IMMUNOCHEMICAL STUDY OF ANTIGENIC SPECIFICITY IN DELAYED HYPERSENSITIVITY

IV. THE PRODUCTION OF UNRESPONSIVENESS TO DELAYED HYPERSENSITIVITY WITH A SINGLE ANTIGENIC DETERMINANT*

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Immunological specificity for delayed skin reactions, as well as for circulating antibody, has been investigated with many haptens coupled to antigenic protein carriers (1). With these antigens, hapten-specific antibodies were formed consistently, whereas delayed reactions were produced only with the immunizing antigen and not with conjugates of the same hapten with a different carrier protein. Thus, there was marked carrier specificity for delayed hypersensitivity reactions, as opposed to the hapten specificity of antibody-induced reactions (2, 3). In contrast to this, when arsanilic acid as the hapten is coupled to an apparently non-antigenic carrier such as polytyrosine, the specificity for delayed hypersensitivity is directed predominantly towards the hapten alone (4). Further confirmation of the specificity of these reactions was shown by the successful desensitization achieved with a variety of conjugates of arsanilic acid (5). Because of the hapten-specific nature of this phenomenon, it was felt that the same system would provide a useful tool to investigate the specificity of immunologic unresponsiveness or tolerance.

A further advantage of polytyrosine-azobenzene arsonate as an antigen for the study of specificity in unresponsiveness is that the sole determinant on the molecule for delayed reactions and circulating antibody is the azobenzene arsonate (ABA) group attached to tyrosine and that the specificity for unresponsiveness would presumably also be directed towards this group. It seemed possible that even tyrosine-azobenzene arsonate itself might induce unresponsiveness in the newborn guinea pig. The following results indicate that unresponsiveness for delayed hypersensitivity to the ABA determinant is specific for the determinant and is not only induced by the complete antigen but also by the hapten and by conjugates of arsanilic acid and unrelated carriers.

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Polytyrosine-Azobenzenearsonate (ABA-polytyr) and Polyhistidine-Azobenzenearsonate (ABA-polyhist).—Arsanilic acid was diazotized in the cold with nitrous acid and conjugated to either polytyrosine or polyhistidine (purchased from New England Nuclear Corporation, Boston) in alkaline solution in a ratio of 1 arsanilate group to 5 tyrosine or histidine residues. The reactions were allowed to proceed for 2 hours, at which time the resulting dark red solutions were dialyzed overnight against running water.

Bovine Serum Albumin-Azobenzenearsonate (ABA-BSA): Guinea Pig Serum Albumin-Azobenzenearsonate (ABA-GSA).—Bovine serum albumin (BSA), crystallized, was obtained from Pentex, Incorporated, Kankakee, Illinois. Guinea pig serum albumin (GSA) was prepared by repeated precipitation with Na₂SO₄. After electrophoresis on cellulose acetate, a very faint α-globulin band was visible in addition to the main albumin band. Arsanilic acid was conjugated to BSA by reaction of 10⁻⁵ moles of the diazotized acid per 10 mg of protein (70 ABA/BSA molecule). The ABA-GSA used for skin testing was prepared by coupling a large excess of arsanilic acid (4 × 10⁻⁴ moles per mg N protein). Conjugation was allowed to proceed in ice cold alkaline solution (pH 9-10) overnight and the deeply colored products dialyzed against running tap water. All transfers were done quantitatively, and final concentrations of conjugated proteins were determined by assuming no loss during the conjugation process.

Preparation of Small Conjugates.—In order to synthesize conjugates of single amino acids structurally similar to the polymers, N-acetylated amino acids were used. This insured that coupling of the ABA group occurred directly to the aromatic ring and not on the α-amino group. The position of the azo link was then identical with that in the amino acid polymers and protein conjugates.

N-acetyltirosine and N-acetylhistidine were purchased from California Biochemical Research Company, Los Angeles. Tyrosyltyrosine was purchased from the New England Nuclear Corporation, and was acetylated by mixing 10⁻⁴ moles with 5 ml of half saturated sodium acetate and slowly adding excess acetic anhydride with stirring. The reaction mixture was allowed to stand overnight in the cold and then made alkaline with NaOH.

One mole of diazotized arsanilic acid was added to one mole (plus 10 per cent excess) of N-acetyltirosine and N-acetylhistidine. Two moles were added per mole of the N-acetyltyrosyltyrosine. After standing overnight at pH 9-10, the deeply colored preparations were purified by alternate precipitation with acid and resolubilization with alkali. These conjugates are designated ABA-tyr, ABA-hist, and diABA-dityr respectively.

Induction of Unresponsiveness.—White male guinea pigs, less than 24 hours old, were injected with 1 ml of the conjugates in saline. This volume was distributed between the four foot-pads, two intramuscular sites, and in the peritoneum. As the newborn guinea pigs were weaned immediately, there was considerable mortality in the first few weeks from this, as well as the possible toxicity of the arsanilate compounds. Even in the control groups, approximately 10 to 30 per cent of the animals died in the first few weeks of life.

Immunization and Testing.—At 6 to 8 weeks the surviving guinea pigs were immunized with antigens emulsified in an equal volume of light mineral oil-arlasel A mixture (8.5 ml to 1.5 ml) containing 5 mg of killed mycobacterium per ml. A total of 0.1 ml of this emulsion containing 100 µg of the antigen was distributed among the four foot-pads. Two weeks later, skin tests were done with the heterologous carrier conjugate, ABA-GSA, to test for hapten-specific delayed sensitivity. Five days later, the guinea pigs were bled, and all experiments were terminated 2 to 3 days later with a test for systemic anaphylaxis by intracardiac injection of a 1.0 ml of a solution of ABA-BSA (1 mg/ml). Anaphylaxis was only recorded if it was observed within 5 minutes after the injection. Hemagglutination tests were carried out on the sera with formalinized sheep cells conjugated with arsanilic acid.
RESULTS

As both ABA-polytyr and ABA-polyhist consistently produced hapten-specific delayed hypersensitivity reactions in the adult guinea pig, it seemed likely that they would also effectively produce unresponsiveness in the newborn. These antigens were given to groups of 1-day-old guinea pigs in a dose containing $2 \times 10^{-6}$ moles of ABA per newborn animal. The delayed responses were examined 2 weeks after immunization of the 5-week-old animals with either of these antigens, and the results are given in Table I. All animals given ABA-polytyr at birth were rendered completely unresponsive, as neither ABA-polytyr nor ABA-polyhist were able to produce sensitization in the adults. On the other hand, when ABA polyhist was given at birth, the unresponsiveness was only partial in the 7-week-old animals, as they retained or had regained some ability to elicit hapten-specific delayed reactions following immunization with both antigens. The skin reactions, though, were not as great as those of the control group which had not been injected at birth, although all lesions were specific for the hapten.

The possibility then arose that the hapten alone might be capable of playing the same role in the newborn, since the delayed reactions suppressed by the complete antigens were hapten specific. Various simple haptens comprising arsanilic acid conjugated to a single tyrosine or histidine group and the slightly more complex bivalent molecule diABA-dityr, as well as unconjugated arsanilic acid itself, were given to 1-day-old guinea pigs. In Table II, the results of skin tests made on guinea pigs, each given $2 \times 10^{-6}$ moles of ABA at birth, are presented. In this experiment, the animals were 6 weeks old when immunized and 8 weeks old when skin tested.

Arsanilic acid itself was incapable of creating unresponsiveness to delayed hypersensitivity reactions, despite the large dose given at birth, which was quite toxic. Of 22 animals injected, only 9 survived for immunization.

### Table I

| At birth $2 \times 10^{-6}$ moles ABA/ | Immunized at 5 weeks 100 μg/ | Delayed reactions in mm of induration and erythema to: 5 μg N ABA-GSA |
| guinea pig | guinea pig | |
| ABA-polytyr | ABA-polytyr | 0, 0, 0 |
| | ABA-polyhist | 0, 0, 0, 0 |
| ABA-polyhist | ABA-polytyr | 9, 15, 10, 0 |
| | ABA-polyhist | 11, 0, 12, 11 |
| Nothing | ABA-polytyr | 23, 20, 22, 18 |
| | ABA-polyhist | 15, 13, 22, 18 |
ABA-tyr induced partial unresponsiveness to delayed reactions produced by immunization with a conjugate of the homologous amino acid polymer, ABA-polytyr. Only 2 of 5 animals produced even small lesions. When the heterologous amino acid polymer ABA-polyhist was the immunizing antigen, the skin lesions formed in all 5 animals were comparable in size to those of the control group.

Similarly, ABA-hist initiated unresponsiveness in 2 of 4 animals immunized with the homologous amino acid polymer as carrier, ABA-polyhist. Again, this unresponsiveness was completely broken in the other 6 animals immunized with the heterologous polymer of tyrosine conjugated to arsanilic acid. The divalent hapten diABA-dityr created unresponsiveness, too, and this, in contrast to the monovalent haptens, was equally effective against immunization by both the homologous and heterologous amino acid polymer conjugates. Four of 7 animals formed detectable skin lesions, but these were considerably smaller than those in the control animals.

Since $2 \times 10^{-6}$ moles of ABA-conjugate at birth induced only partial unresponsiveness, a fivefold larger dose of the two monovalent and the divalent hapten was tried in order to enhance tolerance to the arsanilate group. In this experiment, arsanilic acid itself was not given, as the larger dose was too toxic. The results of tests done on animals given conjugates containing $10^{-5}$ moles of ABA at birth and immunized at 6 weeks are presented in Table III. This larger dose of the monovalent haptens at birth induced complete unresponsiveness in 4 of the 8 animals in each group, and of the remaining 4 in each group most were par-
tially unresponsive. Six of 8 guinea pigs given ABA-dityr at birth were totally unresponsive, and the remaining 2 were partially so. The unresponsiveness is emphasized by the large lesions achieved in control animals in this experiment. Furthermore, in all groups the heterologous amino acid polymer in the immunizing antigen seemed to be no more effective at breaking this unresponsiveness than the homologous polymer, in contrast to results obtained with the lower level of conjugate injection. All animals gave good delayed hypersensitivity reactions to old tuberculin, demonstrating the specificity of the suppression produced.

A further experiment was carried out on guinea pigs given the larger dose,

**TABLE III**

<table>
<thead>
<tr>
<th>Induction of Unresponsiveness with the High Dose of Arsanilic Acid Conjugates (10^{-5} moles ABA) in Guinea Pigs Immunized at 6 Weeks</th>
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<tbody>
<tr>
<td>At birth 10^{-4} moles ABA/ guinea pig</td>
</tr>
<tr>
<td>ABA-tyr</td>
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<td>ABA-polyhist</td>
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<tr>
<td>ABA-hist</td>
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<tr>
<td>ABA-polyhist</td>
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<tr>
<td>di-ABA-dityr</td>
</tr>
<tr>
<td>ABA-polyhist</td>
</tr>
<tr>
<td>Nothing</td>
</tr>
<tr>
<td>ABA-polyhist</td>
</tr>
</tbody>
</table>

10^{-5} moles of ABA, at birth and immunized 2 weeks later than in the previous experiment, at 8 weeks of age, to determine the duration of unresponsiveness. The results given in Table IV show that unresponsiveness could be more readily broken at this age in animals given the monovalent haptens at birth and that both immunizing antigens were equally effective. However, 6 of the 7 guinea pigs given the divalent hapten di-ABA-dityr were still unresponsive at 8 weeks to the arsanilate grouping. Once again, the large lesions induced by old tuberculin in all the animals demonstrate the specificity of the unresponsiveness produced. The duration of unresponsiveness was also studied in newborns given one of two doses of ABA-polytyr containing either 10^{-6} moles or 10^{-4} moles of ABA. Another group was given ABA-BSA in a concentration of 10^{-8} moles of ABA per animal. A fourth group was given 10 mg of polytyrosine alone at birth, and all the animals were immunized at 8 weeks. The results from this experiment are given in Table V.
TABLE IV
Induction of Unresponsiveness with the High Dose of Arsanilic Acid Conjugates (10^{-5} moles ABA) in Guinea Pigs Immunized at 8 Weeks

| At birth 10^{-4} moles ABA/guinea pig | Immunized at 8 weeks 100 µg/guinea pig | Delayed reactions in mm to 5 µg NABA-GSA | Average reaction to O.T.
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<tbody>
<tr>
<td>ABA-hist</td>
<td>ABA-polytyr</td>
<td>0, 14, 15, 14, 18</td>
<td>15</td>
</tr>
<tr>
<td></td>
<td>ABA-polyhist</td>
<td>11, 12, 16, 11</td>
<td>16</td>
</tr>
<tr>
<td>ABA-tyr</td>
<td>ABA-polytyr</td>
<td>14, 11, 0</td>
<td>18</td>
</tr>
<tr>
<td></td>
<td>ABA-polyhist</td>
<td>12, 13, 14</td>
<td>20</td>
</tr>
<tr>
<td>di-ABA-dityr</td>
<td>ABA-polytyr</td>
<td>0, 0, 0, 0</td>
<td>17</td>
</tr>
<tr>
<td></td>
<td>ABA-polyhist</td>
<td>0, 0, 15</td>
<td>18</td>
</tr>
<tr>
<td>Nothing</td>
<td>ABA-polytyr</td>
<td>5, 17, 16, 18</td>
<td>17</td>
</tr>
<tr>
<td></td>
<td>ABA-polyhist</td>
<td>6, 20, 19, 17</td>
<td>17</td>
</tr>
</tbody>
</table>

ABA-polytyr given to newborns at a dose of 10^{-4} moles of ABA rendered the animals still unresponsive to both immunizing antigens at 8 weeks. By this time, however, animals given the tenfold lower dose of ABA-polytyr (10^{-6} moles of ABA) at birth, had become only partially unresponsive. The pattern of breakthrough was similar to that seen in animals given the monovalent haptens at birth. In this instance, the conjugate of the heterologous amino acid polymer...
ABA-polyhist broke through the unresponsiveness in all 3 animals, whereas, the 5 animals immunized with ABA-polytyr remained tolerant.

Each of 12 guinea pigs given (at birth) $10^{-5}$ moles of ABA conjugated to the unrelated carrier BSA were still unresponsive to delayed hypersensitivity at 8 weeks, whether the immunizing antigen was ABA-polytyr or ABA-polyhist. Conversely, 10 mg of the carrier polytyrosine itself given at birth had no effect on suppression of delayed reactions in the adult, even when the ABA-polytyr was the immunizing antigen, thus confirming the hapten specificity for unresponsiveness to delayed reactions in these experiments.

Studies were made on each animal for systemic anaphylaxis and hemagglutinating antibody in all the experiments above, as described in Materials and Methods.

In general, however, antibody production following immunization with ABA-polytyr or ABA-polyhist was so poor and variable that no conclusive evidence concerning unresponsiveness in respect to hapten-specific antibody formation was obtained, and these results will not be discussed further.

**DISCUSSION**

The specificity of unresponsiveness to antibody formation has been well studied (6) and appears to be a function of all the separate determinants on a single antigen molecule. Unresponsiveness has been successfully induced in guinea pigs by the administration of a single injection of a protein antigen at birth (7). Attempts to produce unresponsiveness to some hapten-protein conjugate in guinea pigs by a single injection at birth were unsuccessful; the tolerant state was, however, achieved when these antigens were given to the foetuses *in utero* sometime before parturition (8). Unresponsiveness to haptens has also been produced in rabbits by neonatal injection of the unconjugated carrier but not by injection of the hapten alone (9). These results suggest a significant role for the carrier in the production of hapten-specific unresponsiveness.

In a more direct fashion, the carrier protein has been shown (1) to play a decisive role in the development of delayed sensitivity in most hapten-conjugate systems studied. Similarly, the development of unresponsiveness to delayed hypersensitivity with hapten-protein conjugates may be inferred to require carrier participation. Thus, Chase (10) demonstrated that guinea pigs rendered unresponsive by feeding of dinitrochlorobenzene were unsensitized by inoculation of the specific hapten in incomplete adjuvant when tested by dermal application of the allergen. Immunization with a conjugate of the hapten and a heterologous protein, however, produced a delayed sensitivity to the conjugate. Salvin and Smith (11) produced unresponsiveness in guinea pigs with hapten-protein conjugates and cyclophosphamide treatment and concluded from their studies that while the specificity of antibody is directed towards small molecular
groupings, and the specificity of delayed hypersensitivity is directed towards larger molecular groupings, the specificity of unresponsiveness may be oriented towards the whole antigen molecule. The difficulty inherent in any such analysis stems from the uncertainty as to the nature and number of antigenic groupings concerned in reactions with the usual hapten-protein conjugates (12). Since such antigens usually consist of hapten coupled to a variety of amino acid residues, each in turn adjacent to an equally diverse assortment of amino acids, it becomes apparent that a most complex array of antigenic determinants may be present. In addition, it has been shown recently (13) that guinea pigs immunized with hapten-protein conjugates make a diversity of antibodies, some directed towards determinants including a portion of the carrier molecule as well as the hapten. Conceivably, therefore, the differences in specificity of antibody, delayed hypersensitivity, and unresponsiveness may be more apparent than real and must be analyzed in terms of all the determinant groups involved in any particular response.

In contrast to these complex systems, a conjugate such as ABA-polytyr represents a useful simplification, since there are relatively few determinants towards which an immune response may be directed. These most probably would consist of the azobenzene arsonate group, plus tyrosine, with perhaps some multiple of either of these also being a possibility. As far as delayed hypersensitivity is concerned, the existing evidence makes it appear likely that the azobenzene arsonate group acts as the major component of the antigenic determinant. In particular: (a) delayed hypersensitivity produced by immunization with ABA-polytyr, ABA-polyhist, or ABA-polylysine may be elicited by any conjugate prepared with an unrelated carrier (4); and (b) delayed hypersensitivity produced by immunization with ABA-polytyr may be suppressed by conjugates of heterologous amino acids or carriers (5).

The results presented in this paper suggest that the unresponsiveness produced in respect to delayed hypersensitivity follows a similar pattern of specificity. Thus, a sufficiently high concentration of ABA-tyr, di-ABA-dityr, and ABA-polytyr could produce a specific suppression of delayed sensitivity, even when immunization was attempted with the conjugate of the heterologous carrier, ABA-polyhist. Similarly, ABA-hist or ABA-polyhist could produce unresponsiveness (albeit not as uniformly) to immunization with ABA-polytyr. Arsanilic acid itself at the only dose level used was without apparent effect. These results, therefore, suggest that the unresponsiveness as well as the delayed reaction are largely directed towards the azophenylarsionate group, the only common determinant of the conjugates used.

However, at lower dose levels, evidence for a slight but significant contribution by the amino acid could be seen (Table II). Here the unresponsiveness induced by ABA-tyr and ABA-hist was broken by immunization with ABA conjugated to the heterologous amino acid polymer but less so in animals immunized
with a conjugate of the homologous amino acid polymer. This was even more clear cut in the use of the low dose of ABA-polytyr to produce unresponsiveness (Table V). At 8 weeks all animals immunized with the heterologous conjugate produced excellent hapten-specific delayed sensitivity, while none of the animals immunized with the homologous conjugate responded. The conjugate ABA-BSA proved to have excellent capacity for suppression. Since this conjugate contains many ABA-tyr and ABA-hist groupings, it is not clear whether its effectiveness is also attributable to contributions by the amino acid residues. It would appear, therefore, that at high dose levels unresponsiveness is retained for all challenge antigens, while at lower dose levels breakthrough of tolerance is achieved first with heterologous carriers.

The injection of the carrier polytyr itself seemed to be completely without effect on the development of hapten-specific delayed sensitivity following immunization with ABA-polytyr. These results, in contrast to those of Boyden and Sorkin (9), may perhaps be taken as further evidence that in this system the azobenzene arsonate group represents a complete determinant, whereas other hapten-conjugate systems require significant contributions by the carrier to the determinants.

The ability of the small monovalent conjugates to produce a long lasting unresponsiveness in respect to delayed sensitivity was surprising in view of previous findings that they produced only a transient suppression in animals already sensitized (5). It was expected that in the former case the small conjugates would be rapidly cleared from the guinea pigs, as was inferred in the latter phenomenon. In fact, the observation that the larger conjugate diABA-dityr had a more profound and longer acting effectiveness in suppression, while ABA-polytyr had a still more profound effect, suggested that the size of a conjugate played some role in its ability to produce suppression, most likely related to its ability to persist. Thus, it has been shown that arsanilic acid is rapidly eliminated from mice, while the large conjugate ABA-BSA is taken up by the reticulo-endothelial system and retained for considerable periods (14).

Alternatively, valence of the molecule may be of importance to its effectiveness in suppressing sensitization. It is known that monovalent haptens will reversibly inhibit antibody-mediated (15), as well as cell-mediated, reactions (5). Polyclonal conjugates, on the other hand, react with and use up the existing supply of antibody or sensitized cells and produce a much longer state of unresponsiveness. If tolerance to delayed sensitivity depended on the using up of cells or active sites in a manner akin to the precipitation of antibody, this would offer an alternative basis than persistence for the greater efficacy of the larger conjugates.

Nevertheless, there seemed to be a remarkable discrepancy between the time (5 days) during which $10^{-8}$ moles of ABA-tyr was effective in suppressing delayed sensitivity in a sensitized animal and the time (6 weeks) over which the
same dose would remain effective in producing tolerance to delayed sensitivity when given to a newborn guinea pig. One possible explanation may lie in the different routes of administration of conjugate used to study these phenomena. Tolerance in the newborn was produced by foot-pad, intramuscular and intraperitoneal injections, which might presumably slow down the elimination of the conjugate in contrast to that occurring in the previously sensitized animal where conjugates were given by intracardiac injection. However, preliminary results in sensitized animals indicate that conjugates given in the foot-pad, intramuscularly and intraperitoneally are still only effective for 5 days.

It would, therefore, seem that a more fundamental difference in effect is involved. The most plausible explanation at this stage is that two different target cells are involved in the reaction with ABA-tyr. In the one case a precursor cell is so affected by contact with the conjugate that it is rendered unresponsive or destroyed. Sufficient time must now elapse before new cells appear or old cells can recover to respond to an immunologic stimulus, and this process requiring weeks would determine the duration of tolerance. On the other hand, previously sensitized cells reacting reversibly with the monovalent conjugate are inhibited from reacting with the antigen. When the conjugate is cleared, a process requiring only days, the sensitized cells are free to react with antigen.

The present results demonstrate that tolerance to azobenzene arsonate directed delayed hypersensitivity may be effectively produced by a variety of conjugates of the azobenzene arsonate group. In general, the larger conjugates were more efficient than the small conjugates in producing and maintaining this tolerance.

Furthermore, it appears that the determinant group present in the conjugate given at birth must have the same, or a broader, specificity than that present on the sensitizing antigen in order to induce a long lasting unresponsiveness to delayed hypersensitivity reactions. It is not yet possible to say whether unresponsiveness in this system is dependent on persistence or valence of conjugate.

SUMMARY

Injections of various conjugates of arsanilic acid into newborn guinea pigs produced a specific tolerance in respect to subsequent development of haptenspecific delayed hypersensitivity. In general, larger polyvalent conjugates produced longer lasting and more profound suppression of delayed sensitivity than did the smaller ones. Carrier injections alone were ineffective. At lower doses of conjugate, breakthrough of tolerance occurred first with animals immunized with the heterologous carrier conjugate.

The duration of tolerance produced by injection of monovalent conjugates into neonates is in contrast to the transient inhibition produced by the same conjugates in previously sensitized animals, suggesting that different target cells may be involved in these two phenomena.
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BIBLIOGRAPHY


