THE ROLE OF IMMUNOGENICITY IN THE INDUCTION OF TOLERANCE WITH CONJUGATES OF ARSANILIC ACID*

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(Received for publication 27 October 1969)

The central failure of the immunologic response in tolerant states is considered by most investigators to operate at the level of the antigen-reactive lymphocyte (1, 2).

One widely held explanation for the induction of tolerance considers it to be a result of the direct interaction of antigen with immunocompetent lymphocytes leading to the specific suppression or elimination of those lymphocytes possessing a specific receptor site for the particular antigen. An immune response is felt to result only when antigen is presented to the lymphocyte after a macrophage-associated "processing" step. Recent evidence on the induction of tolerance by in vitro exposure of competent cells to antigen supports this conception (3).

Our own studies on delayed hypersensitivity to the azobenzenearsonate (ABA)[1] group have demonstrated the very restricted specificity of such sensitivity. Thus, guinea pigs immunized with ABA-*N*-acetyltyrosine give positive delayed reactions with ABA conjugates of a wide variety of unrelated carriers (4). The specific requirements for reaction appear to be the ABA group and a small portion, perhaps one amino acid, of the carrier. More recently we have shown that even the aromatic amino acid to which the ABA group conjugates is only partially involved in the specificity of the determinant. For example, conjugates of ABA with either histidine or tyrosine-containing polymers elicit comparably strong delayed reactions in guinea pigs immunized with ABA-*N*-acetyltyrosine or ABA-*N*-acetylhistidine.

It therefore seems that in contrast to many other haptens (5), the remarkably hapten-specific delayed sensitivity directed to the ABA group requres for its induction and elicitation one of the smallest immunogenic groups yet found (4, 6). However, our subsequent studies on the mechanism of delayed reactions (7), along with similar

* Supported by Grant No. AI-01289 from the United States Public Health Service, Bethesda, Maryland 20014.

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[1] *Abbreviations used in this paper:* ABA, azobenzenearsonate; ACA, active cutaneous anaphylaxis; BSA, bovine serum albumin; EA, hen egg albumin; GAT, poly-glutamyl-alanyl-tyrosine; KLH, keyhole limpet hemocyanin; OT, old tuberculin; PCA, passive cutaneous anaphylaxis; polytyr, polytyrosine; R'3, resorcinol-tri-azophenylazophenylarsonate.

studies by Schlossman et al. (8) in a different system, convinced us that an event other than the direct interaction of the ABA determinant with a receptor on the sensitized lymphocyte was involved in the initiation of the delayed reaction. The observation that ABA conjugated to L-amino acid polymers was capable of eliciting reactions, while similar conjugates of D-amino acid polymers were not (7), suggested the existence of a "processing" event prior to the reaction with the sensitized cell. Whatever the ultimate nature of this event, it was felt to be part of a common requirement for immunogenicity that underlies all the cell-associated reactions such as antibody formation, anamnestic response, and delayed hypersensitivity. By contrast, reactions with preformed antibody such as Arthus and anaphylactic reactions, where no processing is involved, could be elicited with nonimmunogenic materials (8).

Because the induction of tolerance is also considered to be a cell-associated event mediated by immunologically specific interaction of lymphocytes and antigen, it was of interest to apply the lessons learned from studies on the mechanism of delayed reactions with immunogenic and nonimmunogenic ABA conjugates to a study of the mechanism of tolerance induction.

The present study provides evidence that only those conjugates of ABA which are inherently immunogenic are capable of inducing tolerance to the ABA group. Since all ABA conjugates used, whether immunogenic or not, were found to react with preformed antibody specific for the ABA group, it was felt that direct interaction with an antibody-like receptor site on the lymphocyte was not a sufficient explanation for induction of tolerance.

Materials and Methods

Preparation of Azobenzenearsonate (ABA) Conjugates.—To facilitate the preparation of a variety of ABA conjugates under mild coupling conditions, the stable diazonium fluoborate salt of arsanilic acid was prepared by a modification of the procedure of Wofsy et al. (9). Arsanilic acid (recrystallized from H_2O) was diazotized in an excess of cold HCl and $NaNO_2$. After 1 hr, enough ice cold 50% fluoboric acid (J. T. Baker Chemical Co., North Phillipsburg, N. J.) to give a final concentration of 20% was added with stirring to the deep yellow solution. After a little further stirring, a voluminous precipitate of pale yellow crystals of the diazonium fluoborate salt precipitated out and was collected by filtration. The precipitate was washed with cold 10% fluoboric acid and then dried by further washing on the filter with acetone and ether. The resulting pale yellow to tan, finely crystalline product was stored in an amber bottle at −20°C and the appropriate amounts weighed out as needed.

ABA-N-Acetyltyrosine and Di-ABA-N-Acetyltyrosine.—The appropriate amount of *N*-acetyltyrosine (Calbiochem, Los Angeles, Calif.) was dissolved in dilute NaOH and chilled in an ice bath. With continuous stirring 1 or 2 molar equivalents of the ABA-diazonium fluoborate in cold aqueous solution were added dropwise. After addition was completed, the pH was adjusted to around 8–9 with dilute NaOH and stirring continued overnight in the cold. The resultant mono- and di-ABA-*N*-acetyltyrosine (ABA-tyr and di-ABA-tyr) were concentrated by precipitation with HCl and purified on a diethylaminoethyl (DEAE)-cellulose (Bio-Rad Laboratories, Richmond, Calif.) column. ABA-tyr was eluted as an orange red band with 0.2 M/Na_2HPO_4; the purple di-ABA-tyr stuck more firmly to the column and required a solution of 0.2 M/Na_2HPO_4 and 1 M NaCl for elution. Both eluates were concentrated by precipitation with HCl and redissolved in the required amount of H_2O by the addition of

NaOH. Their concentrations were determined spectrophotometrically in 0.01 N/NaOH, using ϵ = 10,500 at 490 mμ for ABA-tyr and ϵ = 17,500 at 545 mμ for di-ABA-tyr (10).

Resorcinol-Tri-Azophenylazophenylarsonate (*R'3*) was kindly provided by Dr. Dan Campbell.

ABA-Conjugates of Tyrosine-Containing Polymers.—Poly-L-tyrosine (New England Nuclear Corp., Boston, Mass.), poly-D-tyrosine (kindly provided by Dr. Michael Sela), poly-L-glutamyl-alanyl-tyrosine (kindly provided by Dr. Paul Maurer) and poly-D-glutamyl-alanyl-tyrosine (kindly provided by Dr. Thomas Gill) were dissolved in water with sufficient 1 M/$NaHCO_3$ added to keep the pH at 8 after addition of the diazonium salt. D- and L-polytyrosine required addition of 1 N/NaOH for solubilization but after some of the diazonium salt had coupled, they became sufficiently soluble to permit readjusting the pH to 8–9 for complete conjugation. Sufficient diazonium salt was added in each preparation to give an estimated coupling ratio of one ABA group per three tyrosine residues in polytyrosine (polytyr) and one ABA group for every tyrosine residue in poly-glutamyl-alanyl-tyrosine (GAT). Coupling was allowed to proceed overnight in the cold at pH 8–9. The orange conjugates were concentrated and purified by negative pressure dialysis against 0.05 M borate buffer, pH 8.5.

Poly-ABA-Poly-L-tyrosine.—A two-fold excess of diazonium salt calculated on the basis of moles of tyrosine was added to a cold alkaline solution of poly-L-tyrosine and allowed to react in the cold for 2 days. The deeply colored material was purified by precipitation with acid and dialysis against borate buffer. Spectrophotometric analysis revealed that 70% of the available tyrosine residues were coupled with an ABA group.

ABA-Protein Conjugates.—Coupling to proteins such as bovine serum albumin (BSA) and hen egg albumin (EA) was accomplished by addition of 10^{-5} moles diazonium salt per 10 mg crystalline protein (Pentex, Inc., Kankakee, Ill.). Coupling was allowed to proceed overnight in the cold at pH 8–9 and the resulting conjugate dialyzed against borate buffer.

ABA-Tyraminecarboxymethylcellulose (*ABA-CMC*).—An insoluble conjugate of ABA was made by first attaching tyramine residues to carboxymethylcellulose using a carbodiimide reagent (11). After washing, this material was coupled with ABA groups by addition of excess diazonium salt to a stirred suspension with reaction allowed to proceed overnight. After extensive washing with dilute HCl, water, and acetone, the resulting yellow powder, dissolved in concentrated NaOH, gave an absorption spectrum characteristic of ABA-tyr.

Immunization and Testing of Animals.—Random bred, white male guinea pigs (400 g) were used throughout. The appropriate amounts of material were emulsified in an equal volume of complete Freund's adjuvant (8.5 parts light mineral oil, 1.5 parts Arlacel-A, 5 mg/ml killed mycobacteria) and a total of 0.1 ml injected into the four footpads. 3 wk later, animals were shaved, depilated, and skin tested with appropriate amounts of antigen in 0.1 ml saline solution. Skin tests were examined at 3–4 hr for evidence of Arthus reactivity, then measured at 24 hr for extent of induration and erythema.

Active Cutaneous Anaphylaxis (*ACA*).—Guinea pigs were immunized with 250 μg ABA-EA in adjuvant and boosted 18 days later with 200 μg ABA-EA intradermally. 8 days after booster, they were shaved and depilated, then skin-tested the following day with the various ABA-conjugates in 0.1 ml saline. Immediately after skin tests were applied, 1 ml of a 1% solution of Evans blue dye in saline was injected intravenously. The extent and intensity of the bluing were measured 30 min later.

Passive Cutaneous Anaphylaxis (*PCA*).—Tests similar to those described above were performed on guinea pigs passively sensitized 2–5 days previously by intravenous injection of 2 ml of a hyperimmune rabbit antiserum raised against ABA-EA.

Microcomplement Fixation Analysis.—The method of Wasserman and Levine (12) was used with no essential modification. The antiserum used throughout was prepared by hyper-

immunization of rabbits with ABA-keyhole limpet hemocyanin (KLH). After inactivation at 56°C for 30 min and absorption with packed sheep red cells, it could be used at dilutions above 1:150, at which point it had slight anti-complementary effects. The complement used was a frozen pool of normal guinea pig serum diluted 1:300.

Induction of Tolerance.—Groups of guinea pigs were injected once or twice intraperitoneally with the appropriate amounts of the various conjugates. 5–7 days after the last injection they were immunized with 2×10^{-7} moles (80 μg) ABA-tyr in complete Freund's adjuvant in the footpads. In several experiments using the smaller molecular weight conjugates, five inocula-

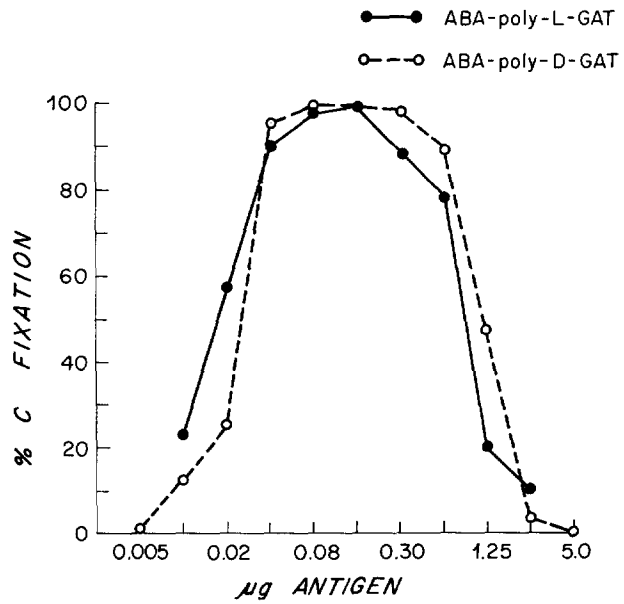

FIG. 1. Complement fixation by ABA-poly-L-GAT and ABA-poly-D-GAT with rabbit anti-ABA-KLH (dilution, 1:1000).

tions were given every other day, starting 1 wk before immunization. 3 wk after immunization, the animals were shaved, depilated, and skin tested with ABA-EA (40 μg) for existence of hapten specific sensitivity and old tuberculin (OT) (1:200) as a nonspecific control. In all experiments, untreated groups of guinea pigs were immunized with ABA-tyr to control the effectiveness of the immunizing action. If these animals reacted poorly, all other groups done at the same time were discarded.

RESULTS

Reaction of ABA Conjugates with Preformed Antibody.—The ability of azobenzenearsonate conjugates to react with preformed antibody in vitro was tested by the microcomplement fixation procedure and the results are seen in Fig. 1 and Fig. 2. The ABA-conjugates of D- and L-GAT were most effective and with an antiserum dilution of 1:1000, were able to give maximum fixation

at around 0.1 μg of antigen. The shape of the complement fixation curves (Fig. 1) was almost identical for the two isomers. The other conjugates were less efficient in fixation of complement and required an antiserum dilution of 1:150 to give measureable fixation curves (Fig. 2). It may be seen that of these, the poly-ABA-poly-L-tyrosine was most effective, with ABA-poly-L-tyrosine and ABA-poly-D-tyrosine, somewhat less so. These three macromolecular conjugates all had maximum fixation at around 0.8 μg antigen. The smaller conju-

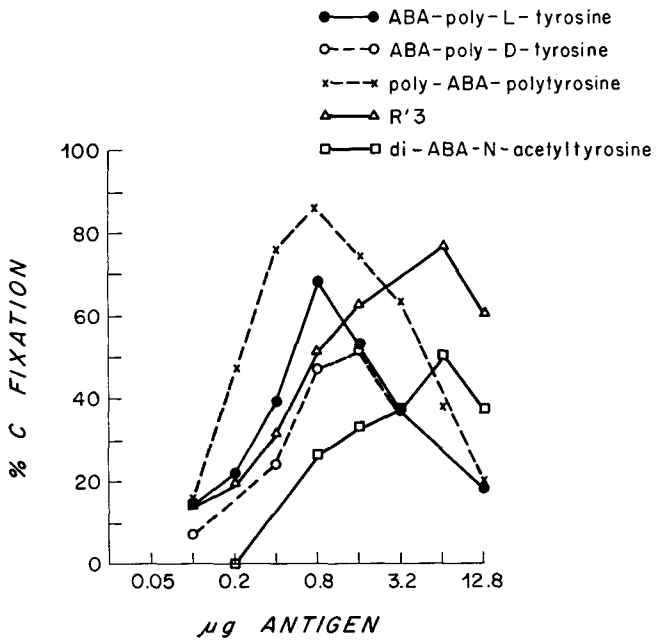

FIG. 2. Complement fixation by ABA-conjugates with rabbit anti-ABA-KLH (dilution 1:150)

gates R'3 and di-ABA-tyr required over 6 μg antigen to achieve maximum fixation while ABA-tyr showed no fixation at all throughout the whole range studied.

In vivo reactivity of the conjugates was tested by their ability to elicit cutaneous anaphylactic reactions in actively and passively sensitized guinea pigs. As may be seen in Table I, 10 μg doses of all conjugates excepting ABA-tyr gave approximately equivalent reactions in all actively sensitized animals tested. ABA-tyr gave small positive reactions in only two of five animals tested. As a control for toxicity, similar tests were carried out in nonimmunized animals. At this test dose, ABA-poly-L-tyrosine, ABA-poly-D-tyrosine, poly-ABA-poly-L-tyrosine and R'3 gave some nonspecific bluing, but this was in all cases less than 5 mm in diameter.

Tests with guinea pigs passively sensitized with rabbit antibody to ABA-EA gave comparable bluing reactions with 1 μg of all the conjugates except ABA-tyr, which did not react, and di-ABA-tyr, which gave only a trace reaction.

TABLE I

In Vivo Reactivity of Azobenzenearsonate Conjugates with Antibody

ABA conjugate used	ACA (10 μg conjugate)	PCA (1 μg conjugate)
ABA-*N*-acetyltyrosine	2/5* (10+±)‡	0/2
Di-ABA-*N*-acetyltyrosine	4/4 (11++)	1/1 (12tr§)
R'3	4/4 (10++±)	2/2 (9+±)
ABA-poly-L-tyrosine	4/4 (11+±)	3/3 (9++)
ABA-poly-D-tyrosine	3/3 (8++)	4/4 (8++)
ABA-poly-L-GAT	3/3 (12++±)	3/3 (8+±)
ABA-poly-D-GAT	3/3 (11+±)	3/3 (10++±)
Poly-ABA-poly-L-tyrosine	4/4 (11++±)	2/2 (9++±)
ABA-BSA	10/10 (10++)	1/1 (6+)

* Number of animals responding/number of animals tested.

‡ Intensity of bluing on a + to +++ scale.

§ tr, trace.

TABLE II

Hapten-Specific Delayed Sensitivity in Guinea Pigs after Immunization with 100 μg of Various ABA Conjugates in Complete Freund's Adjuvant

Conjugate used for immunization (100 μg)	Delayed reaction to	
	ABA-EA (40 μg)	OT (1:200)
ABA-*N*-acetyltyrosine	10/10* (17)‡	10/10 (15)
Di-ABA-*N*-acetyltyrosine	6/19 (14 ery.)§	19/19 (14)
R'3	0/11 (0)	11/11 (15)
ABA-poly-L-tyrosine	8/8 (16)	8/8 (15)
ABA-poly-D-tyrosine	0/6 (0)	6/6 (16)
Poly-ABA-poly-L-tyrosine	0/11 (0)	11/11 (17)
ABA-poly-L-GAT	11/11 (14)	11/11 (15)
ABA-poly-D-GAT	0/6 (0)	6/6 (17)
ABA-BSA	0/12 (0)‖	12/12 (14)

* Number responding/number tested.

‡ Mm of induration and erythema.

§ Erythema only, no induration.

‖ Skin tests were edmatous at 3 hr.

Immunogenicity of Conjugates.—When groups of guinea pigs were immunized with 100 μg amounts of the various ABA conjugates in complete Freund's adjuvant (Table II), intense hapten-specific sensitivity developed only in those animals immunized with ABA-tyr, ABA-poly-L-tyr, and ABA-poly-L-GAT. 6 of 19 animals immunized with di-ABA-tyr developed erythematous reactions and

all animals immunized with ABA-BSA developed Arthus type reactivity and circulating antibody, but no demonstrable hapten-specific sensitivity. R'3, ABA-poly-D-tyrosine, ABA-poly-D-GAT and poly-ABA-poly-L-tyrosine were ineffective at this dose. A reasonably large dose of skin test antigen (40 μg EA) was used throughout to elicit even mild degrees of sensitivity and OT reactions indicated that all animals received an immunizing stimulus and were in suffi-

TABLE III

Induction of Hapten-Specific Unresponsiveness in Guinea Pigs Pretreated with ABA Conjugates and Immunized with 2×10^{-7} Moles ABA-tyr in Complete Freund's Adjuvant

Number of guinea pigs	ABA conjugate given	Average delayed reaction to	
		ABA-EA (40 μg)	OT (1:200)
6	ABA-*N*-acetyltyrosine (2 mg × 2)	15 ery.* (4/6)‡	17§
9	Di-ABA-*N*-acetyltyrosine (2 mg × 2)	11	16
4	R'3 (2 mg × 2)	15	15
6	ABA-poly-L-tyrosine (2 mg)	0	14
5	ABA-poly-D-tyrosine (2 mg)	14	15
10	ABA-poly-L-GAT (2 mg)	11 ery. (7/10)	16
12	ABA-poly-D-GAT (2 mg)	12	15
6	Poly-ABA-poly-L-tyrosine (2 mg × 2)	11	19
6	ABA-BSA (2 mg)	15 ery. (6/6)	16
16	Controls	15	15

* Erythema only, no induration.

‡ Number positive/number tested.

§ Mm of induration and erythema.

ciently good health to give a delayed reaction where sensitivity existed. In those cases where delayed reactivity was found, reactions were intensely indurated and erythematous. The nonindurated erythematous reactions occasionally seen after immunization with di-ABA-tyr were similar in time course and appearance to very mild delayed reactions, but were not further studied.

Induction of Unresponsiveness.—Because of the toxicity of some ABA conjugates after intravenous injection, other routes were chosen to insure wide distribution of antigen. The larger macromolecular conjugates were administered in 1 ml of saline distributed among the four footpads and intraperitoneally

1 wk before immunization. A second intraperitoneal injection of the smaller conjugates ABA-tyr, di-ABA-tyr, R'3, as well as poly-ABA-poly-tyr were given 5 days before immunization. The results in Table III show that in comparison to large reactions of 15 mm induration and erythema in control animals, total suppression was achieved by pretreatment with 2 mg of ABA-poly-L-tyr and profound suppression by the same dose of ABA-L-GAT and ABA-BSA. In the latter two groups, while hapten-specific reactions when present were large and delayed in onset, they were almost entirely erythematous and without induration. Two injections of ABA-tyr also produced a pronounced depression of reactivity to an average of 15 mm erythema and slight induration in four of six animals reacting. All other conjugates given produced only slight or no change

TABLE IV

Induction of Hapten-Specific Unresponsiveness in Guinea Pigs Given Repeated Injection of Small ABA Conjugates and Immunized with 2×10^{-7} Moles ABA-tyr

Number of guinea pigs	ABA conjugate given	Average delayed reaction to	
		ABA-EA (40 μg)	OT (1:200)
7	ABA-*N*-acetyltyrosine (0.5 mg × 5)	slight ery.*	14‡
6	Di-ABA-*N*-acetyltyrosine (0.5 mg × 5)	14	14
7	R'3 (0.5 mg × 5)	14	15
6	ABA-diazonium fluoborate (1 mg × 3)	14 ery.*	15
4	Controls	15	16

* Erythema only.

‡ Mm induration and erythema.

in the hapten-specific response. In all groups, tuberculin reactions were unaffected by pretreatment.

In a separate experiment, the intraperitoneal injection of 100 mg of the insoluble ABA-tyraminecarboxymethylcellulose produced no significant change in hapten-specific sensitivity. Reactions to 40 μg ABA-insulin in guinea pigs pretreated with the insoluble conjugate were 21 mm induration and erythema, identical in size and intensity to a similarly immunized and tested control group.

Since low molecular weight materials are very rapidly excreted by the guinea pig (13), it was felt desirable to test the ability of the smaller conjugates to induce tolerance after more prolonged administration. From Table IV it may be seen that five injections of a total of 2.5 mg ABA-tyr spread over 10 days did, in fact, produce a more profound suppression of hapten-specific response than did 4 mg given in two injections (Table III). Similar treatment with di-ABA-tyr and R'3, however, were without effect. An additional group was

treated with 10^{-5} moles of the ABA-diazonium salt given intravenously in three divided doses every other day. These animals showed a marked suppression of sensitivity, with resultant delayed reactions exhibiting erythema only. Again, tuberculin reactivity in all groups was comparable to controls.

DISCUSSION

Because of the small likelihood that nature would develop two different types of molecule to recognize the same antigenic specificity, it is considered reasonable by most immunologists that the recognition site on immunocompetent cells resembles an antibody molecule at least insofar as its combining site is concerned. If this assumption is valid, it can be further argued that in the special case of delayed hypersensitivity after immunization with ABA-tyr, the recognition site is directed chiefly to the ABA group with some small contribution by the aromatic ring of tyrosine. This is predicated on the observation of the hapten-specific nature of reactivity after immunization with ABA-tyr (4) in which comparable skin reactions may be elicited with ABA conjugates of a variety of carriers containing histidine or tyrosine.

The results reported in Table I show that by in vitro or in vivo tests, antibody from rabbits or guinea pigs possesses the same degree of hapten-specificity. In these instances all ABA conjugates, regardless of the type of carrier used, could be shown to react with preformed antibody. The one exception for which there is adequate precedent (14) is the monovalent conjugate of *N*-acetyltyrosine and presumably this failure cannot be attributed to lack of combination with antibody so much as inability to produce intermolecular complexes.

In contrast to the equivalent reactivity of ABA conjugates with preformed antibody, they divided into two quite distinct categories in terms of ability to immunize for hapten-specific delayed sensitivity (Table II). Those functioning as immunogens have a single ABA group per tyrosine moiety. Nonimmunogens result from (*a*) conjugation of the ABA group to intrinsically nonimmunogenic polypeptides of D-amino acids, or (*b*) clustering of more than one ABA group on each aromatic residue or crowding a sterically permissible maximum number of groups along a chain.

The reasons for failure of D-polypeptide conjugates to function as immunogens has been discussed before (15) and may be due to difficulties in metabolization or processing. Crowding of ABA groups may also provide an inhibition by steric block to some necessary processing event. The di-ABA-tyr occupied the only intermediate position in that about one-third of the animals developed mild delayed reactivity. This could represent a response to a slight contamination with ABA-tyr.

ABA-BSA represents an immunogen in a different sense giving rise to ABA-specific antibody but not delayed sensitivity. The reasons for this dichotomy between ABA-BSA and ABA-tyr are intriguing and suggest that the nature of

the immunogen responsible for antibody or delayed hypersensitivity is different. Preliminary work has shown that in ABA conjugates made with BSA, remarkably little ABA couples to tyrosine or histidine and most of it goes on free amino groups. Thus, the dose of ABA-BSA used for immunization may contain insufficient ABA-tyr or ABA-hist to induce hapten-specific delayed sensitivity but enough of the determinant required to initiate antibody synthesis.

The results on induction of tolerance with the various conjugates demonstrate the same marked difference in behavior from that seen with preformed antibody. With one exception, there was a direct correspondence between the ability of a conjugate to sensitize when given in complete adjuvant and its ability to induce unresponsiveness when given in saline. True, the most effective immunogen ABA-tyr was not as effective a toleragen on a weight comparison basis with ABA-poly-L-tyr or ABA-L-GAT. There was evidence, however, that this could be attributed to differences in degree of persistence between monomer and polymer, in that more prolonged administration of the ABA-tyr did, in fact, result in a more profound unresponsiveness.

The one seeming exception was ABA-BSA which produced a suppression in responsiveness despite its inability to sensitize. As already indicated above, this might have been a quantitative phenomenon with enough determinants to produce tolerance present in the 2 mg dose, but not enough to immunize in the 100 μg dose. Further studies of this apparent discrepancy are contemplated.

The effectiveness of ABA diazonium salts injected directly is intriguing and bears considerable similarity to unresponsiveness produced by feeding or intravenous injection of contact sensitizers (16). There is little data, however, to determine whether this effect is produced by virture of direct reaction of diazonium salt with immunocompetent cells or via coupling first to circulating serum proteins.

In general, these results provide little support for the concept that tolerance involves a direct interaction of lymphoid cells with an antigenic determinant. In the present case, it would seem likely that since the specific determinant involved in immunization and elicitation is the ABA group, plus a part of the tyrosine residue, the receptor on the immunocompetent precursor cell should be complementary for this small moiety. Theoretically, therefore, it would seem likely that all D- and L-polymer pairs of ABA conjugate used would have an equal chance of binding to the receptor as they do with antibody. The observed differences in effectiveness of small (ABA-tyr) and large (ABA-poly-L-tyr) conjugates might well be due to inherent differences in distribution and persistence of antigen. But the physicochemical similarity of D- and L-polymer conjugates precludes such an explanation for the observed differences in activity of these later compounds. The failure of R′3 and poly-ABA-poly-L-tyrosine to induce unresponsiveness is also inexplicable in these terms.

In the earlier study on elicitation of hapten-specific delayed sensitivity by

immunogens and not by nonimmunogens (7) we argued for a preliminary processing event by some biologic system capable of distinguishing D- and L-amino acid polymers as well as sterically hindered conjugates. So too in the present case, we feel that the prerequisite of an antigen-processing event prior to induction of tolerance may well be the most satisfactory explanation of the data. While no clear concept of what such processing may involve, emerges from this work, one reasonable possibility is that it consists of an enzymatic event in or on some cell. Since the function of biologic catalysts (enzymes) is markedly affected by optical isomerism and steric factors, this remains a reasonable possibility for the failure of D-amino acid polymers and heavily conjugated carriers. However, such a conception raises further questions as to the nature of the processing events occurring for immunization or for tolerance. Do these occur in different sites or by different mechanisms as a consequence of the differences in mode of injection for immunization or suppression? Or do they in fact represent the same phenomenon but occur at different rates; immunization representing a slow release of processed antigen from a depot and tolerance resulting from a more rapid burst of processed material? Until more is known about the actual cell types involved in induction and suppression of delayed sensitivity, processing must remain at best a loose conception suggesting only a necessary manipulation of antigen prior to its effecting induction.

SUMMARY

A number of azobenzenearsonate (ABA) conjugates have been prepared and tested for ability to react with antibody, to sensitize for hapten-specific delayed hypersensitivity and to induce hapten-specific unresponsiveness. All conjugates tested by in vitro or in vivo methods show a capacity to react with preformed antibody. Conjugates of L-amino acid polymers are immunogenic and induce tolerance. Conjugates of D-amino acid polymers or conjugates with high density of ABA groups are nonimmunogenic and fail to induce tolerance.

Since paired D- and L-polymer conjugates react comparably with preformed antibody but differ markedly in tolerance induction, it is argued that combination with an antibody-like receptor molecule on the surface of an immunocompetent cell is not a sufficient condition for tolerance. The lack of effectiveness of sterically crowded conjugates as well as D-polymer conjugates argues for a preliminary biologic "processing" of antigen necessary for induction of immunity or tolerance. Such a processing event might well involve enzymatic attack on the antigen.

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