IMMUNOCHEMICAL STUDIES ON THE ANTIGENIC DETERMINANTS REQUIRED TO ELICIT DELAYED AND IMMEDIATE HYPERSENSITIVITY REACTIONS*

By STUART F. SCHLOSSMAN, M.D., SHLOMO BEN-EFRAIM,‡ PH.D., ARIEH YARON,§ PH.D., AND HERBERT A. SOBER, PH.D.

(From the Department of Medicine, Harvard Medical School, Beth Israel Hospital, Boston, and the Laboratory of Biochemistry, National Cancer Institute, National Institutes of Health, Public Health Service, Department of Health, Education, and Welfare, Bethesda, Maryland)

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The injection of an antigen into an animal may induce the formation of at least two apparently separate, immune responses. The immediate response is associated with circulating antibody. The delayed response, on the other hand, is thought to represent a form of immunity unrelated to conventional circulating antibody. While many studies have characterized the chemical nature of the antigenic determinants involved in the immediate response, less is known about the molecular basis for the delayed response.

The delayed response has been more difficult to investigate for several reasons. There is no quantitative in vitro test system nor have the components mediating the response been isolated or characterized. Elucidation of the molecular basis for the delayed response has been hampered by the lack of a chemically well defined system which would permit a study of the antigenic determinants involved. Heretofore, most studies have utilized chemically heterogeneous, hapten-substituted proteins and polypeptides.

Previous studies of the immunologic specificity of delayed hypersensitivity reactions to hapten-protein conjugates have been interpreted as showing that the determinants for the delayed response involve broad areas of the immunizing antigen (1–7). In contrast to this the determinants required to react with antibody and elicit the immediate skin response is thought to be limited mainly to the haptenic portion of the immunizing molecule. Leskowitz, (8), on the other hand, in a more chemically defined, polytyrosine–azobenzene arsonate system, suggested that the determinant for the delayed response was primarily the hapten with but a small portion of the carrier

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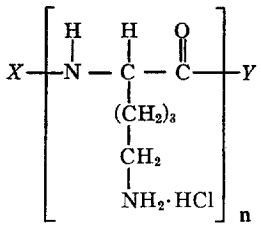
‡ Visiting Scientist, Laboratory of Immunology, National Institute of Allergy and Infectious Diseases, National Institute of Health from the Israel Institute for Biological Research, Nes-Ziona, Israel.

§ Visiting Scientist, National Cancer Institute, National Institute of Health, from Department of Biophysics, Weizmann Institute of Science, Rehovoth, Israel.

molecule involved. Because of poor production of antibody no comparisons were made between the size of the antigenic determinants required to elicit delayed or immediate hypersensitivity reactions.

Recently, a chemically defined, homologous series of hapten-substituted oligopeptides was prepared and its immunogenic properties were studied (9). It was shown that guinea pigs could be sensitized with α,N-DNP-oligo-L-lysyl peptides equal to or larger in size than the heptamer, that smaller members of this series were not immunogenic, and that the same compound, the octamer, could induce the formation of both delayed and immediate sensitivity in the same animal. Although this study demonstrated that both immune responses could be provoked by the same chemical structure, it remained unresolved whether the two responses were only the result of different biological mechanisms or whether they also reflected specificity to different portions of the same molecule.

In the present study, the chemical nature of the antigenic determinants for both immune responses was investigated with the following hapten derivatives of oligolysines:



1. α,N-DNP-oligo-L-lysine butylamide, designated as α-DNP$(Lys)_n$BuAm where X is $(NO_2)_2C_6H_3$-, and Y is $CH_3(CH_2)_3NH$-,
2. Oligo-L-lysine butylamide, designated as $(Lys)_n$BuAm where X is H-, and Y is $CH_3(CH_2)_3NH$-,
3. α,N-DNP-oligo-L-lysine, designated as α-DNP$(Lys)_n$ where X is $(NO_2)_2C_6H_3$-, and Y is -OH,
4. Oligo-L-lysine, designated as $(Lys)_n$ where X is H-, and Y is -OH.

Individual members of these homologous series were used to immunize strain 2 and Hartley guinea pigs. Sensitive animals were subsequently challenged with immunogenic and nonimmunogenic oligomers as well as related hapten-substituted proteins to study their ability to elicit delayed and immediate sensitivity.

Materials and Methods

Hapten-Substituted Lysine Oligopeptides.—Individual members of two homologous series of α-DNP$(Lys)_n$BuAm and $(Lys)_n$BuAm (n = 4, 5, 6, 7, 8, and 9; n = number of lysine residues in one oligolysine molecule) were prepared from α-DNP$(Lys)_{\bar{8}.4}$BuAm and $(Lys)_{\bar{8}.4}$ BuAm ($\bar{n}$ = average degree of polymerization) respectively by chromatographic separation on CM-cellulose and desalting, as described (9) except that, with compounds not containing the dinitrophenyl group, the preparative scale chromatographic effluent was monitored at 2200 A. Chain length was determined from the ratio of α-N-DNP-lysine to total lysine content

and from chromatographic position as reported earlier (9). An example of the analytical chromatography of one of the purified oligomers is given in Fig. 1.

Hapten-Substituted Polylysines.—Poly-ε,*N*-benzyloxycarbonyl-L-lysine butylamide (Yeda, Rehovoth, Israel, purchased Foreign Research Agreement 235103) (preparation No. Ly-50) was used for the preparation of the following two hapten-substituted polymers. Ly-50 was

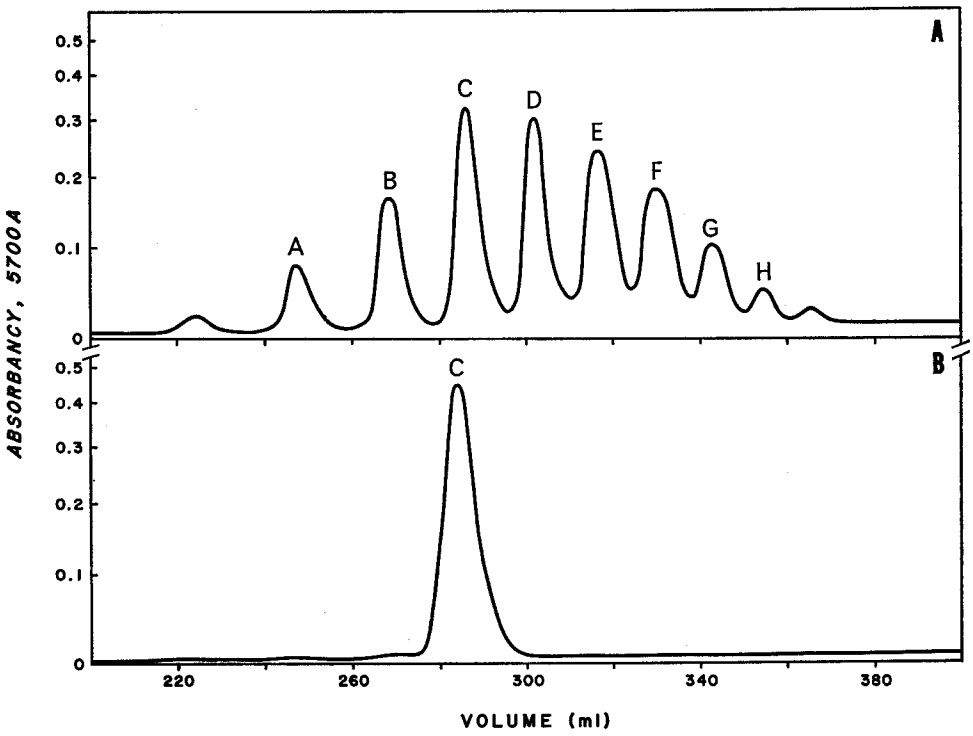


FIG. 1. (A) Analytical chromatography of unfractionated $(Lys)_{\overline{8.4}}$ BuAm peptides (6 mg) on a CM-cellulose column (0.9 x 50 cm). Material was applied to the column in 10 ml of 0.01 M lithium acetate buffer, pH 5.0. A linear gradient of 600 ml in 0.01 M lithium acetate, pH 5.0, to 1.0 M LiCl was used for elution. The procedure was performed in a Beckman model 120 amino acid analyzer at a flow rate of 30 ml/hr, and the effluent was continuously monitored by ninhydrin determination and recorded (9).

(B) Chromatography of an aliquot of purified peak C (0.85 mg) prepared by preparative chromatography from the $(Lys)_{\overline{8.4}}$BuAm mixture. Peak C is $(Lys)_6$BuAm. Conditions for chromatography were similar to (A).

dinitrophenylated and the benzyloxycarbonyl groups were removed (unblocked) with HBr in glacial acetic acid (9), yielding α-DNP$(Lys)_{\overline{8.4}}$BuAm. Ly-50 was unblocked yielding $(Lys)_{\overline{8.4}}$BuAm. α-DNP-$(Lys)_{\overline{11}}$ was prepared by the polymerization of ε,*N*-benzyloxycarbonyl, α-*N*-carboxy-L-lysine anhydride in dimethylformamide using ε,*N*-benzyloxycarbonyl-L-lysine benzyl ester (molar ratio of anhydride to ester:8) as the initiator. The polymer was dinitrophenylated and unblocked with HBr as in (9) except that the unblocking was carried out for 24 hr at room temperature to ensure complete removal of the benzyl ester group.

α-DNP(Lys)$_{\overline{18}}$ was prepared in a similar fashion. α-DNP-D-(Lys)$_{\overline{60}}$ and α-DNP(Lys)$_{\overline{60}}$ were the same preparations used in (9). α-DNP(Lys)$_{\overline{18}}$ was randomly substituted with benzyloxycarbonyl groups at the free epsilon amino position in following fashion: α-DNP(Lys)$_{\overline{18}}$ (105 mg) was dissolved in 10 ml of 0.2 M $NaHCO_3$ and to this was added 17 mg carbobenzoxy chloride (K & K Laboratories, Inc., Plainview, New York) in 1 ml ether with shaking in the cold (overnight). The material was neutralized, diluted to low conductivity, placed on CM-cellulose eluted with 0.1 M HCl, and lyophilized as previously described (9). The material was designated α-DNP-ϵ-(CBZ)$_{\bar{n}}$(Lys)$_{\overline{18}}$.

Polylysine.—(Lys)$_{\overline{8\text{-}10}}$ was a chromatographic cut containing octa, nona-, and decalysine in approximately equal quantities. (Lys)$_{\overline{1250}}$ was derived from a high molecular weight poly-ϵ,*N*-benzyloxycarbonyl-L-lysine prepared with triethylamine as the initiator. (Lys)$_{\overline{10\text{-}30}}$ was a fraction from a partial hydrolyzate of (Lys)$_{\overline{1250}}$, obtained by controlled alcohol precipitation. This fraction consisted of polylysine molecules containing an average of 10 to 30 lysine residues.

Hapten-Substituted Proteins.—ϵ,*N*-DNP–substituted bovine plasma albumin (ϵ-DNP-BPA) was prepared from BPA (Armour Laboratories, Chicago) (300 mg in 10 ml of 10% sodium carbonate buffer, pH 10.5) by reaction at room temperature for 2 hr with 1-fluoro-2,4-dinitrobenzene in dioxane. The yellow product was dialyzed against twice daily changes of 0.01 M Na-phosphate, pH 7.3, for 5 days. An average of 9 DNP groups were added per mole of BPA (10).

An unresolved mixture of α-*N*-DNP-L-lysyl chains, $\bar{n}$ = 8.4, was coupled to succinylated BPA by the carbodiimide procedure (11) and the product designated as α-DNP-BPA. Succinic anhydride (250 mg) was added to BPA (1 g) in 0.14 M $NaHCO_3$ (30 ml) and held at room temperature for 1 hr and in the cold for 5 hr. The reaction mixture was dialyzed exhaustively against 0.14 M Na-phosphate, pH 6.5, in isotonic saline, and finally against distilled water. The succinylated BPA product was recovered by lyophilization. A solution of 1-ethyl, 3-(2-morpholinyl-(4)-ethyl) carbodiimide metho-*p*-toluene sulfonate (44.7 mg in 0.5 ml 1.0 N LiCl) (12) was slowly added to a well mixed solution of succinylated BPA (106 mg in 1 ml 1.0 N LiCl). To this was added α-DNP-(Lys)$_{\overline{8.4}}$BuAm (37 mg in 0.47 ml 1.0 N LiCl) and the reaction mixture left overnight at room temperature. Unreacted α-DNP-(Lys)$_{\overline{8.4}}$BuAm was removed by repeated dialysis against 2.5 M NaCl. α-DNP-BPA was finally equilibrated with 0.01 M Na-phosphate, pH 7.5, in isotonic saline by dialysis. The amount of incorporation of α-DNP-lysyl side chains to the succinylated BPA conjugate (molecular weight taken as 66,000) was calculated from the protein content (Kjeldahl analysis), and the α-DNP-lysine content (molar extinction at 3600 A of 16,800) (9). The α-DNP-BPA had an average incorporation of four α,N-DNP-(Lys)$_{\overline{8.4}}$BuAm chains per mole of BPA. A benzyloxycarbonyl derivative of BPA was prepared as outlined in (15) and designated CBZ-BPA.

Adsorbent.—CM-cellulose (standard Selectacel, lot No. 1264, 0.6 Meq/g) was obtained from Schleicher and Schuell Company, Keene, New Hampshire. The dry adsorbent was sieved and material of mesh size 100 to 230 was used. The adsorbent was successively washed with 0.25 M LiCl, 0.25 M LiOH, 1 M HCl, ethyl alcohol, and 0.25 M LiCl in 0.25 M LiOH according to the method of Peterson and Sober (13) and finally washed with starting buffer until the effluent conductivity and pH matched that of the starting buffer. During the washing procedure fines were discarded.

Spectrophotometry.—A Beckman DU spectrophotometer with silica cells of 1 cm light path was used for all spectrophotometric determinations which were made in 0.01 M sodium phosphate buffer, pH 7.0, at 3600 A.

Immunization.—Freund's complete and incomplete adjuvant were purchased from Difco Laboratories, Inc., Detroit. Freund's complete adjuvant was also prepared with killed mycobacteria strain H37 RV, 10 mg/ml in Bayol F with Arlacel A (85:15). Bayol F and Arlacel A were obtained from Atlas Chemical Industries, Inc., Wilmington, Delaware.

Guinea pigs of the Hartley and of inbred strain 2 weighing 300 to 400 g were used. Approximately 70% of NIH Hartley guinea pigs can be sensitized to α-DNP(Lys)$_{\overline{n}}$peptides, whereas only 25% of commercially available Hartley guinea pigs can be similarly sensitized.

The α-DNP(Lys)$_n$BuAm peptides and related peptides were diluted in buffered saline and emulsified with an equal volume of Freunds complete adjuvant. Each animal was injected with a total of 0.4 ml containing 0.1 mg of the material to be tested. The injections were distributed equally in the hind foot-pads.

The Hartley strain was reinjected at the end of 1 and 2 wk with 0.1 mg of the antigen made up in incomplete Freund's adjuvant (0.5 ml) and administered subcutaneously in the dorsum of the neck. Blood samples were obtained at 3, 4, and 5 wk by cardiac puncture.

Inbred strain 2 guinea pigs were reinjected in the dorsum of the neck with 0.1 mg of the material mixed in 0.4 ml of Freund's complete adjuvant approximately 3 wk after the first injection and following a second intradermal skin test. Blood samples were obtained by cardiac puncture 10 and 20 days after the first injection and 10 days following the second injection.

Skin Tests.—The flanks of the guinea pigs were carefully shaved and 1 hr later, 0.1 ml of a buffered saline solution usually containing 10 μg of the test antigen was injected intradermally. The test sites were observed at 3 to 6, 24, and 48 hr. The immediate (Arthus) reaction was graded according to diameter as follows: 0, absent; +, 5 to 10 mm; ++, 10 to 15 mm; +++, 15 to 20 mm; and ++++, greater than 20 mm. In general, immediate reactions showed edema, erythema, hemorrhage, and necrosis; whereas delayed reactions were characterized by only erythema and induration. The reactions were classified as "pure delayed" if no immediate reaction was observed and if no appreciable change occurred in the extent of the reaction between 24 and 48 hr. They were classified as "pure immediate" if visible at 3 to 6 hr and disappeared by 24 hr. In control Hartley guinea pigs (animals not injected at all or injected with adjuvant alone) or in nonresponding animals, test substances frequently produced a pale erythematous reaction no larger than 5 mm at 3 hr and which disappeared by 24 hr. Such reactions were not observed in control guinea pigs of strain 2.

Hartley guinea pigs were first tested 2 to 3 wk after the beginning of immunization. Nonresponding animals were checked again after additional immunizing injections. Guinea pigs of inbred strain 2 were tested 10 and 20 days after the beginning of immunization.

Passive Cutaneous Anaphylaxis (PCA).—The PCA tests were performed in Hartley guinea pigs as detailed in a previous communication (9).

RESULTS

The Contribution of Peptide Chain Length to the Immunological Specificity of Immediate and Delayed Sensitivity.—Sixteen out of seventeen Hartley strain guinea pigs immunized with a mixture containing homologous oligomers of a hapten-oligopeptide series, α-DNP(Lys)$_{\overline{8.4}}$BuAm, responded in 4 wk with delayed and immediate skin reactivity. Eight of these animals were used to test the ability (Table I) of individual members of the series to elicit these responses. The individual oligomers differed only in the number of lysyl residues, each containing a single α-*N*-DNP substitution at the *N* terminal end of the peptide and terminating at the carboxyl end with a butylamide group.

It is apparent from the results in Table I that the tetra-, penta-, and hexamer were only capable of eliciting an immediate skin reaction. On the other hand, the octamer and nonamer not only elicited an immediate reaction but, in addition, a delayed reaction appeared which was intense at 24 to 48 hr. The hep-

tamer, occupying an intermediate position, while producing a strong immediate reaction in all animals tested, developed only a weak delayed response in one out of eight guinea pigs. The smaller oligomers, the tetra-, penta-, and hexamer, while not immunogenic themselves (9) were, nevertheless, able to elicit PCA reactions, as well as immediate skin responses in sensitized animals. To rule out the possibility that skin reactivity was a result, in some way, of the heterogeneity in chain length of the immunizing material, guinea pigs were sensitized with single members of that same homologous series, α-DNP(Lys)$_8$BuAm and α-DNP(Lys)$_9$BuAm. As shown in Table II, exactly the same effects were obtained, namely, that the tetra-, penta-, and hexamer were only able to provoke

TABLE I

Skin Reactivity of Hartley Guinea Pigs Sensitized with α-DNP $(Lys)_{\overline{8.4}}$ BuAm

Test material	Animal No. 1		2		3		4		5		6		7		8		Totals	
	I*	D‡	I	D	I	D	I	D	I	D	I	D	I	D	I	D	I	D
α-DNP(Lys)$_4$BuAm	2+	0	2+	0	0	0	0	0	3+	0	0	0	0	0	2+	0	4/8	0/8
α-DNP(Lys)$_6$BuAm	2+	0	2+	0	2+	0	0	0	3+	0	0	0	2+	0	2+	0	6/8	0/8
α-DNP(Lys)$_6$BuAm	3+	0	3+	0	2+	0	0	0	3+	0	2+	0	3+	0	3+	0	7/8	0/8
α-DNP(Lys)$_7$BuAm	4+	+	4+	0	3+	0	4+	0	3+	0	3+	0	4+	0	3+	0	8/8	1/8
α-DNP(Lys)$_8$BuAm	4+	4+	4+	2+	4+	4+	4+	3+	4+	4+	4+	0	4+	0	4+	4+	8/8	6/8
α-DNP(Lys)$_8$BuAm	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	2+	4+	3+	4+	4+	8/8	8/8
α-DNP(Lys)$_{\overline{8.4}}$BuAm	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	8/8	8/8

* Immediate (Arthus) skin reaction at 3 to 6 hr.

‡ Delayed skin reaction at 24 to 48 hr.

immediate responses, whereas the octamer and nonamer could elicit both responses in both strains of guinea pigs.

Following immunization, strain 2 guinea pigs develop (at 2 wk) a stage of delayed sensitivity prior to the development (at 7 to 10 wk) of immediate sensitivity and circulating antibody. 2 wk following immunization with α-DNP(Lys)$_{\overline{8.4}}$BuAm, animals were skin tested with individual peptide members of this homologous series. As is indicated in Table III, all animals developed sensitivity to the immunizing antigen, α-DNP(Lys)$_{\overline{8.4}}$BuAm and to α-DNP-(Lys)$_9$BuAm. In 60% of the animals, α-DNP(Lys)$_8$BuAm was able to elicit a delayed reaction. On the other hand, oligomers smaller than or equal in size to the heptamer did not elicit delayed reactions. At 10 wk, however, when these animals had developed both immediate and delayed skin reactivity, they were again skin tested with individual oligomers. At this time, it is again apparent (Table III) that the immediate skin response, but not the delayed response, can be elicited with the tetramer, hexamer, and heptamer. However,

both the nonamer and immunizing antigen could now elicit both immediate and delayed skin reactivity.

Further evidence of the contribution of peptide chain length to the immunological specificity of the immediate and delayed skin reaction was obtained in

TABLE II

Skin Reactivity of Sensitized Hartley and Strain 2 Guinea Pigs

Test material	Hartley guinea pigs* sensitized with α-DNP(Lys)$_8$BuAm‡		Strain 2 guinea pigs§ sensitized with α-DNP(Lys)$_9$BuAm‡	
	Immediate	Delayed	Immediate	Delayed
α-DNP(Lys)$_4$BuAm	3/4	0/4	3/7	0/7
α-DNP(Lys)$_5$BuAm	3/4	0/4	—	—
α-DNP(Lys)$_6$BuAm	3/4	0/4	4/7	0/7
α-DNP(Lys)$_7$BuAm	3/4	1/4	4/6	0/6
α-DNP(Lys)$_8$BuAm	4/4	4/4	—	—
α-DNP(Lys)$_9$BuAm	4/4	4/4	7/7	7/7

* Tested at 4 wk.

‡ Different oligomers were used to conserve material. Both oligomers have been shown to be immunogenic (9).

§ Tested at 10 wk.

TABLE III

Skin Reactivity of Strain 2 Guinea Pigs Sensitized to α-DNP(Lys)$_{\overline{8.4}}$BuAm

Test material	Tested at 2 Wk		Tested at 10 Wk	
	Immediate	Delayed	Immediate	Delayed
α-DNP(Lys)$_4$BuAm	0/10	0/10	3/7	0/7
α-DNP(Lys)$_5$BuAm	0/10	0/10	—	—
α-DNP(Lys)$_6$BuAm	0/10	0/10	5/7	0/7
α-DNP(Lys)$_7$BuAm	0/10	0/10	5/7	0/7
α-DNP(Lys)$_8$BuAm	0/10	6/10	—	—
α-DNP(Lys)$_9$BuAm	0/10	10/10	7/7	7/7
α-DNP(Lys)$_{\overline{8.4}}$BuAm	0/20	20/20	7/7	7/7

animals sensitized to (Lys)$_{\overline{8.4}}$BuAm. The individual members of this series were tested for their ability to elicit skin reactions. These oligomers also differ from one another only in the number of lysyl residues, but each contained a free *N* terminal, α-amino group (instead of the DNP group) and terminated at the carboxyl end with butylamide. As can be seen in Table IV, the tetra-, penta-, hexa-, and heptamer could elicit only the immediate response in Hartley guinea pigs. In strain 2 animals no skin reaction at all was elicited with the

hexamer and heptamer. However, in contrast, the octamer and nonamer could elicit both the immediate and delayed responses in Hartley animals, and, when tested at 2 wk, only the delayed response in strain 2 guinea pigs.

The Role of Hapten and Oligolysyl Carrier in the Specificity of the Delayed and Immediate Skin Responses.—Hartley and strain 2 guinea pigs sensitized to α-DNP(Lys)$\overline{_{8.4}}$BuAm were skin tested with various polylysyl peptides, DNP-substituted polylysyl peptides, and DNP-substituted proteins to further investigate the specificity of the delayed and immediate skin responses. At the time of testing Hartley animals showed both immediate and delayed skin sensitivity to the immunizing antigen, whereas strain 2 animals only displayed the delayed reaction. The results in Table V indicate that compounds, e.g., α-

TABLE IV

Skin Reactivity of Hartley and Strain 2 Animals Sensitized to (Lys)$\overline{_{8.4}}$BuAm

Test material	Hartley guinea pigs*		Strain 2 guinea pigs‡	
	Immediate	Delayed	Immediate	Delayed
$(Lys)_4$BuAm	3/3	0/3	—	—
$(Lys)_5$BuAm	3/3	0/3	—	—
$(Lys)_6$BuAm	3/3	0/3	0/5	0/5
$(Lys)_7$BuAm	3/3	0/3	0/5	0/5
$(Lys)_8$BuAm	3/3	3/3	0/5	5/5
$(Lys)_9$BuAm	3/3	3/3	0/5	5/5
(Lys)$\overline{_{8.4}}$BuAm	6/6	6/6	0/25	25/25

* Tested at 4 wk.

‡ Tested at 2 wk.

DNP(Lys)$\overline{_{60}}$, α-DNP-BPA, α-DNP(Lys)$\overline{_{18}}$, and (Lys)$\overline{_{8.4}}$BuAm, possessing either the dinitrophenyl or butylamide group as well as a large oligo-L-lysyl carrier were able to elicit both delayed and immediate skin reactions. These materials contain hapten-substituted peptides of chain length equal to or longer than the octamer, an oligomer known to elicit both delayed and immediate skin reactions (9). Furthermore, it should be emphasized that in α-DNP(Lys)$\overline{_{60}}$, the α-DNP$(Lys)_8$ moiety is only part of a much larger oligo-L-lysyl carrier, yet the 60-mer appeared as effective in eliciting the delayed response as the sensitizing agent, α-DNP(Lys)$\overline{_{8.4}}$BuAm. In contrast to this, ε-DNP-BPA and α-DNP-D(Lys)$\overline{_{80}}$ both containing the dinitrophenyl group, the first with an ε-*N*-DNP substitution and the second with a poly-D-lysyl carrier, could only elicit immediate responses in Hartley guinea pigs and no delayed sensitivity response at all in strain 2 animals. Perhaps because of their small size neither α-DNP-lysine nor α-$DNP(Lys)_2$ were able to elicit skin responses. Hapten-free poly-L-

TABLE V

Cross-Reactions in Hartley and Strain 2 Guinea Pigs Sensitized to α-DNP(Lys)$_{8.4}$BuAm

Test material	Hartley guinea pigs*		Strain 2 guinea pigs‡	
	Immediate	Delayed	Immediate	Delayed
ϵ-DNP-BPA	6/6	0/6	0/8	0/8
α-DNP-BPA	6/6	6/6	0/5	5/5
α-DNP-D-(Lys)$_{60}$	2/6	0/6	0/6	0/6
α-DNP(Lys)$_{60}$	6/6	6/6	0/7	7/7
α-DNP(Lys)$_{18}$	3/3	3/3	0/5	5/5
(Lys)$_{8-10}$	0/3	0/3	0/5	0/5
(Lys)$_{10-30}$	0/6	0/6	0/7	0/7
(Lys)$_{1250}$	—	—	0/2	0/2
(Lys)$_{8.4}$BuAm	6/6	6/6	0/6	6/6
α-DNP-Lysine	0/4	0/4	0/2	0/2
α-DNP-(Lys)$_2$	0/4	0/4	—	—
α-DNP(Lys)$_{8.4}$BuAm	22/22	22/22	0/51	51/51

* Tested at 4 wk.

‡ Tested at 2 wk.

TABLE VI

*Skin Reactivity of Hartley Strain Guinea Pigs Immunized with Various Hapten Substituted and Unsubstituted Poly-L-Lysines**

Skin test material	Immunizing material			
	α-DNP(Lys)$_{8.4}$BuAm	(Lys)$_{8.4}$BuAm	α-DNP(Lys)$_{11}$	(Lys)$_{8-10}$, $_{10-30}$
α-DNP(Lys)$_{8.4}$BuAm	I + D	I + D	I + D	0
(Lys)$_{8.4}$BuAm	I + D	I + D	0	0
α-DNP(Lys)$_{11}$	I + D	0	I + D	0
(Lys)$_{8-10}$	0	0	0	0
(Lys)$_{10-30}$	0	0	0	0

* I, immediate (Arthus) type hypersensitivity; and D, delayed type hypersensitivity.

lysine preparations with a wide distribution of chain lengths, were also unable to provoke skin reactions in this system (Table V).

Cross-Reactions of the Hapten Poly-L-Lysine System.—The data concerning immunogenicity and cross-reactions in Hartley strain guinea pigs to various hapten poly-L-lysines and poly-L-lysines are summarized in Table VI. 3 wk after immunization approximately 70% of these guinea pigs developed both delayed and immediate skin reactivity to α-DNP(Lys)$_{8.4}$BuAm, α-DNP(Lys)$_{11}$, and (Lys)$_{8.4}$BuAm. All animals showing immediate skin reactivity contained

circulating antibody as determined by PCA reaction or quantitative precipitation test. Eight animals each were immunized with $(Lys)_{\overline{8-10}}$ and $(Lys)_{\overline{10-30}}$, but none developed sensitivity ($0/16$) even after prolonged immunization and skin testing. The results obtained represent the cross-reactions observed in groups of 6 guinea pigs sensitized to the various immunizing antigens. As is apparent in Table VI, animals sensitized to α-DNP$(Lys)_{\overline{8.4}}$BuAm cross-react with compounds containing the butylamide group or the dinitrophenyl group but not to poly-L-lysine itself. Animals sensitized to $(Lys)_{\overline{8.4}}$BuAm will cross-react only with compounds containing the butylamide group but not with α-DNP$(Lys)_{\overline{11}}$ or poly-L-lysines. Sera from animals immunized with $(Lys)_{\overline{8.4}}$BuAm were set up in PCA tests with CBZ-BPA and α,DNP-ϵ,$(CBZ)_{\overline{n}}$ $(Lys)_{\overline{18}}$ as the antigens to rule out the remote possibility that residual benzyloxycarbonyl groups accounted for the immunogenicity of the $(Lys)_{\overline{8.4}}$BuAm rather than the butylamide group. These PCA tests were negative. In a similar fashion, animals sensitized to α-DNP$(Lys)_{\overline{11}}$ can cross-react only with compounds containing the dinitrophenyl group, but not with either poly-L-Lys BuAm or poly-L-lysines.

DISCUSSION

Previous studies have shown that both delayed and immediate sensitivity could be induced by the same chemically well defined antigen (9). The present observations suggest that these two responses, in fact, reflect specificity to different portions of the immunizing antigen. Furthermore, they indicate that the determinant required to elicit the delayed response involves the hapten attached to a larger portion of the immunizing antigen than is required to react with conventional antibody and elicit the immediate response.

Support for these two views is derived from the experiments with Hartley strain guinea pigs (Tables I and II) which show that in animals sensitized to a dihaptenic oligopeptide mixture, α-DNP$(Lys)_{\overline{8.4}}$BuAm, or to a single member of the mixture, α-DNP$(Lys)_8$BuAm, only the immediate skin reaction was elicited by the homologous tetra-, penta-, or hexamer whereas the octamer or nonamer could elicit both delayed as well as immediate hypersensitivity reactions. Similar results were obtained in the absence of the α-DNP group with the monohaptenic-substituted oligo-L-lysines, $(Lys)_{\overline{8.4}}$BuAm (Table IV).

Further support for these findings was obtained in strain 2 animals sensitized to either α-DNP$(Lys)_{\overline{8.4}}$BuAm, α-DNP$(Lys)_9$BuAm, or $(Lys)_{\overline{8.4}}$BuAm. Tested at a time (2 wk) when only the delayed reaction appeared, they responded only to the appropriate octamer or nonamer (Tables II, III, and IV) but not to any smaller hapten-substituted oligomer. However, 8 wk later, after development of immediate sensitivity and circulating antibody, the smaller peptides were now only able to elicit the immediate response whereas both delayed and immediate responses were elicited by the larger oligomers. Whenever immediate skin sensitivity was observed PCA reactions for circulating antibody could be obtained.

That the hapten is an integral part of the determinant for both immediate as well as delayed skin reactivity, is shown by the failure of unsubstituted poly-L-lysine to elicit either delayed or immediate responses in animals sensitized to α-DNP(Lys)$_{\overline{8.4}}$BuAm, (Lys)$_{\overline{8.4}}$BuAm, or to α-DNP(Lys)$_{\overline{11}}$ (Table VI). Immediate (Arthus) type cross-reactions occurred only when both the sensitizing and the test antigens shared a common haptenic determinant. In contrast, delayed type cross-reactions occurred only when the test antigen and sensitizing antigen both have the same haptenic determinant attached to an oligo-L-lysyl carrier of 7 or more lysyl residues. This view is supported by the observations that ϵ-DNP-BPA and α-DNP-D-(Lys)$_{\overline{80}}$ were able to elicit only immediate but not delayed skin reactivity in animals sensitized to α-*N*-DNP substituted, oligo-L-lysine carriers (Table V) whereas the appropriate monohaptenic (BuAm-free) α-DNP-L-(Lys)$_{\overline{n}}$[α-DNP(Lys)$_{\overline{60}}$ and α-DNP(Lys)$_{\overline{18}}$] were able to elicit both immediate and delayed reactions in animals sensitized to the dihaptenic antigen, α-DNP(Lys)$_{\overline{8.4}}$BuAm (Table V).

At most, 2 lysyl residues seems to be the difference between the ability to elicit the immediate or delayed skin sensitivity reactions in this study. The increased net positive charge resulting from the additional two ϵ-amino groups may permit the immunogenic octamer, α-DNP(Lys)$_8$BuAm, to remain at a local site for a longer time than the inactive hexamer and for this reason display a delayed response. It should be noted that several findings are in conflict with this interpretation. Firstly, the concentration of α-DNP(Lys)$_6$BuAm can be increased without the appearance of a delayed response. Secondly, the concentration of α-DNP(Lys)$_8$BuAm can be decreased as much as 100-fold and a delayed response can still be elicited. Finally, neither ϵ-DNP-BPA nor α-DNP-D(Lys)$_{\overline{80}}$ can elicit a delayed response, whereas both α-DNP-BPA and α-DNP-L(Lys)$_{\overline{60}}$ can elicit such a response (Table V).

Karush and Eisen (14) have suggested that delayed hypersensitivity reactions are mediated by circulating antibodies of high binding affinity but present in serum in extremely low concentrations. They have calculated that the determinant for the delayed response in a bovine DNP-gamma globulin system need be no more than a single DNP-lysyl group plus one apolar amino acid side chain to provide sufficient affinity to react with high affinity antibody. It seems worthwhile to consider this hypothesis even though there is nothing in the present work which bears directly on this question.

The work reported herein indicates that hapten conjugated to smaller or unrelated peptides is still capable of eliciting the immediate response. These findings are consistent with the view that the hapten is the major factor in providing sufficient binding affinity to react with circulating antibody and induce the immediate reaction. However, why the hexamer, α-DNP(Lys)$_6$BuAm, can elicit an immediate but not a delayed response in sensitized animals is not at all clear. To invoke an antibody as the mediator of a delayed response would require that the combining site of this antibody be directed toward a much

larger area of the immunizing antigen, and that the eighth and ninth lysyl residue in the active oligo-L-lysyl derivatives supply a critical addition to the overall binding energy. Although there is no direct evidence for this effect, by virtue of its ability to assume a different or more stable attachment, the octamer may bind more firmly to the antibody than does the hexamer. Thus, the addition of two lysyl residues in the peptide chain may be the sufficient condition to convert the inactive hexamer into the active octamer.

We have previously reported that Hartley and strain 2 guinea pigs were sensitized with α-DNP(Lys)$_{\bar{n}}$ peptides if $\bar{n}$ was equal to or greater than 7 (9). It is apparent from the current studies that the ability to elicit the delayed response parallels the immunogenic capacity of these hapten-substituted peptides. The immediate response, on the other hand, can be elicited by non-immunogenic peptides. This finding may indicate that the delayed response depends on the local biosynthesis of sufficient "antibody" (cell-bound or free) to form "antigen-antibody complexes" capable of eliciting observable tissue damage, whereas the immediate Arthus response depends only on the diffusion of preformed antibody into the site of test injection. The delayed skin response may then be analogous to a local secondary response wherein immunogenic peptides can stimulate sensitized lymphocytes to produce cell-bound or free "antibody." Under these circumstances, the need for a larger determinant in order to elicit the delayed response does not necessarily imply a larger combining site. The combining sites for immediate and delayed hypersensitivity reactions may indeed be the same, but the delayed response may depend on the formation of an additional and special site on the antibody molecule which is directly involved in tissue binding.

SUMMARY

Hartley and strain 2 guinea pigs were sensitized to chemically defined α-DNP(Lys)$_{\bar{n}}$BuAm, α-DNP(Lys)$_{\bar{n}}$, and (Lys)$_{\bar{n}}$BuAm peptides and skin tested with individual members of these homologous series, related peptides and hapten-substituted proteins. The immediate skin response (Arthus) could be elicited with hapten-substituted tetra-, penta-, or hexamers, whereas both immediate and delayed skin responses could be provoked by the octamer or nonamer. The hapten is an integral part of the determinant for both immediate and delayed skin reactivity, since poly-L-lysine was unable to elicit either delayed or immediate reactions in sensitized animals. Arthus type cross-reactions occurred only when the sensitizing and test antigen shared a common haptenic determinant. In contrast to this, in this system, delayed type cross-reactions occurred only when the test antigen and the sensitizing antigen contained both a large oligo-L-lysine carrier as well as the same haptenic determinant.

These observations imply that the mediation of the delayed response requires a larger determinant than is necessary to elicit the immediate response. The role of high affinity antibody as the mediator of the delayed response is

discussed in terms of the size of the antigenic determinants required to elicit this response.

It was found that the ability to elicit the delayed response paralleled the immunogenic capacity of these peptides, whereas the immediate response could be elicited by nonimmunogenic peptides. This finding suggests that the delayed response may require the continued biosynthesis of antibody and may be analogous to a local in vivo secondary response.

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