IMMUNOCHEMICAL STUDY OF ANTIGENIC SPECIFICITY IN DELAYED HYPERSENSITIVITY

V. Immunization with Monovalent Low Molecular Weight Conjugates*

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Arsanilic acid conjugated to poorly antigenic carriers was shown to be capable of producing good hapten-specific delayed hypersensitivity but poor hapten-specific antibody production (1). This hapten-specific delayed hypersensitivity was only transiently suppressed by intravenous injections of monovalent conjugates of arsanilic acid and *N*-acetyltyrosine (2). On the other hand, intramuscular, intradermal, and intraperitoneal injections of such conjugates into newborn guinea pigs produced an unresponsiveness in respect to hapten-directed delayed hypersensitivity lasting for many weeks (3). Because of this ability to produce tolerance, it became of interest to determine whether these simple monovalent conjugates could also function as complete antigens in the production of hapten-specific delayed hypersensitivity. The results demonstrate that the azobenzenearsonate (ABA) group conjugated to a variety of simple aromatic compounds is capable of acting as a complete antigen in the production of delayed hypersensitivity but rarely, if at all, in respect to antibody production.

Materials and Methods

Reagents.—Arsanilic acid, obtained from Eastman Kodak Co., Rochester, New York, was recrystallized 2 X from water prior to use. The *N*-acetyl derivatives of L-tyrosine, L-histidine, and L-tryptophane were purchased from California Corporation for Biochemical Research, Los Angeles, as chromatographically homogeneous and were used without further treatment. D-tyrosine, obtained from Mann Research Laboratories, New York, and L-tyrosyl-L-tyrosine, obtained from New England Nuclear Corp., Boston, were acetylated with acetic anhydride and sodium acetate (4) prior to use.

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Samples of a polymer of D-glutamic acid, D-alanine, and D-tyrosine (D-GAT)[1] and a polymer of the L-isomers (L-GAT) were very kindly provided by Dr. Paul Maurer, Seton Hall College of Medicine, Jersey City, New Jersey.

Samples of *p*-cresol and 3,5-xylen-1-ol were purchased from K and K Laboratories, Jamaica, New York; and N, *N*-dimethylaniline was obtained from Matheson, Coleman, and Bell, Cincinnati. All were used without further purification.

Preparation of Conjugates.—The required amount of arsanilic acid was diazotized in cold acid solution by addition of sodium nitrite. This was added to a slight excess of the appropriate amine acid or phenol derivative in ice cold alkaline solution. Conjugation was allowed to proceed overnight. The deeply colored products were recovered by repeated precipitation with excess acid and resolubilization in alkali.

The trivalent conjugate resorcinol-tri-azophenylazophenylarsonate (R'3) was kindly provided by Dr. Dan Campbell, California Institute of Technology, Pasadena, California.

Immunization and Skin Testing.—Random bred, white male guinea pigs (400 g) were injected in the four foot-pads with an emulsion of the conjugate in complete Freund's adjuvant (1). 2 wk later they were shaved and depilated and skin tested with conjugates of arsanilic acid and guinea pig serum albumin (ABA-GSA) and insulin (ABA-ins). Skin sites were examined at 2 hr for signs of Arthus reaction and at 24 hr the diameter of induration and erythema measured with a ruler.

5 days after skin testing, animals were bled and after another 2 days challenged by intracardiac injection of 1 mg of a conjugate of arsanilic acid and bovine serum albumin (ABA-BSA). Anaphylaxis was graded as slight (snuffling and nose rubbing), moderate (dyspnea, gasping, prostration), and fatal. Sera were analyzed for content of antibody by hemagglutination of formalized sheep red cells conjugated with arsanilic acid (5).

Only occasional, very low titers of antibody were found by this procedure, and these result, will not be further discussed.

RESULTS

Since the most likely antigenic determinants involved in the delayed sensitivity produced by immunization with polytyrosine azobenzenearsonate were the azobenzenearsonate (ABA) group itself or ABA-tyrosine (or some multiple of

TABLE I

Hapten-Specific Delayed Sensitivity Produced by Immunization with Conjugates of Arsanilic Acid, Tyrosyl-Tyrosine, and Tyrosine

Immunizing agent	No. of guinea pigs	Average delayed reaction in mm to 5 μg N ABA-GSA	Anaphylaxis* with 1 mg ABA-BSA
di-ABA-dityr (1×10^{-6} moles ABA)	5	17	3/5‡ (2F, 1S)
ABA-tyr (2×10^{-6} moles ABA)	6	18	2/6 (1F, 1S)

* F, fatal; M, moderate; and S, slight.

‡ No. positive/No. challenged.

[1] D-GAT stands for a polymer of D-glutamic acid, D-alanine, and D-tyrosine.

these groups), it was decided to test the simplest units available for ability to function as a complete antigen. In Table I are seen the results of an experiment in which guinea pigs were immunized with conjugates consisting of either one or two ABA-tyrosine units. As may be seen, both the tyrosyl-tyrosine and the tyrosine conjugate produced excellent hapten-specific delayed sensitivity as judged by reaction with a conjugate of arsanilic acid and an unrelated carrier, ABA-GSA.

Only occasional antibody production, as demonstrated by systemic anaphylaxis, was seen with either conjugate.

To determine whether the reactions observed represented classical delayed hypersensitivity, an attempt was made to transfer this sensitivity passively with cells (6). A group of Hartley strain guinea pigs weighing 400 to 500 g were

TABLE II

Passive Transfer of Hapten-Specific Delayed Hypersensitivity in Guinea Pigs with Washed Peritoneal Exudate Cells

Group	No. of guinea pigs	Average delayed reaction in mm to 50 μg N ABA-insulin	1:100 O.T.
Donors	12	14 (10)*	11 (12)
Recipients	6	10 (4)	10 (5)
Control	7	0	5

* No. of guinea pigs giving positive reactions.

immunized with 2×10^{-6} moles of di-ABA-dityr. 10 days later, each guinea pig received an intraperitoneal injection of 15 ml sterile light mineral oil; 2 days later, the peritoneal cavity of each animal was lavaged under light ether anesthesia with 15 ml sterile heparinized Hank's solution, and collected through a multiply perforated trochar into a siliconized tube. After centrifugation and washing with heparinized Hank's solution approximately 0.2 to 0.4 ml of packed cells was obtained from each donor. The cells from two donors were pooled and injected intravenously into each Hartley strain recipient weighing 350 g, then donors, recipients, and normal untreated Hartley pigs were skin tested with 50 μg of ABA-insulin and 0.1 ml of 1:100 old tuberculin (O. T.). The reactions read at 24 hr shown in Table II demonstrate that delayed sensitivity to both O.T. and the ABA group were transferable by sensitized cells.

It next became of interest to determine whether conjugates with other amino acids or simple aromatic nuclei would also be effective. The results in Table III demonstrate that while arsanilic acid itself is ineffective at the dose used, conjugates of it with the aromatic amino acids tyrosine, histidine, and tryptophane are all effective in provoking hapten-specific delayed sensitivity. In addition, the positive results obtained with conjugates of *p*-cresol, *p*-dimethyl-

aniline and 3,5-xylenol indicate that the amino acid function is not a necessity. Rather it would seem than an azo bond linkage with any suitable acceptor is the necessary prerequisite for production of an antigenic determinant.

Of all the conjugates studied, only the R'3 dye failed to sensitize. The reason for this failure is not known but may be related to the observation that in our hands very heavily conjugated polytyrosine in which every tyrosine moiety is conjugated with an ABA group sensitizes only poorly. This phenomenon has

TABLE III

Hapten-Specific Delayed Sensitivity Following Immunization with Conjugates of Arsanilic Acid

Immunized with 2×10^{-6} moles	No. of guinea pigs	Average delayed reaction in mm to:			Immunizing conjugate (2×10^{-7} moles)	Anaphylaxis* with 1 mg ABA-BSA
		1 μg N ABA-GSA	1 μg N ABA-insulin	(2×10^{-7} moles) ABA–N-acetyl tyr		
Arsanilic acid	5	0	—‡	0	sl. ind.	0/5§
ABA–*N*-acetyltyrosine	3	12	16	sl. er.	—	0/3
ABA–*N*-acetylhistidine	9	15	8	sl. er.	sl. er.	4/9 (2F, 1M, 1S)
ABA–*N*-acetyltryptophane	4	15	13	0	0	1/4 (1F)
ABA–*N*-benzoyltyrosine	6	13	19	sl. ind.	9 er. sl. ind.	1/6 (1M)
ABA–*p*-cresol	5	12	15	0	0	0/5
ABA–*p*-dimethyl aniline	5	11	—	0	sl. ind.	2/5 (2M)
ABA–3,5-xylenol	5	12	10	0	0	0/5
R' 3 dye	6	0	0	0	sl. ind.	0/6

er., erythema; ind., induration; and sl., slight.

* F, fatal; M, moderate; and S, slight.

‡ —, not done.

§ No. positive/No. challenged.

been reported by others (7) for the dinitrophenyl polylysine system as well. Since R'3 has three ABA groups on a single resorcinol nucleus, it is possible that steric interference may occur at the cellular site at which the sensitized event occurs.

Previous studies confirming the work of Benacerraf et al. (8) demonstrated that carriers consisting of polymers of D-amino acids did not function as antigens in producing sensitization to the ABA group. It has been suggested that the failure of such carriers to function is attributable to their indigestibility by enzymes of the reticuloendothelial system. If this were the reason for failure to function, one might expect that production of an antigenic determinant by prior digestion might lead to sensitization. In Table IV are seen the results of an experiment to test this hypothesis. It may be seen that conjugates of ABA and

the polymer of L-glutamic acid, L-alanine, and L-tyrosine produce excellent hapten-specific delayed sensitivity as well as hapten-specific antibody production. Similarly, the monomer ABA-tyrosine gives excellent hapten-specific delayed sensitivity at comparable dose levels, but no antibody production as detected by active anaphylaxis. In contrast, the conjugate of the polymer of D-glutamic acid, D-alanine, and D-tyrosine was totally ineffective. The conjugate

TABLE IV

Hapten-Specific Delayed Hypersensitivity Following Immunization with Conjugates of Arsanilic Acid and D- and L-Amino Acids

Immunizing agent	No. of guinea pigs	Average delayed reaction in mm to:		Anaphylaxis* with ABA-BSA 1 mg
		1 μg N ABA-GSA	1 μg N ABA-insulin	
ABA–L-GAT				
(3×10^{-8} moles ABA)	5	10	12	5/5‡ (2F, 2M, 1S)
ABA–*N*-acetyl L-tyr				
(2×10^{-7} moles ABA)	6	12	17	0/5
(2×10^{-8} moles ABA)	6	8	14	0/6
ABA–D-GAT				
(3×10^{-7} moles ABA)	6	0	0	0/6
(3×10^{-8} moles ABA)	6	0	0	0/6
ABA–*N*-acetyl D-tyr.				
(2×10^{-7} moles ABA)	6	10	14	0/6
(2×10^{-8} moles ABA)	6	5	10	0/6
ABA–poly-D-lysine	9	11	9	4/9 (4F)

* F, fatal; M, moderate; and S, slight.

‡ No. positive/No. challenged.

of ABA and D-tyrosine alone, however, produced a degree of hapten-specific delayed sensitivity only slightly less than that produced with the L-tyrosine conjugate, again with no detectable antibody. These results are consistent with the concept that digestion of large antigens to smaller determinants is required but do not prove that it is an essential step. Of interest in this connection was the finding that a conjugate of poly-D-lysine was effective in producing both hapten-specific delayed sensitivity and antibody, thus suggesting caution in the designation of antigenicity on the basis of optical isomerism alone.

In one further experiment, it was seen that optical isomerism was of significant importance in eliciting a hapten-specific delayed reaction (Table V).

TABLE V

Hapten-Specific Delayed Reactions Produced by Conjugates of D- and L-Polymers in Guinea Pigs Immunized with Conjugates of D- and L-Monomers

Guinea pig	Immunizing agent	Delayed reaction in mm to:			Anaphylaxis* with ABA-BSA 1 mg
		1 μg ABA-GSA	20 μg ABA-L-GAT	20 μg ABA-D-GAT	
1	ABA–*N*-acetyl L-tyrosine				
	2×10^{-6} moles	10 ind. er.‡	20 ind. er.	0	0/5§
2	2×10^{-6} "	12 " "	21 " "	0	
3	2×10^{-6} "	11 " "	17 " "	0	
4	2×10^{-6} "	12 " "	15 " "	0	
5	2×10^{-6} "	12 " "	15 " "	0	
1	ABA–*N*-acetyl D-tyrosine				
	2×10^{-6} moles	7 er.	15 er. sl. ind.	0	0/4
2	2×10^{-6} "	14 er. sl. ind.	16 ind. er.	0	
3	2×10^{-6} "	10 er. sl. ind.	15 " "	0	
4	2×10^{-6} "	14 ind. er.	15 " "	0	
1	di-ABA–di-*N*-acetyl tyrosine				
	1×10^{-6} moles	9 er. sl. ind.	14 ind. er.	0	1/4 (1M)
2	1×10^{-6} "	9 ind. er.	14 " "	0	
3	1×10^{-6} "	10 " "	15 " "	0	
4	1×10^{-6} "	10 " "	15 " "	0	
1–6	Nothing	All negative	All negative	All negative	

* F, fatal; M, moderate; and S, slight.

‡ er., erythema; ind., induration; and sl., slight.

§ No. positive/No. challenged.

When guinea pigs were sensitized with a conjugate of either D- or L-tyrosine, delayed skin reactions could be elicited equally well with the conjugate of L-GAT. However, in no instance was the conjugate of D-GAT effective in the production of delayed lesions. It would therefore appear that optical isomerism was not involved in the specificity of the sensitizing process but that it somehow affected the expression of this response. This could conceivably be attributable to a physical effect such as the masking of ABA groups in the D-isomer polymers by some helix formation not possible in the L-isomer.

DISCUSSION

The ability of very small molecules to behave as antigens has been demonstrated recently by Borek and Stupp (9) for conjugates of hexatyrosine and by Plescia et al. (10), who produced antibodies to DNA using oligoribonucleotides. To our knowledge, other than simple chemicals which produce contact dermatitis by conjugation in vivo with macromolecular carriers, no instances of antigenicity of materials with a molecular weight of only 400 have been reported.

The three most rigorous criteria for classic delayed hypersensitivity have now been applied to the reactions following immunization with small conjugates. These are (*a*) characteristic time course and gross appearance; (*b*) histologic appearance; and (*c*) passive transfer by white cells from a sensitized donor. In all three respects the reactions studied conform to the designation delayed sensitivity.

Several possible hypotheses may be offered to explain the development of sensitivity following immunization with the materials reported here.

1. Small conjugates such as ABA–*N*-acetyltyrosine are incorporated into a larger protein molecule which functions as the antigen. The equivalent antigenicity of conjugates of *N*-aceyl-D-tyrosine as well as other unnatural substances such as *p*-dimethylaniline and 3,5-xylenol aruges against this.

2. The ABA group transfers from its aromatic carrier to a neighboring protein which becomes the antigen. The azo bond is a covalent linkage, and there is no evidence to indicate rupture of this bond in vivo. Furthermore, the nonantigenicity of the D-glutamyl–alanyl-tyrosine conjugate in which transfer should be as likely an event cannot be reconciled with this assumption.

3. The small conjugate acts as a complete antigenic determinant. Since available evidence suggests that at least protein antigens are broken down in vivo into antigenic determinants (11), it might be argued that the antigenic determinant alone is the functioning entity and that in the present instance the stage involving digestion is bypassed with the use of the monovalent conjugates. This might also explain why conjugates of the indigestible polymers made from D-amino acids are ineffective, while the conjugates of the simple D-amino acid work.

A further point of some interest is the almost total absence of hapten-specific antibody production following immunization with simple monovalent conjugates. Despite uniform production of strong hapten-specific delayed reactions, only rarely could antibody be demonstrated by active systemic anaphylaxis and not at all by in vitro tests. This might be taken as further substantiation of the contention that antibody formation and delayed sensitivity are separate processes, each favored by a different type of determinant (12) or antigenic carrier (13).

To date, experiments with a large variety of simple conjugates of the sort tested here have turned up only two other haptens which give hapten-specific delayed hypersensitivity analogous to that obtained with arsanilic acid (14). Many of the more commonly used haptens such as *p*-aminobenzoic acid, sulfanilic acid, 2,4-dinitrofluorobenzene and *p*-iodoaniline when conjugated to simple carriers do not work. It seems apparent, therefore, that very special properties are necessary for a hapten to be able to sensitize and elicit a response requiring little or no contribution by its carrier. Further studies will be required to determine the chemical structures essential to such function.

SUMMARY

Hapten-specific delayed hypersensitivity was produced by immunization of guinea pigs with arsanilic acid conjugated to *N*-acetyltyrosine or other small aromatic molecules. Such hapten-specific delayed sensitivity could be passively transferred by peritoneal exudate cells. While a conjugate made from a polymer of D-amino acids was ineffective in producing sensitization, the conjugate made with D-tyrosine was effective, suggesting that the inability of D-amino acid polymers to be broken down by enzymes might be bypassed by use of the monomer. The effectiveness of such monomers in producing delayed sensitivity, but not antibody production, is consistent with a hypothesis that different types of antigenic determinants are involved in the production of each.

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