STUDIES ON DELAYED HYPERSENSITIVITY

I. Inferences on the Comparative Binding Affinities of Antibodies Mediating Delayed and Immediate Hypersensitivity Reactions in the Guinea Pig*

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Delayed allergic reactions are characterized by their slow evolution, and by their ability to be passively transferred by lymphoid cells and not by serum (1). These characteristics have been alternatively interpreted as indicating that delayed hypersensitivity is mediated either by "antibodies" fixed on cells or, as has been hypothesized by Karush and Eisen (2), by circulating antibodies of comparatively high binding affinity present in serum in extremely low concentrations. At present, the antibodies mediating delayed hypersensitivity have not been identified, and accordingly their properties cannot be studied directly. The availability of antigens of comparatively well defined structures, *i.e.* lightly coupled hapten–poly-L-lysine conjugates (3) has permitted the present experiments which provide indirect evidence pertaining to the specific binding energy characteristics of delayed hypersensitivity.

Earlier studies have shown that elicitation of delayed allergic reactions in hapten systems is markedly dependent upon carrier specificity, whereas the elicitation of immediate allergic reactions appeared to require only hapten binding (4–7). This striking difference in specificity between immediate and delayed reactions may be explained in three alternative ways: (*a*) the combining site dimensions may be larger for antibodies mediating delayed reactions than for conventional serum antibodies (*cf.*, reference 5); (*b*) the combining site dimensions may be the same for both, but antibodies mediating delayed reactions may be comparatively poorly fitted to antigen, and thus require a larger area of specific contact to provide sufficient binding energy to initiate the biological reaction; and (*c*) delayed reactions may require comparatively high binding energies for their elicitation, and accordingly require compara-

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tively close specific contact between antigen and antibody over a large surface area to provide this energy.

This paper reports experiments with several haptenic antigenic systems which demonstrate that: (*a*) Arthus reactions also manifest carrier specificity, although to a smaller extent than do delayed reactions; (*b*) desensitization by injection of minute doses of antigen results in moderate specific desensitization of delayed hypersensitivity without desensitization of Arthus reactivity to the same antigenic determinant; (*c*) insoluble antigen-antibody complexes prepared from high affinity guinea pig antibodies can elicit specific delayed skin reactions in sensitized guinea pigs; and (*d*) homologous conjugates of structurally similar haptens show considerably less cross-reactivity in delayed reactions than in Arthus reactions. These experimental results are interpreted as indicating that delayed hypersensitivity reactions in the guinea pig are mediated by "antibodies" of comparatively high binding affinities. High binding affinities are achieved for these antibodies more likely by closer adaptation between antigen and antibody, than by a larger area of specific contact.

Materials and Methods

Haptens and Conjugates.—Crystalline potassium allylmercaptomethylpenicillin was a gift from the Upjohn Co., Kalamazoo, Michigan. Crystalline potassium benzylpenicillin and sodium dimethoxyphenylpenicillin were gifts from Bristol Laboratories, Syracuse, New York. Dinitrofluorobenzene (DNFB) and *p*-nitrobenzenesulfonyl chloride were purchased from Eastman Organic Chemicals, Rochester, New York. The latter was recrystallized from ether-petroleum ether, mp, 77–78°C, *ε-N-(Dinitrophenyl)-aminocaproyl chloride* (*DNP-EACCl*). ε-*N*-(DNP)-aminocaproic acid (DNP-EACA) was prepared by reaction of 1 M equivalent of DNFB with 1.25 M equivalents of ε-aminocaproic acid in dioxane-water (25 per cent) maintained at pH 10.5 in a pH-stat, 25°C, 90 minutes. The product was precipitated at pH 2, and recrystallized from ethanol-water, mp, 135–137°C. 2.5 gm DNP-EACA was heated with 5 ml thionyl chloride in a boiling water-bath for 10 minutes. The reaction mixture was triturated repeatedly with cool ether-petroleum ether (1:2, v/v), and DNP-EACCl separated as a yellow crystalline solid, mp, 54–57°C. *ε-N-p-nitrobenzenesulfonamidocaproic* acid (Nisyl-EACA) was prepared by reaction of Nisyl chloride with 1.2 M equivalents of ε-aminocaproic acid in 25 per cent acetone-water solution, 25°C, for 2 hours with the pH maintained at 11 by additions of 1 N NaOH in a pH-stat. The product was precipitated from the reaction mixture at pH 2.5, and crystallized from ethanol-water, mp, 134–135°C, ϵ_M at λ_{max} 265 mμ, 11,300. PLL_{316}·HBr[1] (Lot L-16) was purchased from Pilot Labs., Watertown, Massachusetts. GPA[1] was prepared from pooled normal guinea pig serum by starch block electrophoresis. *Penicilloyl-PLL_{316} conjugates* (APO_{20}-PLL_{316}, BPO_{24}-PLL_{316}, DPO_{21}-PLL_{316}) were prepared by reacting PLL_{316} with 0.10 M equivalents of each penicillin (per mole ε-NH_2) in aqueous solution, pH 11.5 for 90 minutes. *DNP_{20}-PLL_{316} and $Nisyl_{20}$-PLL_{316}* were prepared by reacting PLL_{316} with 0.08 M equivalents of the active chloride (per mole-ε-NH_2) in 10 per cent dioxane-water at pH 10.0 for 90 minutes. *$(DNP\text{-}EAC)_{21}$-PLL_{316}*[1] was prepared by reacting PLL_{316} with 0.09 M equivalent of

[1] Abbreviations.—*Haptenic groups*: APO, allylmercaptomethylpenicilloyl; BPO, benzylpenicilloyl; DPO, dimethoxyphenylpenicilloyl; DNP, dinitrophenyl; DNP-EAC, ε-*N*-(DNP)-aminocaproyl; Nisyl, *p*-nitrobenzenesulfonyl. *Carriers*: PLL_{316}, poly-L-lysine (subscript refers

DNP-EACCl (per mole ϵ-NH_2) in 50 per cent dioxane-water at pH 10.0 for 90 minutes. *DNP_{24}-GPA and $Nisyl_{23}$-GPA* were prepared by reacting GPA with 1 M equivalent (with respect to ϵ-NH_2) of the active chlorides in 10 per cent dioxane-water at pH 10.0 for 90 minutes. Conjugates were purified by prolonged dialysis. They were assayed by duplicate microKjeldahl analyses (8) for nitrogen, penamaldate analyses (9) for penicilloyl concentration, and spectrophotometric analyses [ϵ_M at λ_{max} 360 mμ, 17,400 for DNP, and ϵ_M at λ_{max} 265 mμ, 11,300 for Nisyl] with corrections for the optical contributions of the hapten carriers.

Immunization and Skin Reactions.—Albino Hartley strain guinea pigs weighing 400 to 500 gm were immunized. Primary immunizations were with 100 μg of antigen (as carrier protein) emulsified in 0.2 ml saline and 0.2 ml complete Freund's adjuvant (Difco Laboratories, Detroit) and injected into the hind foot-pads. Booster schedules are described in the text. For experiments with PLL antigens, responder and non-responder animals were differentiated on the basis of their responses to a skin test just prior to booster (3), and non-responders were discarded. Skin tests were performed and graded as follows: animals were injected intradermally in the posterior-lateral areas with 10 μg antigen in 0.1 ml saline. Arthus reactions were read at 2 hours. For both GPA and PLL conjugates they were graded according to extent of hemorrhage: 1+, stippled hemorrhage; 2+, up to 6 mm diameter hemorrhage; 3+, 6 to 9 mm diameter hemorrhage. All positive reactions showed edema, 15 mm diameter for PLL conjugates and 20 mm diameter for GPA conjugates. Controls (immunized with complete Freund's adjuvant alone) gave negative reactions to GPA conjugates, and 5 to 7 mm diameter edema without hemorrhage to PLL conjugates. Delayed reactions were read at 22 to 24 hours. They were graded according to diameter (mm) of erythematous induration for GPA conjugates: 1+, 10 to 15; 2+, 15 to 20; 3+, 20 to 25; 4+, >25. They were graded according to extent of tissue damage for PLL conjugates: 1+, 7 to 11 mm diameter nodule; 2+, 10 to 15 mm indurated erythematous nodule with superficial skin necrosis and some hemorrhage; 3+, 12 to 17 mm markedly indurated papule with large, central punched out ulcer. Controls (immunized with adjuvant alone) gave negative reactions to GPA conjugates, and 5 to 7 mm diameter flat papules to PLL conjugates. The difference in appearance of skin reactions to GPA and PLL conjugates is probably due mainly to the limited diffusion in skin of the highly cationic PLL conjugates. Combined (Arthus and delayed) reactions were seen. Arthus and delayed components can be easily read separately in guinea pigs, as Arthus reactions are at a maximum at 2 hours and are essentially gone at 24 hours when delayed reactions are read. Delayed reactions do not appear until at least 6 to 8 hours after skin testing.

Double Diffusion-in-Gel was done by Ouchterlony's Petri dish method (10) as described previously (6).

Insoluble Antigen-Antibody Complexes.—Ten guinea pigs were immunized with DNP_{24}-GPA (day 1, 100 μg in adjuvant, days 29 and 37, 200 μg in saline intradermally) and bled from the jugular veins on day 43. The pooled serum contained 1.6 mg of antibody protein per ml precipitated by DNP_{24}-GPA (85 μg antigen protein per ml at equivalence), as determined by quantitative precipitin analyses (8) using Eisen's spectrophotometric method of analysis of precipitates (11). Insoluble antigen-antibody complexes in antibody excess were prepared by precipitation of 12.5 ml pooled serum with 500 μg DNP_{24}-GPA. From precipitin curves, it was determined that the precipitate contained approximately 500 μg antigen and 11.6 mg antibody protein. The precipitate was washed 3 times with ice cold saline, and finely suspended in 5.0 ml of saline.

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to average degree of polymerization); GPA, guinea pig serum albumin. *Conjugates*: *e.g.* BPO_{24}-PLL_{316} or DNP_{24}-GPA, benzylpenicilloyl-poly-L-lysine or dinitrophenyl-guinea pig albumin (subscripts refer to average number of haptenic groups per mole conjugate).

RESULTS

Carrier Specificity in Arthus and Delayed Skin Reactions.—A group of guinea pigs was immunized with DNP_{20}-PLL_{316} and another group with DNP_{24}-GPA. Each group was skin tested with both antigens. Table I shows that there was almost complete absence of cross-reactivity in delayed reactions between the 2 conjugates, although 2 of the 6 animals showed trace but definite delayed allergic cross-reactions. These trace reactions were not residua of prior Arthus reactions, as they could be seen also in other animals tested 8 days after immunization where Arthus reactions were absent. Thus, carrier specificity is a marked, but not an absolute, requirement for delayed skin reactions. Table I shows also that each of the 2 groups of guinea pigs gave considerably more intense Arthus reactions to the immunizing conjugate than to the other conjugate. These Arthus reactions were hapten-specific (and not specific for carrier alone or for possible impurities in the conjugates) as: (*a*) PLL_{316}, GPA, and alkalai denatured GPA did not elicit skin reactions in these animals, as had been found previously (3, 6); (*b*) precipitin reactions between sera from DNP_{24}-GPA sensitized animals and DNP_{24}-GPA were completely inhibited by the univalent hapten DNP-EACA at 3×10^{-3} M concentration. These reciprocally controlled experiments (Table I) using conjugates of 2 carriers which are themselves non-antigenic (*i.e.* PLL and GPA) thus unambiguously demonstrate carrier specificity for Arthus reactions. As can be seen in Table I, carrier specificity was considerably less marked for Arthus than for delayed reactions.

TABLE I

*Arthus and Delayed Allergic Cross-Reactivity Between DNP_{20}-PLL_{316} and DNP_{24}-GPA**

Immunizing antigen‡	Guinea pig No.	Testing antigens‡			
		Arthus reactions§		Delayed reactions§	
		DNP_{20}-PLL_{316}	DNP_{24}-GPA	DNP_{20}-PLL_{316}	DNP_{24}-GPA
DNP_{20}-PLL_{316}	1	2+	1+	3+	Trace
	2	2+	1+	3+	Neg.
	3	3+	1+	3+	Neg.
DNP_{24}-GPA	4	1+	3+	Neg.	3+
	5	1+	3+	Neg.	3+
	6	1+	3+	Trace	3+

* Immunization: Day 1, 100 μg antigen in adjuvant; day 23, 100 μg antigen in saline intradermally; skin tested on day 30.

‡ See footnote 1 for abbreviations.

§ See Materials and Methods section for grading of reactions.

Effect of Structural Modifications of Hapten on Arthus and Delayed Cross-

Reactivities.—Groups of guinea pigs were immunized with various hapten-PLL conjugates and tested for Arthus and delayed cross-reactivity to equally coupled PLL conjugates of structurally related haptens. Table II shows typical results obtained with the comparatively large penicilloyl haptens. These haptens have identical thiazolidine carboxylic acid groupings and differ only in the structures

TABLE II

*Arthus and Delayed Allergic Cross-Reactions Among Homologous Penicilloyl-Polylysine Conjugates**

Immunizing antigen	Guinea pig No.	Testing antigen					
		Arthus reactions‡			Delayed reactions‡		
		APO_{20}-PLL_{316}	BPO_{24}-PLL_{316}	DPO_{21}-PLL_{316}	APO_{20}-PLL_{316}	BPO_{24}-PLL_{316}	DPO_{21}-PLL_{316}
APO_{20}-PLL_{316}	1	3+	3+	2+	3+	2+	1+
	2	2+	2+	2+	3+	2+	1+
	3	1+	1+	1+	3+	2+	1+
	4	2+	2+	1+	3+	2+	1+
	5	2+	2+	1+	2+	2+	Neg.
BPO_{24}-PLL_{316}	6	1+	2+	1+	1+	3+	Trace
	7	3+	3+	2+	1+	3+	Trace
	8	3+	3+	1+	3+	3+	Trace
	9	3+	3+	3+	2+	3+	1+
	10	1+	1+	1+	Trace	3+	Trace
DPO_{21}-PLL_{316}	11	2+	2+	3+	1+	1+	3+
	12	2+	2+	3+	Trace	1+	3+
	13	1+	2+	3+	Trace	1+	3+
	14	Trace	Trace	Trace	1+	1+	3+
	15	2+	2+	3+	Trace	1+	3+

* Immunization: Day 1, 100 μg antigen in adjuvant; day 30, 50 μg antigen in saline intradermally; skin tested on day 37.

‡ See footnote 1 for abbreviations and Materials and Methods section for grading of reactions.

of their N-7 side chains (Fig. 1). In general, there was considerable less cross-reactivity in delayed reactions than in Arthus reactions. The BPO and APO hapten conjugates cross-reacted almost completely in Arthus reactions, but showed considerably decreased delayed allergic cross-reactions. The APO and DPO hapten conjugates gave only slight delayed cross-reactions, and moderately strong Arthus cross-reactions. Individual animals showed this specificity difference between immediate and delayed reactions to different extents. Table III shows representative results obtained with the smaller haptens (DNP and Nisyl, see Fig. 1). Here, too, the equally coupled DNP_{20}-PLL_{316} and $Nisyl_{20}$-

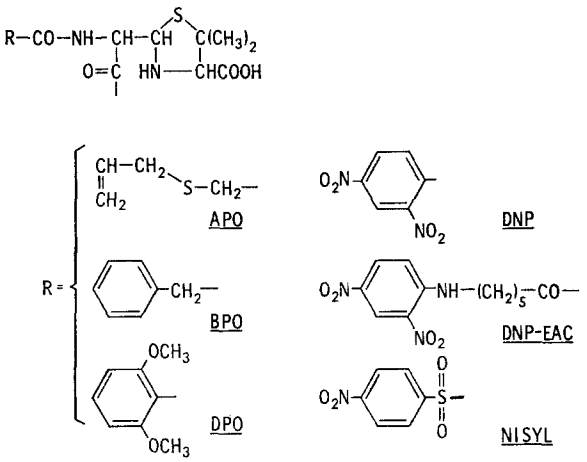

FIG. 1. Structural formulas of haptens used in this study

TABLE III

*Arthus and Delayed Allergic Cross-Reactions Between Homologous Polylysine Conjugates of the Dinitrophenyl and p-Nitrobenzenesulfonyl Haptenic Groups**

Immunizing antigen*	Guinea pig No.	Testing antigens			
		Arthus reactions‡		Delayed reactions‡	
		DNP_{20}-PLL_{316}	$Nisyl_{20}$-PLL_{316}	DNP_{20}-PLL_{316}	$Nisyl_{20}$-PLL_{316}
DNP_{20}-PLL_{316}	1	2+	2+	3+	1+
	2	2+	2+	3+	1+
	3	2+	2+	3+	2+
	4	1+	1+	3+	2+
$Nisyl_{20}$-PLL_{316}	5	1+	1+	2+	3+
	6	1+	2+	1+	3+
	7	1+	1+	Trace	3+
	8	2+	3+	1+	3+

* Immunization: Day 1, 100 μg antigen in adjuvant; skin tested on day 17.

‡ See footnote 1 for abbreviations and Materials and Methods section for grading of reactions.

PLL_{316} conjugates showed considerably stronger cross-reactions in Arthus reactivity than in delayed hypersensitivity. These specificity differences were seen at all reaction intensities. PLL itself failed to elicit Arthus and delayed reactions in guinea pigs immunized with hapten-PLL conjugates.

Precipitin reactions could not be carried out with the PLL conjugates, as the conjugates non-specifically precipitate serum proteins. Accordingly, 2 groups of

guinea pigs were immunized with DNP_{24}-GPA and with $Nisyl_{23}$-GPA. They were bled on the 16th day after immunization, and skin-tested on the 17th day. Table IV shows that the DNP and Nisyl-GPA conjugates cross-reacted to a moderate degree in Arthus reactions, but did not cross-react in delayed reactions. GPA and alkali denatured GPA did not elicit skin reactions. The differences between delayed and Arthus cross-reactivities were more marked in the GPA system than in the PLL system (*cf.* Table III). This occurs probably because carrier structure varies slightly among different GPA conjugates

TABLE IV

*Arthus and Delayed Allergic Cross-Reactivity Between Homologous Dinitrophenyl and p-Nitrobenzenesulfonyl Conjugates of Guinea Pig Serum Albumin**

Immunizing antigens‡	Guinea pig No.	Testing antigens			
		Arthus reactions‡		Delayed reactions‡	
		DNP_{24}-GPA	$Nisyl_{23}$-GPA	DNP_{24}-GPA	$Nisyl_{23}$-GPA
DNP_{24}-GPA	1	2+	1+	2+	Neg.
	2	2+	1+	3+	Trace
	3	2+	1+	3+	Neg.
	4	2+	1+	2+	Neg.
$Nisyl_{23}$-GPA	5	1+	1+	Neg.	3+
	6	1+	1+	Neg.	3+
	7	1+	1+	Neg.	2+
	8	1+	1+	Neg.	3+

* Immunization: Day 1, 100 μg antigen in adjuvant; skin tested on the 17th day.

‡ See footnote 1 for abbreviations and Materials and Methods section for grading of reactions.

(due to conjugation of different amine residues, and/or different degrees of denaturation of carrier) whereas the relatively simple carrier structure of lightly coupled PLL conjugates probably does not vary significantly. The sera from DNP_{24}-GPA and $Nisyl_{23}$-GPA sensitized animals were tested for cross-reactivity in specific precipitation (double diffusion-in-gel against 100 μg per ml concentrations of DNP_{24}-GPA and $Nisyl_{23}$-GPA). The 4 $Nisyl_{23}$-GPA sera gave equally intense precipitin lines against both antigens. The 4 DNP_{24}-GPA sera gave intense lines against DNP_{24}-GPA and faint lines against $Nisyl_{23}$-GPA. These precipitin lines were hapten-specific, as precipitation failed to occur in agar plates containing the homologous univalent hapten Nisyl-EACA or DNP-EACA at 3×10^{-3} M concentration. Thus, cross-reactivity in precipitation is comparable to extent of cross-reactivity in Arthus reactions, and considerably greater than the extent of cross-reactivity in delayed reactions (*cf.* Table IV).

Effect of Structural Modification of Hapten on Allergic Cross-Reactivity in Active Cutaneous Anaphylaxis.—Groups of guinea pigs were sensitized with DNP and Nisyl conjugates of GPA and PLL_{316}, and they were tested for cross-

TABLE V

Allergic Cross-Reactions (Active Cutaneous Anaphylaxis) Between Homologous Dinitrophenyl and p-Nitrobenzenesulphonyl Conjugates‡*

Immunizing antigen	Guinea pig No.	Concentrations (μg/ml)					
		DNP_{20}-PLL_{316}			$Nisyl_{20}$-PLL_{316}		
		1	10	100	1	10	100
DNP_{20}-PLL_{316}	1	Trace	4 PM	8 PM	Trace	4 PM	8 PM
	2	5 PM	8 PM	8 M	5 PM	8 PM	8 M
	3	5 P	7 PM	10 M	5 P	6 PM	7 PM
$Nisyl_{20}$-PLL_{316}	4	Trace	4 PM	7 PM	Trace	4 PM	9 PM
	5	Trace	5 P	10 PM	5 PM	6 PM	9 M
	6	5 PM	9 PM	9 PM	5 PM	9 PM	10 PM

Immunizing antigen	Guinea pig No.	Concentrations (μg/ml)			
		DNP_{24}-GPA		$Nisyl_{23}$-GPA	
		1	10	1	10
DNP_{24}-GPA	7	11 PM	15 M	8 P	13 M
	8	8 P	8 M	5 P	5 P
	9	—	15 MS	—	15 MS
$Nisyl_{23}$-GPA	10	8 M	10 M	8 M	9 M
	11	12 PM	15 M	8 PM	14 M
	12	—	15 MS	—	15 MS

* Evans blue (0.5 ml of 1 per cent in saline) injected intravenously, immediately after, antigen solutions in 0.1 ml saline injected intradermally. Reaction (read in 15 minutes) intensities based on intensity of blue color (P, pale; PM, pale-moderate; M, moderate; MS, moderate-strong; S, strong) and reaction diameter (mm). Antigen solutions in non-sensitized animals and saline diluent in sensitized animals gave 1 × 2 mm blue streaks.

‡ Immunization: Day 1, 100 μg antigen in adjuvant; day 14, 50 μg antigen in saline intradermally; skin tested on day 20.

reactivity between homologous DNP and Nisyl conjugates in active cutaneous anaphylaxis reactions. The results in Table V show considerable specific cross-reactivity between the DNP and Nisyl conjugates of homologous carriers, comparable in extent of cross-reactivity to Arthus reactions and to precipitation-

in-gel, and considerably greater than the extent of cross-reactivity in delayed reactions (*cf.* Tables III, and IV).

Desensitization of Arthus and Delayed Skin Reactivity with Small Doses of Antigen.—Guinea pigs were doubly sensitized with 100 μg each of BPO_{24}-PLL_{316} and DNP_{20}-PLL_{316} in complete Freund's adjuvant and boosted on day 15 with 100 μg of each antigen in saline injected intradermally. The animals

TABLE VI

*Specific Desensitization of Arthus and Delayed Skin Reactivity with Small Doses of Antigen (DNP_{20}-PLL_{316})**

Guinea pig No.	Skin test dose of DNP_{20}-PLL_{316}							
	Arthus reactions‡				Delayed reactions‡§			
	Before desensitization		After desensitization		Before desensitization		After desensitization	
	2 μg	10 μg	2 μg	10 μg	2 μg	10 μg	2 μg	10 μg
1	1+	3+	1+	2+	3+	3+	1+	1+
2	1+	2+	1+	2+	3+	3+	2+	3+
3	1+	2+	1+	2+	3+	3+	1+	2+
4	Trace	1+	Trace	1+	1+	3+	Trace	1+
5	Trace	1+	Trace	2+	1+	3+	Trace	1+
6	Trace	1+	Trace	1+	1+	3+	Trace	2+
7	Trace	1+	Trace	1+	1+	3+	Trace	1+

* Immunization: Day 1, 100 μg of BPO_{24}-PLL_{316} and DNP_{20}-PLL_{316} in complete adjuvant; day 15, 50 μg of both antigens intradermally.

‡ Skin tested on day 21, desensitized with total dose of 4.0 μg (0.5 μg every hour for 8 hours) DNP_{20}-PLL_{316} on day 24, and skin tested 15 minutes after last desensitizing dose of antigen.

§ These 7 animals were tested also with BPO_{24}-PLL_{316} and showed little or no decrease in skin reactivity to BPO_{24}-PLL_{316} after desensitization with DNP_{20}-PLL_{316}. Another group of guinea pigs similarly immunized but given 8 hourly injections of saline (instead of DNP_{20}-PLL_{316}) showed no decrease in skin reactivity to the antigens.

were skin tested with both antigens on day 21. On day 24, they were desensitized by 8 intramuscular injections of 0.50 μg DNP_{20}-PLL_{316} given at 1 hour intervals, and skin-tested again with both antigens 15 minutes after the last desensitization injection. Skin reactions were read at 2 hours for Arthus reactions, and at 20 hours for delayed reactions. Some typical results are shown in Table VI. All 7 guinea pigs given this course of desensitization showed a moderate decrease in delayed skin reactions to DNP_{20}-PLL_{316}, but no decrease in Arthus skin reactivity to this antigen. Quantitative considerations of antibody binding by DNP_{20}-PLL_{316} are consistent with the failure of this small dose of antigen (4 μg) to desensitize guinea pigs for Arthus reactivity. A 4 μg

dose of DNP_{20}-PLL_{316} can absorb a maximum of about 360 μg of antibody protein,[2] which is less than 6 per cent of the total antibodies present and is thus negligably small.[3] Desensitization of delayed reactivity was specific, as simultaneous testing with BPO_{24}-PLL_{316} showed only a slight decrease in skin reactivity. Some non-specific decrease in delayed allergic skin reactivity is known to accompany specific desensitization of delayed hypersensitivity (13). This

TABLE VII

*Elicitation of Delayed Allergic Skin Reactions with Insoluble Antigen-Antibody Complexes**

Guinea pigs tested	Guinea pig No.	Delayed reactions‡	
		DNP_{24}-GPA, 10 μg (alone)	DNP_{24}-GPA, 10 μg (as antigen-antibody complex)
Controls (immunized with ovalbumin)	1	Negative	5 mm Nodule
	2	Negative	5 mm Nodule
	3	Negative	5 mm Nodule
	4	Negative	5 mm Nodule
Immunized with DNP_{24}-GPA§	5	15 mm (Skin necrosis)	12 mm (Skin necrosis)
	6	14 mm (Skin necrosis)	10 mm (Skin necrosis)
	7	17 mm (Skin necrosis)	12 mm (Skin necrosis)
	8	13 mm (Skin necrosis)	10 mm (Skin necrosis)
	9	17 mm (No necrosis)	12 mm (No necrosis)

* Complexes were prepared from high affinity guinea pig anti-(DNP_{24}-GPA) antibodies (see Materials and Methods section).

‡ Skin tested on day 50. Delayed reactions read at 24 hours. Complexes evoked 7 to 12 mm erythematous edema with trace hemorrhage in both immunized and control animals at 2 hours. DNP_{24}-GPA did not evoke skin reactions in controls at 2 hours, and evoked 1+ and 2 + typical Arthus reactions in immunized animals.

§ Immunization: Day 1, 100 μg antigen in adjuvant; days 30 and 40, 50 μg in saline intradermally.

desensitization of delayed skin reactivity was not due to the initial skin tests (3 days prior to desensitization) as preliminary experiments showed that guinea pigs immunized and skin tested in an identical manner, but injected 8 times with

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[2] Succinylated DNP_{20}-PLL_{316} (which does not non-specifically precipitate serum) can specifically precipitate a maximum of 60 to 90 μg antibody per μg antigen (as PLL base) in marked antibody excess from high titred late immunization guinea pig anti-DNP sera.

[3] Three days after the last skin tests, the 7 guinea pigs (Table VI) were bled, and the serum pool was assayed for anti-DNP antibodies by quantitative precipitin reaction with succinylated DNP_{20}-PLL_{316}. The pool contained 250 μg per ml antibody protein. This figure is within the range reported by Kantor *et al.* (126 to 2460 μg per ml) (12). These animals weighed about 600 gm, with a serum volume of about 25 ml, and a total antibody protein content of at least 6250 μg.

saline (instead of being desensitized), gave essentially identical skin tests on the 21st and 24th days.

Elicitation of Delayed Reactions with Insoluble Antigen-Antibody Complexes.— DNP_{24}-GPA–sensitized guinea pigs were skin-tested simultaneously with DNP_{24}-GPA alone, and with DNP_{24}-GPA in the form of insoluble antigen-antibody complexes. The complexes were prepared in moderate antibody excess from a late immunization, high–titred guinea pig anti-(DNP_{24}-GPA) serum pool (see Materials and Methods), and accordingly contained serum antibodies of comparatively high binding affinity. Table VII shows that the antigen-antibody complexes evoked specific delayed allergic skin reactions in the sensitized guinea pigs and only trace irritation reactions in controls. These reactions appeared in 8 hours and reached their maxima at 24 hours. They were macroscopically indistinguishable in appearance from typical delayed allergic reactions elicited in the same animals by free DNP_{24}-GPA. They were generally of smaller diameter than were the reactions elicited by free DNP_{24}-GPA, but both reactions were equally as indurated. The intensity of delayed reactions elicited by free and complexed antigen generally paralleled one another. Elicitation of delayed skin reactions by toxoid-human antitoxin complexes in human beings immunized with diphtheria toxoid has been reported by Lawrence and Pappenheimer (14). Uhr and Pappenheimer (13) reported elicitation of delayed skin reactions by complexes of toxoid and horse antitoxin in guinea pigs sensitized with diphtheria toxoid. In the present experiments, a haptenic system was utilized in order to increase the probability that both delayed hypersensitivity and serum antibody are directed against the same antigenic determinants. Also, antigen-antibody complexes were prepared from high affinity, homologous antibodies in order to permit interpretation in terms of the relative binding affinities of delayed versus conventional serum antibodies.

DISCUSSION

We infer, from the foregoing experimental results, that antibodies mediating delayed hypersensitivity (hereafter called "delayed antibodies") are on the average, of higher binding affinities than are conventional serum antibodies, at least in the experimental systems studied here. We note, however, that the experimental data supporting this view are of an indirect nature (rather than direct measurements of binding affinities of these antibodies), and accordingly this view is subject to the uncertainty generally inherent in inferential scientific methods. The arguments leading to this view are presented below.

Firstly, it was shown that a minute dose of antigen (*i.e.* 4 μg DNP_{24}-PLL_{316}) can specifically desensitize hyperimmunized guinea pigs for delayed hypersensitivity (to a moderate extent) without desensitizing for Arthus reactivity. Considering the comparatively simple and well defined structure of the antigen used (*i.e.* DNP_{24}-PLL_{316}), and the demonstration that both Arthus and delayed

hypersensitivity are directed against an antigenic unit comprised of the DNP group plus part of the PLL carrier (Table II), it is reasonable to conclude that both delayed and Arthus reactions in this system were specific for generally the same structural unit of antigen. (In contrast, protein antigens are made up of several structurally distinct antigenic units, different ones of which may induce delayed and immediate hypersensitivity, *cf.* references 4 and 5). In addition, it is reasonable to provisionally assume that specific desensitization of delayed hypersensitivity depends upon absorption of delayed antibodies by antigen, since this process shows structural specificity (see text and reference 13) and since desensitization occurs immediately after injection of antigen (13). Accordingly, the ability of delayed antibodies to bind low concentrations of antigen in the presence of comparatively high concentrations of late immunization conventional serum antibodies of the same specificity, would indicate that delayed antibodies have a higher average binding affinity to antigen than do conventional serum antibodies. The possibility that part of the high binding affinity of delayed antibodies may be non-specific appears unlikely, but cannot be excluded by the present experiments.

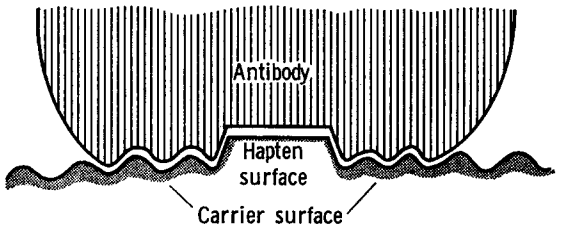
The present observation that insoluble antigen-antibody complexes prepared from high affinity guinea pig serum antibodies (late immunization, high titred serum, precipitation in moderate antibody excess) can elicit specific delayed allergic skin reactions provides additional support to the view that delayed antibodies can compete successfully with high affinity conventional serum antibodies for antigen, and accordingly delayed antibodies are of higher average binding affinity.

These data do not necessarily indicate that all delayed antibody molecules are of higher binding affinity than all conventional serum antibody molecules. The observation that complete desensitization of delayed hypersensitivity was not achieved with the 4 μg dose of antigen might suggest that not all delayed antibody molecules are of unusually high binding affinity, although other explanations for this observation (*e.g.* continued synthesis of delayed antibody) are at present equally plausible.[4] Delayed antibodies may show heterogeneity with regard to binding affinities (*cf.* Gell and Silverstein, references 7 and 15) as do conventional serum antibodies (16). In any case, the present data indicate that at least a large percentage of delayed antibodies are of comparatively high binding affinity.

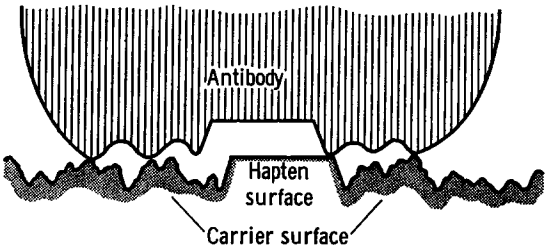
The physical basis for the higher average binding affinities of delayed antibodies may be either that delayed antibodies have larger antibody-combining

[4] In a similar experiment, guinea pigs were desensitized by the same schedule but with a total dose of 32 μg of DNP_{20}-PLL_{316}. Here, too, a moderate reduction of delayed hypersensitivity was achieved, but complete desensitization was not observed. The failure to achieve complete desensitization of delayed hypersensitivity with this higher antigen dosage may be due to continued synthesis of delayed antibodies during the latent period, and following the rapid removal of antigen complexed with conventional serum antibodies. This possibility is under investigation.

site dimensions than do conventional antibodies, or that the delayed antibody-combining sites are more closely fitted to the antigenic unit. As to the first possibility, comparative measurements of combining site dimensions for delayed and conventional antibodies are not available, so this possibility remains unresolved. However, the finding (Table I) that Arthus reactions are also dependent for elicitation upon carrier specificity[5] (although to a smaller extent than delayed reactions) decreases the probability that delayed antibodies are



A. Specific binding of immunizing hapten conjugate by "delayed antibody"



B. Specific binding of hapten conjugate of heterologous carrier by "delayed antibody"

FIG. 2. The postulated role of carrier specificity in delayed hypersensitivity.

specific for a larger antigenic unit than are conventional antibodies (*cf.* references 4 and 5). The findings (Tables II and III) that lightly coupled polylysine conjugates of structurally similar haptens cross-react to a smaller extent in delayed than in immediate hypersensitivity reactions suggest rather that the delayed antibody-combining sites are more closely fitted to the antigenic unit than are conventional antibodies.[6]

[5] Carrier specificity has been indicated also for rabbit antihapten serum antibodies (17–21), and for human anti-BPO skin-sensitizing antibodies (22). Specificity toward tertiary structural configurations of the carrier protein has been indicated for rabbit precipitating anti-BPO antibodies (20).

[6] These findings could also be explained by postulating that delayed antibody-combining sites may be more rigid than are the combining sites of conventional serum antibodies.

From the foregoing discussion, the marked requirement for carrier specificity in delayed hypersensitivity would be to provide the high binding affinities inferred for delayed antibodies. Aside from the additional attractive forces provided by specific antibody binding to the carrier part of the antigenic unit, a proper fit between the carrier part of the antigenic unit and the antibody-combining site would allow the haptenic part of the antigenic unit to establish close contact with its structurally complementary area of the combining site. Stated conversely, for closely fitted and/or relatively inflexible antibody-combining sites, the steric repulsive forces between the combining site and a dissimilar carrier part of the antigenic determinant would prevent the haptenic part of the antigenic unit from making close contact with its complementary area of the antibody-combining site, thus reducing the binding energy contribution of hapten. (Fig. 2).[7]

The inferences that delayed antibodies are of comparatively high binding affinities, and that delayed antibody-combining sites are comparatively closely fitted to the hapten, along with the known marked requirement for carrier specificity (4–7), support the view (2) that delayed hypersensitivity reactions require antigen-antibody reactions of comparatively high binding affinity for their initiation.[8] The reason for the requirement for high binding affinity is unknown at present. Two general possibilities are: (*a*) high binding affinity may be required to permit the formation of stable antigen-antibody complexes from antigen and extremely low concentrations of circulating serum antibody

[7] Karush and Eisen (2) have calculated that an antigenic "determinant equivalent to the DNP-lysyl group plus one apolar amino acid side chain could exhibit an affinity more than sufficient to meet the minimum requirement (for delayed hypersensitivity reactions) for a ΔF° of -14 kilocalories." This unit is probably considerably smaller than the dimensions of antibody-combining sites (23, 24, 20). When this unit is part of an antigenic determinant containing a dissimilar carrier, the maximum binding energies of "DNP-lysyl plus one apolar side chain" may not be realized due to steric repulsion of the carrier part preventing close approximation of the haptenic part of the antigenic determinant to the closely fitted and/or rigid antibody-combining site. For this reason, delayed hypersensitivity reactions even to large apolar haptens (which would themselves be theoretically capable of providing sufficient binding energies for delayed reactions) may always be markedly dependent upon carrier specificity, unless the carrier surface is sufficiently separated from the Hapten.

[8] Although unlikely, the possibility cannot be excluded that although delayed reactions are mediated by high affinity antibodies, they might not require unusually high binding forces for their initiation. Thus it may be considered that binding forces provided by hapten alone might be sufficient to initiate delayed reactions if steric repulsive forces from dissimilar carrier did not interfere with hapten binding. In an attempt to evaluate this possibility experimentally, guinea pigs were immunized with DNP_{24}-GPA and tested for delayed cross-reactivity to $(DNP\text{-}EAC)_{21}\text{-}PLL_{316}$. In this conjugate, the carrier is the random-coil PLL and the DNP group is separated from the $\epsilon\text{-}NH_2$ by a ϵ-aminocaproyl chain (Fig. 1), and accordingly the probability of steric repulsion from dissimilar carrier is less. Here, too, carrier specificity was marked; the animals gave 4+ delayed reactions to DNP_{24}-GPA and failed to react to $(DNP\text{-}EAC)_{21}\text{-}PLL_{316}$. However, these results do not rule out the possibility under discussion since there is free rotation about the amide linkage (of hapten to carrier), and the DNP group might accordingly exist rotated close to the PLL carrier surface.

as has been proposed by Karush and Eisen (2); and (*b*) high binding affinity may be required to allow for cell membrane alterations following binding of antigen by cell-fixed antibody. Finally, it is noted that in addition to high binding affinity, an antibody molecule may have to possess other unique structural features in order to mediate delayed hypersensitivity reactions.

SUMMARY

Experiments carried out with several well defined antigenic systems (hapten conjugates of poly-L-lysine and guinea pig serum albumin) in guinea pigs demonstrated that:

1. Arthus reactions also manifest carrier specificity, although to a smaller extent than do delayed hypersensitivity reactions.

2. Desensitization by injection of minute doses of antigen results in moderate specific desensitization of delayed hypersensitivity without desensitization of Arthus reactivity to the same antigenic determinant.

3. Insoluble antigen-antibody complexes prepared from high affinity guinea pig antibodies can elicit specific delayed skin reactions in sensitized guinea pigs.

4. Homologous conjugates of structurally similar haptens show considerably less cross-reactivity in delayed reactions than in immediate hypersensitivity reactions to the same antigenic determinant.

These experimental results are interpreted as indicating that delayed hypersensitivity reactions in the guinea pig are mediated by "antibodies" of comparatively high binding affinities. High binding affinities are achieved for these antibodies more likely by closer structural adaptation between antigen and antibody than by a larger area of specific contact.

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