying amounts of DNFB were diluted in 1,4-dioxane and added dropwise to the polylysine solutions in order to obtain preparations with different average numbers of hapten groups per molecule. The concentration of dioxane never exceeded 5 per cent by volume. After 1 to 2 hours at room temperature, glycine hydrochloride was added in amounts 10 times that of the polymer present and the pH was again adjusted to 9.8 for 1 to 2 hours. Those preparations of molecular weight 65,000 or higher, were dialyzed for 3 days in the cold against 0.005 M phosphate buffer pH 7.5. The polylysine preparation containing 20 lysyl residues was precipitated twice by adjusting the pH to 6 and adding 4 to 5 volumes of absolute alcohol. Precipitates were washed five times with absolute ethanol before redissolving in saline.

The average number of hapten groups per molecule of polymer conjugate was calculated from their optical densities at 360 mμ related to the molar extinction coefficient of ϵ-DNP

lysine (17,400). Polymer concentrations were calculated from Kjeldahl nitrogen determinations.

Preparations of DNP-polylysine made with the 182,000 mol wt polymer varied from 32 to 450 DNP groups per molecule of polymer. Those prepared from the 65,000 mol wt polylysine varied from 5.4 to 139 groups per molecule. The polylysine containing only 20 lysyl residues was used to prepare two conjugates having an average of 0.35 to 5.8 DNP groups per molecule respectively. Two DNP copolymer preparations used contained 17.6 and 52 DNP groups per molecule respectively. It should be pointed out that all degrees of conjugation represent averages and that the polydispersity of each preparation could not be estimated. One preparation of DNP-polylysine (mol wt 182,000, 32 groups per molecule) was tested for purity by the Analytica Corp., New York, by acid hydrolysis and chromatography according to the method of Spackman, Stein, and Moore (15). Less than 0.3 per cent material other than lysine was present and this fraction did not contain DNP groups.

DNP-fibrinogen and DNP–guinea pig albumin (DNP-GPA) used in the precipitin and immunoelectrophoretic studies were prepared as described previously (16).

Immunization.—Male and female albino guinea pigs of the Hartley strain, weighing 400 to 500 gm, were immunized. The antigens were diluted in normal saline to proper concentration, and then emulsified with an equal volume of Difco complete Freund's adjuvant. The amount of antigen to be used for each animal was distributed in the four foot-pads in a volume of 0.1 ml per foot-pad.

Skin Tests.—Guinea pigs were skin-tested generally on the 7th day when the flank was carefully shaved and 10 μg of the antigen to be tested was injected intradermally in a volume of 0.1 ml of saline. Non-immunized animals were used as controls and when injected with DNP-polylysine preparations, a non-specific inflammatory reaction appeared in 2 to 3 hours and persisted for 24 hours reaching a size of not greater than 6 × 6 mm. This reaction always disappeared completely without hemorrhage or necrosis. Non-specific inflammatory reactions were not observed, to DNP-copolymer.

Skin reactions were observed at 4 and 24 hours and the nature of the reactions, immediate or delayed, were graded as previously described (16). Briefly, immediate reactions (4 hours) were graded as follows: ±, slight edema; +, definite edema; ++, severe edema and slight hemorrhage; +++, severe edema with necrosis and hemorrhage; ++++, severe hemorrhagic necrosis. The delayed reactions were more extensive and showed mainly redness and induration; they were considered positive if greater than 6 × 6 mm, and the size of the reactions measured and recorded. Immediate reactions appeared within 1 hour and were maximum at 3 to 4 hours. Delayed reactions appeared after 6 hours and reached maximum intensities at 18 to 24 hours. Guinea pigs were tested weekly for 3 weeks after immunization and reacting animals were tested in the 4th week with a drop of 0.25 per cent DNFB in an acetone–olive oil vehicle (4:1). None of the animals demonstrated contact reactions on the following day and they were promptly bled to death from the carotid artery; sera were separated and tested for antibodies. At the end of one month, all non-reacting animals were injected with 200 μg of DNP-guinea pig albumin intravenously prior to sacrifice, and observed for symptoms of anaphylaxis. No animals without skin reactivity demonstrated anaphylaxis.

Capillary Precipitin Tests.—Capillary precipitin tests were performed according to the method of Swift, Wilson, and Lancefield (17). In animals immunized with DNP-conjugates, DNP-fibrinogen was used as antigen in a concentration of 250 μg/ml. Copolymer was used in concentration of 200 μg/ml. Tubes were first incubated at 37°C for 1 hour and refrigerated overnight at 4°C to be used the next morning. Precipitates were judged to be present or absent with no quantitation attempted. All positive sera were subjected to immunoelectrophoresis to confirm the presence of precipitating antibody.

Immunoelectrophoresis.—A modification of Scheidegger's technic (18) for agar gel immuno-

electrophoresis was employed. 2 per cent agar in a barbital buffer pH 8.6, ionic strength, 0.075, was poured on 3¼ by 4 inch lantern slides which had been precoated around the periphery with 1 per cent agar and allowed to dry. In all experiments, approximately 0.01 ml of serum was placed in the center well. After electrophoresis for 105 minutes at 24 ma and 200 to 250 volts, 0.1 ml of antigen solution (DNP–guinea pig albumin, 200 μg/ml) was placed in each trough and the slides were incubated in a moist chamber for 2 days. After washing in normal saline, the slides were dried and stained with amido black 10B by the method of Uriel and Scheidegger (19).

Passive Cutaneous Anaphylaxis (PCA).—Those guinea pig sera tested for PCA activity were diluted in saline and tested using the technique described by Ovary (20). The antigen used to test antibodies directed against the DNP group was DNP-guinea pig albumin, 400 μg/ml. DNP-polylysine could not be used because of non-specific histamine release by this agent. Copolymer was used in final concentration of 100 μg/ml and did not provoke reactions in control animals. All antigens were diluted in equal volumes of 1 per cent Evans blue dye and 1 ml of this solution injected intravenously. Each serum was tested at 3 dilutions in 4 guinea pigs weighing 200 to 250 gm. An unknown serum was considered devoid of detectable antibody only when the test animal gave a positive response at a site prepared with a standard anti-DNP serum.

Antibody Protein Determination.—Serum antibody protein was calculated from antibody nitrogen determinations performed according to the quantitative methods of Heidelberger *et al.* (21).

RESULTS

Immunogenicity of DNP-Polylysine.—As shown in Table I, three different sized polylysine polymers obtained from separate sources, when conjugated with varying numbers of DNP groups, proved to be antigenic in guinea pigs. The most extensive experience in this study was with the largest polymer of 182,000 molecular weight. Delayed skin sensitivity with this preparation developed in 37 of the 115 animals immunized. This became evident in the first intradermal challenge, 7 days after immunization. With only one exception, all animals eventually to react would do so upon the first challenge. Conversely, no new "recruits" appeared from the negative animals, even though they were stimulated weekly by intradermal challenge with 10 μg of antigen. The appearance of precipitating antibody followed skin reactivity in all animals tested. Some of the animals showing positive reactions to DNP-polylysine were also skin-tested with polylysine and found to be negative to the unconjugated polymer.

Immunoelectrophoretic patterns were obtained with sera from animals with skin reactivity and positive capillary precipitin tests. Several patterns, shown in Fig. 1, illustrate the migration of specific antibody in the region associated with the 7S gamma globulins. Antibody protein determinations on selected groups of sera were performed; content varied from 0.126 mg/ml to 2.46 mg/ml as shown in Table I.

—The remaining animals with negative skin reactions showed no signs of anaphylaxis upon intravenous challenge with DNP—guinea pig albumin and therefore showed no evidence of immune response.

TABLE I
Antigenicity of DNP Polylysine

Group	Immunizing antigen				No. of reactions/No. tested		
	Av. no. lysyl residues	Av. mol wt	groups/ molecule	Amount	Skin* 24 hrs.	Precipitating antibody	
				μg			*mg prot./ml*
1	1420	182,000	32	1	3/5	3/5	
2	1420	182,000	32	10	4/6	4/6	
3	1420	182,000	32	10	3/12	3/12	
4	1420	182,000	32	100	4/12	4/12	(0.126) (0.804) (0.186) (0.320)
5	1420	182,000	32.5	100	5/10	5/10	
6	1420	182,000	44	1	1/6	1/6	
7	1420	182,000	44	10	2/6	2/6	
8	1420	182,000	44	100	4/6	4/6	(2.46) (1.52) (1.2)
9	1420	182,000	44	1000	1/6	1/6	
10	1420	182,000	115	1	1/6	N.D.	
11	1420	182,000	115	10	2/6	2/6	
12	1420	182,000	115	100	2/6	2/6	
13	1420	182,000	115	1000	2/6	2/6	
14	1420	182,000	221	50	1/7	1/7	
15	1420	182,000	225	100	N.D.	0/12	
16	1420	182,000	225	100	1/6	1/6	(0.720)
17	1420	182,000	450	50	1/9	1/9	
Total	1420	182,000			37/115	36/121	
18	316	66,000	5.4	50	4/8	4/8	
19	316	66,000	44	50	4/8	3/7	
20	316	66,000	83	50	3/8	3/7	
21	316	66,000	137	50	3/10	3/10	
Total	316	66,000			14/34	13/32	
22	20	3,300	0.35	50	3/8	3/8	
23	20	3,300	5.8	50	2/7	2/7	
Total	20	3,300			5/15	5/15	

N.D., not done.

* All guinea pigs showing no skin reactivity to DNP-polylysine were intravenously injected with 200 μg of DNP guinea pig albumin. None developed signs of anaphylaxis.

Effect of Antigen Dosage on the Number of Responding Guinea Pigs.—The effect of varying antigen dosages on the $\frac{\text{No. of responding animals}}{\text{No. of immunized animals}}$ (per cent response) was studied over a range of 4 logs, from 1 μg to 1 mg. Preparations of

DNP-polylysine were selected so that the percentages of lysyl residues conjugated with DNP groups were similar (2.5 to 8 per cent). The variation over the whole dosage range was small; from 25 to 41 per cent of animals immunized, responded with skin reactions and antibody production as shown in Fig. 2.

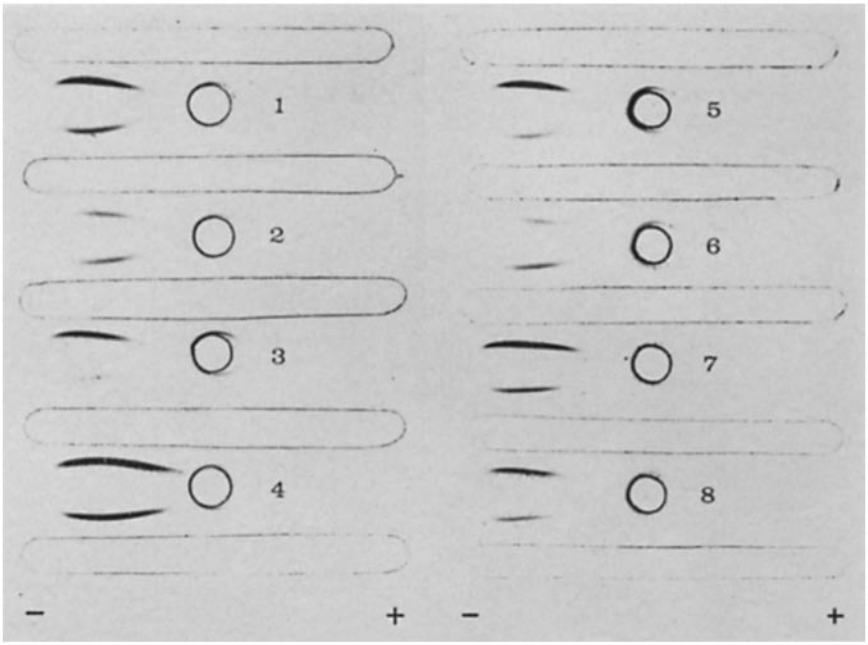

FIG. 1. Immunoelectrophoretic Patterns Obtained with Sera from Animals Immunized with DNP-Polylysine and DNP Copolymer. Sera from guinea pigs immunized with: (center wells, top to bottom)

Wells 1 to 4. DNP-polylysine 182,000 mol wt, 44 groups/mol

Wells 5 and 6. DNP-polylysine 66,000 mol wt, 83 groups/mol

Wells 7 and 8. DNP-copolymer 65,000 mol wt, 17.6 groups/mol

All troughs contained 0.1 ml of DNP-GPA in concentration of 200 μg./ml.

It appeared that immunization with 100 μg of DNP-polylysine was responsible for the greatest response, but since only 12 animals were immunized with 1 mg of antigen, the sample size does not permit significant comparisons.

Effect of Percentage of Lysyl Residues Conjugated with DNP Groups on the Number of Responding Guinea Pigs.—Since polylysine itself is not antigenic, the addition of increasing numbers of hapten groups to the molecule was suggested as a means to increase the percentage of immunized animals that develop immune responses. Accordingly, guinea pigs were immunized with different preparations of DNP-polylysine (mol wt 182,000) containing increasing

numbers of DNP groups per molecule of polymer. When polylysine containing 1420 lysyl residues was conjugated with about 250 to 400 DNP groups, the preparation became insoluble so that the percentage conjugation was limited by solubility properties. As shown in Fig. 3, contrary to expectation, the percentage of responding animals decreased as the percentage conjugation of the immunizing DNP-polylysine was increased. Only 9 per cent of guinea pigs developed skin reactivity and antibodies when immunized with DNP-polylysine, which was 21 per cent or higher conjugated compared to a 42 per cent

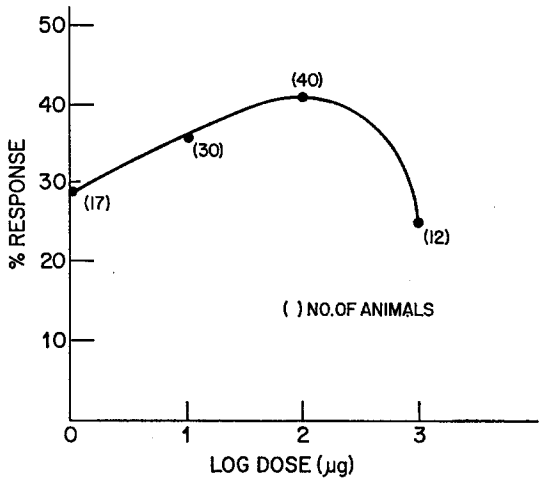

FIG. 2. Effect of antigen dosage on the percentage* response of guinea pigs immunized with DNP-polylysine.

$$* \text{ Percentage response} = \frac{\text{No. of responding animals}}{\text{No. of immunized animals}}.$$

immune response when injected with antigen only 2.1 per cent conjugated. Furthermore, repeated weekly challenges with the immunizing antigen failed to increase the number of reacting animals.

Antigenicity of DNP-Copolymer Glu-Lys.—Fifteen guinea pigs were immunized with DNP-copolymer as shown in Table II. As in the case of immunization with DNP-polylysine, approximately one-third of the animals developed delayed skin reactions to the immunizing antigen. Four of the five responding animals were also sensitive to the carrier copolymer alone. Antibodies directed against the DNP group were shown by the reactivity of three sera (from skin-reactive animals) which precipitated with DNP-fibrinogen. The same three animals developed fatal anaphylaxis when injected intravenously with 100μg of copolymer glu-lys. From this experiment it is evident that

animals responding to immunization with DNP-copolymer developed delayed skin reactivity to the immunizing conjugate and to the carrier copolymer glu-lys alone, as well as antibodies directed against the hapten and against the carrier polymer. None of the animals were found to produce antibodies to the copolymer alone.

Immune Response of Guinea Pigs Immunized Independently with DNP-Polylysine and Copolymer Glu-Lys.—It has been shown that immunization of guinea pigs with DNP-copolymer glu-lys produced immune responses to DNP and to the copolymer in the same fraction of the animals. In the following

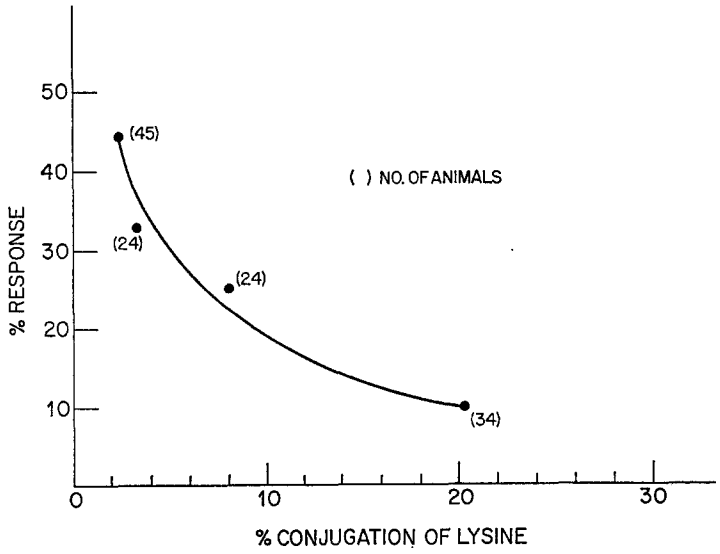

FIG. 3. Effect of percentage of lysyl residues conjugated with DNP groups on the per centage response of guinea pigs immunized with DNP-polylysine.

experiments, illustrated in Table III, the responses of animals immunized with both DNP polylysine and unconjugated copolymer glu-lys were studied to determine whether the same fraction of immunized animals responding to one antigen would also respond to the other, when both antigens are injected independently. Three groups of guinea pigs were first immunized in 2 foot-pads with DNP-polylysine; 2 weeks later, the same animals were immunized in the other 2 foot-pads with copolymer glu-lys. One week after the second immunization, they were skin-tested with DNP-polylysine and copolymer glu-lys. The following week all animals were bled for antibody studies.

Two animals in the first experiment developed delayed skin sensitivity to DNP-polylysine and antibodies directed against the DNP group. The same two animals also developed delayed reactivity to the copolymer glu-lys and

antibodies directed against the copolymer determined by the PCA method. The four remaining animals did not respond to either antigen. In the second group of animals, only one of the two animals demonstrating sensitivity to DNP-polylysine was also sensitive to copolymer. In the third group the same two animals showed immune response to the copolymer glu-lys and to DNP-polylysine. The fourth group of guinea pigs were immunized with both antigens simultaneously in order to investigate the possible effect of prior immunization with DNP-lysine on the ability of animals to respond to the copolymer glu-lys.

TABLE II

Immunogenicity of DNP-Copolymer Glu-Lys

Immunizing antigen				No. of reactions/No. tested				
				24 hr. skin reactions		Precip. antibody		Anaphylaxis
Exp.	Av. mol wt	DNP groups/mol	Amount	DNP-Copolymer	Copolymer	DNP fib.	Copolymer	Copolymer
			μg	mm	mm			
I	65,000	52	100	2*/7 18 × 20 25 × 25	1*/7 18 × 20	N.D.	N.D.	N.D.
II	65,000	17.6	50	3‡/8 18 × 23 18 × 22 20 × 23	3‡/8 12 × 20 8 × 10 12 × 15	3‡/8	0/8	3‡/8
Total				5/15	4/15			
Per cent				33.3	27.0			

N.D., not done.

* Same animals.

‡ Same animals.

The same animals responded to both antigens in a manner similar to the groups immunized sequentially. When all groups are combined, only 1 of 49 animals immunized to both DNP-polylysine and copolymer responded to DNP-polylysine alone. None produced antibodies to copolymer glu-lys alone; eleven animals responded to both antigens and 34 did not respond at all.

These data clearly indicate that factors predisposing an animal to develop antibodies to DNP-polylysine are also operative with respect to the development of an immune response to the copolymer glu-lys.

Specificities of Skin Reaction in Animals Immunized with DNP Conjugates.— Although both DNP-polylysine and copolymer glu-lys have been shown to induce delayed and immediate sensitivities in the same fraction of immunized guinea pigs, it remained to demonstrate the presence or absence of immunologi-

TABLE III

Immune Response of Guinea Pigs Immunized Independently with DNP-Polylysine and Copolymer Glu-Lys

Exp.	Immunizing Antigen				Interval between immunizations	Number of Reactions/Number Tested						
						Skin				Precip. antibody		PCA
		Av. mol wt	DNP groups/mol	Amount		DNP-Polylysine		Copolymer		DNP-fibrinogen	Copolymer	Copolymer
				μg	wks							
I	1st, DNP-polylysine	182,000	115	10	2	2*/6	20 × 20	2*/6	13 × 14			
	2nd, copolymer glu-lys	65,000	—	50			17 × 18		10 × 11	2*/6	0/6	2*/6
II	1st, DNP-polylysine	182,000	115	100	2	2‡/6	18 × 20	1‡/6	13 × 14			
	2nd, copolymer glu-lys	65,000	—	50			20 × 20		Trace	2‡/6	0/6	1‡/6
III	1st, DNP-polylysine	182,000	115	1000	2	2§/6	12 × 14	2§/6	8 × 16			
	2nd, copolymer glu-lys	65,000	—	50			13 × 14		12 × 13	2§/6	0/6	2§/6
IV	1st, DNP-polylysine	182,000	44	50	None	2‖/15		2‖/15		2‖/15	0/15	2‖/15
	2nd, copolymer glu-lys	65,000	—	50								
V	1st, DNP-polylysine	182,000	450	100		4¶/16	18 × 20	4¶/16	12 × 15	N.D.	N.D.	N.D.
			44	10	None		20 × 22		11 × 12			
	2nd, copolymer glu-lys	65,000	—	25			20 × 24		10 × 12			
							17 × 22		15 × 16			

Summary of Immune Responses in Animals Immunized with Copolymer Glu-Lys and DNP-Polylysine

Immune response to:	No. of animals reactive/No. tested
Copolymer alone	0/49
DNP-polylysine alone	1/49
Both copolymer and DNP-polylysine	11/49

*, ‡, §, ‖, ¶ = Same animals.

cal cross-reactivity between these two antigens. Two groups of guinea pigs were immunized with DNP-polylysine and DNP-copolymer glu-lys respectively. One week later, all animals were challenged with 10 μg of the following antigens: DNP-copolymer, copolymer, DNP–guinea pig albumin, and DNP-polylysine (Table IV). In the first group, three of eight animals immunized with DNP-copolymer reacted to the immunizing antigen with both immediate and delayed reactions; similar reactions were obtained when challenged with copolymer alone. Challenge with DNP-GPA or DNP-polylysine resulted only

TABLE IV

Specificities of the Skin Reactions in Animals Immunized with DNP Conjugates

Immunizing antigen	Guinea pig No.	Challenging antigen (10 μg)							
		DNP-copolymer		Copolymer		DNP-GPA		DNP-polylysine	
		4 hrs.	24 hrs.	4 hrs.	24 hrs.	4 hrs.	24 hrs.	4 hrs.	24 hrs.
DNP-copolymer glu-lys. Av. mol wt., 65,000; 17.6 DNP groups/mol; 50 μg injected in complete adjuvant	1	2+	20 × 22	±	18 × 20	3+	—	2+	—
	2	—	—	—	—	—	—	—	—
	3	2+	18 × 20	±	18 × 20	3+	—	2+	—
	4	+	15 × 17	±	12 × 12	3+	—	2+	—
	5	—	—	—	—	—	—	—	—
	6	—	—	—	—	—	—	—	—
	7	—	—	—	—	—	—	—	—
	8	—	—	—	—	—	—	—	—
DNP-poly-L-lysine Av. mol wt., 182,000; 32.5 DNP groups/mol; 50 μg injected in complete adjuvant	1	+	18 × 20	—	—	3+	tr	2+	16 × 20
	2	—	—	—	—	+	—	2+	22 × 25
	3	—	—	—	—	—	—	—	—
	4	—	—	—	—	—	—	—	—
	5	±	—	—	—	3+	—	3+	20 × 20
	6	+	—	—	—	—	—	3+	20 × 30
	7	—	—	—	—	—	—	—	—
	8	—	—	—	—	—	—	—	—
	9	+	—	—	—	2+	—	3+	22 × 24
	10	—	—	—	—	—	—	—	—

in immediate reactions without delayed components, illustrating both the marked specificity of the antibodies for the DNP group and the previously described carrier specificity requirements of delayed reactions (16).

The second group, immunized with DNP-polylysine, consisted of ten animals, of which five demonstrated immediate and delayed reactions to the immunizing antigen. Immediate reactions directed against the DNP group were also observed in four animals when tested with DNP-GPA or DNP-copolymer glu-lys; one delayed response was also observed to DNP-copolymer glu-lys. Neither immediate nor delayed reactions were evident in animals challenged with the copolymer glu-lys. The single delayed reaction to DNP-copolymer observed might be explained by the fortuitous presence of several

contiguous lysyl residues (some with DNP groups) in the copolymer molecule so that the reaction could have been due to the DNP-polylysine determinant. The absence of immune responses to copolymer glu-lys in animals immunized with DNP-polylysine establishes the lack of immunological cross-reactivity between these two antigens.

DISCUSSION

Evidence has been presented which establishes that conjugation of the immunogenically silent polylysine molecule with dinitrophenyl groups results in a synthetic antigen capable of inducing anti-DNP antibodies in guinea pigs. The possibility that protein contamination of the polylysine preparation was responsible for the antigenicity can be discounted for several reasons: Three different polylysine preparations obtained from separate manufacturers all produced potent conjugates. Animals immunized with one conjugate demonstrated immediate and especially delayed reactions to all DNP-polylysine preparations. This is strong evidence for the immunological specificity of DNP-polylysine as the marked carrier specificity requirements of delayed reactions have been well established (16). Finally, amino acid analysis by Moore-Stein column chromatography revealed less than 0.3 per cent material other than lysine, eluted in a region between neutral and acidic amino acids, and containing no DNP groups.

Precautions were taken to insure that none of the dinitrofluorobenzene used to conjugate polylysine did remain in the preparation to react with guinea pig tissue proteins and produce a potent antigen. These included: (*a*) the addition of large excesses of glycine to the reaction mixture at pH 9.8 immediately after conjugation and prior to dialysis; (*b*) testing all reactive animals for contact sensitivity to DNFB. There were no positive reactions. A positive reaction would have been the most sensitive evidence for the presence of free DNFB in the immunizing materials. The capacity to show an immune response to DNP-polylysine seems to be limited at most to 40 per cent of guinea pigs, irrespective of dosage or level of conjugation, and to behave as an all-or-none-response, since the positive guinea pigs all produced appreciable amounts of anti-DNP antibodies while the negative animals showed no signs of anaphylaxis when challenged with DNP protein conjugates. It is reasonable to assume that these different immunological responses reflect constitutional differences presumably genetic among the random bred guinea pigs. However, since it is well known that all guinea pigs can normally produce anti-DNP antibodies (5) when immunized with DNP conjugates with foreign or autologous proteins, it can be assumed that none of these animals lack the capacity to recognize the dinitrophenyl group as antigen or to make specific antibodies against it. The constitutional difference revealed by these experiments must reside in the presence or absence of another mechanism operative at an earlier stage. In

this regard, the immune responses of the guinea pigs immunized with DNP copolymer glu-lys or with both DNP-polylysine and copolymer glu-lys are highly informative. It appears indeed that only those guinea pigs capable of reacting to one antigenic specificity, DNP, were able to react to the other glu-lys although immunologically unrelated. The constitutional difference which is being postulated must therefore operate at a stage previous to the formation or selection of the immunological specificity. The response of the same animals to DNP-polylysine and copolymer glu-lys must depend upon some common property of these compounds, which may be their lysine content. If one considers that antigens must be initially broken down by macrophages into smaller fragments capable of initiating the process of antibody production (22) the importance of lysine in both polymers suggests that the absence or presence in guinea pig macrophages of an enzyme capable of splitting lysyl peptide bonds might explain the results observed in our experiments.

While there are no data to substantiate any of these hypotheses, the use of single synthetic antigens such as DNP-polylysine with known specificity should prove very useful to explore further the problems raised by these experiments and to allow a genetic approach to some of the aspects of antibody synthesis. As an initial step in this direction, an attempt should be made to select guinea pig strains capable of uniform response to DNP-polylysine.

The antigenicity of DNP-polylysine appeared to decrease with the degree of conjugation either because highly conjugated polylysine, by virtue of a change in charge or structure, does not gain access as easily to areas where the immune process is initiated, or because with higher degrees of conjugation the probability increases of producing sequences of contiguous lysyl residues conjugated with DNP groups. If, for enzyme cleavage of the lysyl peptide bond, the ϵ amino nitrogen is required to be unconjugated, as it is for trypsin cleavage (23), then several non-metabolizable fragments would result from *in vivo* breakdown. These fragments might produce a state of immune paralysis similar to that observed with the polysaccharide polymers, also believed to be metabolized poorly, if at all.

The first explanation involves a "passive" lack of antigenicity whereas the second denotes an "active" process. This issue is amenable to experimental investigations which are currently in progress.

The antigenicity in guinea pigs of various DNP-polylysine preparations demonstrated in these experiments contrasts with the recent observations of Parker, Kern, and Eisen (24) who reported an absence of immune responses in guinea pigs immunized with poly-L-lysine which had been conjugated either with 2,4-dinitrophenyl groups or with benzyl penicilloyl groups. Large doses (1 mg) of highly conjugated (22 per cent) DNP-polylysine were given to a group of five animals in their studies. Constitutional variations among guinea pigs could easily explain negative results based on only 5 animals. On the

evidence presented, the role of a high degree of conjugation decreasing antigenicity might also explain the absence of immune response observed by these investigators. The antigenicity in guinea pigs of DNP conjugates of polylysine averaging only 20 lysyl residues warrants caution in the use of such polymers conjugated with benzyl penicilloyl in human penicillin sensitivity studies as it has been suggested (25).

SUMMARY

Dinitrophenyl conjugates of poly-L-lysine, varying in percentage conjugation and molecular weight have been found to induce skin reactivity and precipitating antibodies in guinea pigs. At best, 40 per cent of immunized animals developed delayed and immediate responses to DNP-polylysine, which is believed to reflect constitutional differences among the animals assayed. Only those animals capable of responding to DNP-polylysine, responded to an immunologically distinct poly-α-amino acid consisting of glutamyl and lysyl residues ("copolymer glu-lys"). The percentage of animals responding to the DNP-polylysine antigen decreased as the degree of DNP conjugation increased.

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