Characteristics of Human Antibody to Diphtheria Toxin

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Human antibody to diphtheria toxin was analyzed with an ammonium sulfate radioimmunoassay. In 15 immune sera with titers of between 0.03 and 25 units/ml, the avidity was similar to that of U.S. standard antitoxin. The proportion of the post-immunization anti-diphtheria antibody specific for fragment A was variable, but in the 10 sera tested constituted a substantial fraction of the antibody. Approximately 2% of healthy adult males tested failed to make an anti-diphtheria toxin response after two booster doses of 1.5 Lf adsorbed diphtheria-tetanus toxoid, although these nonresponders made precipitating antibody to tetanus toxoid and had normal immunoglobulin levels.

The single polypeptide chain of intact diphtheria toxin (62,000 daltons) contains a specific protease-sensitive site (1, 5). The nicked toxin produced upon limited proteolysis consists of two peptides which are held together by a disulfide bond. The toxin and nicked toxin have the same in vivo toxicity. After incubation of nicked toxin with a thiol-reducing agent, the two polypeptides, fragment A (24,000 daltons) and fragment B (38,000 daltons), may be separated. Fragment A catalyzes the adenosine diphosphate ribosylation of transerase II, whereas intact or nicked toxin, and fragment B, have no enzymatic activity. Fragment B, however, is apparently required for the passage of fragment A into cells, since only toxin and nicked toxin, but not fragment A, are toxic in vivo (2, 6).

Both diphtheria toxin and fragment A are soluble in 45% saturated ammonium sulfate, which permits the primary binding test of Farr (4) to be used to measure antibody produced to these antigens. We have applied this method to the study of some of the parameters of the human immune response to diphtheria toxoid.

MATERIALS AND METHODS

Diphtheria toxin. Partially purified diphtheria toxin (2,000 Lf/ml, lot D-249) was purchased from Connaught Medical Research Laboratories, Toronto, Canada. Approximately 85% of the toxin in this preparation was nicked toxin. Pure diphtheria toxin and fragment A of diphtheria toxin were prepared from this material by using diethylaminoethyl (DEAE)-cellulose and Sephadex G-100 column chromatography as described by Collier and Kandel (1). No contamination could be detected in the purified toxin by electrophoresis on polyacrylamide gels in the presence of sodium dodecyl sulfate (SDS gels) under conditions capable of detecting 1% of the total sample applied. Fragment A contained no contaminants detectable by immunodiffusion (Fig. 1) or SDS gels (Fig. 2). Protein content of pure diphtheria toxin and of fragment A was estimated by the optical density at 280 nm, assuming an extinction coefficient of 10.4 (E1%280).

Diphtheria toxin and fragment A were labeled by an adaptation of the chloramine-T method of Hunter and Greenwood (7). To 5 mCi of carrier-free Na125I were added 0.7 ml of diphtheria toxin in PBS (0.1 M NaCl, 0.05 M sodium phosphate, pH 7.35) and 0.1 ml of chloramine-T (1 mg/ml in water). After a 10-min incubation period at room temperature, the reaction mixture was applied to a 20-ml bed volume Sephadex G-25 column equilibrated in PBS, and eluted at 1 ml/min to separate the radioactively labeled protein from the other reactants. The fraction of effluent counts eluting with the protein was 99% when 0.35 mg of diphtheria toxin was labeled and 21% when 0.7 mg of fragment A was labeled. On SDS gels the 125I diphtheria toxin counts appeared in a single band coincident with the carrier diphtheria toxin, and the 125I fragment A counts appeared as two discrete bands; 56% of the counts were coincident with the carrier fragment A and 45% ran at a mobility appropriate for fragment A dimers. After reduction, 12% of the toxin counts ran as intact toxin, 38% as fragment A, and 50% as fragment B. The counts in labeled reduced fragment A appeared in two bands; 96% of the counts were coincident with the carrier fragment A, and 4% ran as fragment A dimers. For assays of anti-fragment A activity, frag-
ment A (1.0 µg/ml) was incubated for 10 min at room temperature in 0.1 M dithiothreitol before dilution to the 5 ng/ml assay concentration.

The 111In label in both fragment A and diphtheria toxin was completely precipitable with excess antibody in the ammonium sulfate test system, but over a 3-month period of storage in the PBS containing 0.1% NaN₃ and 0.5% human serum albumin the fraction precipitable decreased to 92%. In the absence of specific antibody, 6% of the label precipitated in the ammonium sulfate test system.

Diphtheria toxoid. A solution of purified toxin (8.4 mg/ml in 0.15 m sodium borate buffer, pH 7.9) in 0.07% formaldehyde (8) was incubated for 0, 8, 20, and 40 hr at 37 C. At the end of the incubation period, samples of treated toxin were diluted 1/200 into diluent (0.25% human serum albumin in PBS) for toxicity testing, and into SDS gel buffer containing 7% mercaptoethanol. In intradermal skin tests on rabbits, 0.05 ml of a 42-ng/ml dilution of the 0-hr sample produced a 3.0-cm diameter zone of erythema after 48 hr, the 8-hr sample produced trace erythema, and the 20- and 40-hr samples gave no reaction. On SDS gels, mercaptoethanol-treated samples gave a distribution of intact toxin, fragment A, and fragment B similar to the original preparation, whereas the 8-hr sample dissociated only slightly, and the 40-hr sample apparently contained no molecules which could be dissociated.

Immunodiffusion. Plates for Ouchterlony immunodiffusion (10) consisted of a 0.8-mm thick layer of 1% agar in buffer (0.14 M NaCl, 0.005 M sodium phosphate, pH 7.4), covered with a plastic template; the wells contained 50 µlitters, and were 0.85 cm apart. Horse anti-diphtheria toxin (National Drug Co., Philadelphia, Pa.), diluted to 100 units/ml was used for the analysis of diphtheria toxin and fragments.

Anti-tetanus antibodies were detected in human sera by reaction with tetanus toxoid (fluid toxoid, approximately 15 Lf/ml, Lederle Laboratories, Pearl River, N.Y.). Serum samples which did not produce a precipitin line after 72 hr of incubation at 23 C were considered to be negative.

Electrophoresis. Electrophoresis in 10% acrylamide gels containing 0.1% SDS was done as described by Weber and Osborn (13). Samples to be reduced were incubated for 30 min at 23 C in buffer containing 7% mercaptoethanol and 0.1% SDS before electrophoresis.

Immunizations. Immunizations consisted of intramuscular injections of 0.5 ml of adsorbed combined diphtheria and tetanus toxoids, containing approximately 1.5 Lf of diphtheria toxoid per dose (Merrell-National Laboratories, Swiftwater, Pa.).

Antibody assays. Test sera were diluted 1/20 initially in PBS. Subsequent dilutions were made in 1/11 normal rabbit serum (NRS), which contained an amount of carrier protein equivalent to 1/20 human serum, as determined by the optical density at 280 nm (OD₂₅₀) of the redissolved precipitate formed in 45% saturated ammonium sulfate (SAS). All dilutions of U.S. standard diphtheria antitoxin (lot A-27, 6 units/ml) were made in 1/11 normal rabbit serum.

Assays were done in polystyrene test tubes (12 by 75 mm). Labeled antigen (0.6 ml in 0.5% human serum albumin-PBS) was added to 0.5 ml of dilutions of the test sera with an automatic dispenser (Labindustries). After a 1-hr incubation period, 1.0 ml of 90% SAS in water was added, mixed thoroughly, and held for an additional 30 min. Samples were then centrifuged in a swinging-bucket multiple-place head for 45 min at 1,200 x g. The supernatant fluids were subsequently poured off, and the tubes were inverted to drain. The 111In in the precipitate was counted in an automatic well counter (Nuclear-Chicago model 4330) using polystyrene test tubes (16 by 150 mm) as adaptors for the assay tubes. All operations were carried out at room temperature.

Percent of radioactivity precipitated was computed by the formula: counts in sample – NRS counts/counts precipitated by hyperimmune serum – NRS counts. No correction was made for reduced nonspecific precipitation in the presence of increased specific precipitation. All measurements were the average of duplicate determinations. Samples were counted until at least 25,000 decays were counted in the 100% samples; a 1-min counting period was used for 111In diphtheria toxin, and a 10-min period was used for 111In fragment A. Unless otherwise noted, antitoxin titers were measured by using 2.5 ng of 111In toxin per ml as the antigen.
Because of the differences in gamma globulin concentration among normal human sera, it was necessary to ascertain that these differences do not greatly affect the apparent titer for a given concentration of antibody. Assays of 1/1000 dilution of an antiserum diluted in several different concentrations of carrier demonstrated that 1/1 normal rabbit serum was sufficient to produce maximal specific precipitation of antigen. Doubling the carrier concentration resulted in the precipitation of an additional 2% of the total amount of antigen, resulting from an increase in the nonspecific precipitation. Decreasing the carrier concentration by 1/2 caused a decrease of 5% in the total antigen precipitated. Since in a series of 23 unselected normal human sera the total protein precipitated in the assay conditions differed by no more than +62% and -29% from the mean, normal variation in serum immunoglobulin concentrations is unlikely to produce significant error. Substantial error could result, however, if the test serum had abnormally low immunoglobulin concentrations. To preclude this possibility, the OD₅₅₀ of the redissolved precipitate was measured in samples with a low apparent titer at a 1/2 dilution.

**RESULT**

**Assay of standard diphtheria antitoxin.** Assays of U.S. standard diphtheria antitoxin were done by using diphtheria toxin and fragment A (Fig. 3). The antigen binding capacity (ABC 33%) per unit is defined as the amount of antigen precipitated by one unit of antitoxin at the concentration of antitoxin required to precipitate 33% of a specified amount of antigen in the ammonium sulfate assay. The ABC 33% at 2.5 ng of diphtheria toxin per ml is 0.87 µg/unit, the ABC 33% at 25 ng of diphtheria toxin per ml in 4.3 µg/unit, and the ABC 33% at 2.5 ng of fragment A per ml is 0.35 µg/unit. The ratio of the antigen binding capacity for diphtheria toxin to the antigen binding capacity for fragment A is 2.48 and is similar to the ratio of the molecular weights of diphtheria toxin and fragment A, which is 2.67. This result implies that the antigenic sites recognized by the standard antitoxin are present on both parts of the molecule at approximately the same density (sites/dalton) on each of the polypeptide chains. The ratio of the ABC 33% at different antigen concentrations is a measure of the avidity of the antiserum; in this case the ratio ABC 33% at 2.5 ng/ml:ABC 33% at 25 ng/ml is 0.20 and may be used as a reference figure for comparison with experimental sera. Accuracy of the technique was estimated by assaying 10 individually diluted samples at different concentrations. At 6.0 x 10⁻³ units/ml,
the standard deviation was 3.0%; at $1.2 \times 10^{-3}$ units/ml, 6.3%; at $2.4 \times 10^{-4}$ units/ml, 10.3%; and at $4.8 \times 10^{-5}$ units/ml, 25.4%. The lower limit of the useful range of the assay, if only a single determination is done, is taken to be $2.5 \times 10^{-4}$ units/ml; since 0.025 ml of serum is used in the assay, this corresponds to $1.0 \times 10^{-5}$ units/ml in the test serum. Additional accuracy may be achieved by the use of multiple determinations. Serum anti-diphtheria toxin levels as low as $2 \times 10^{-4}$ units/ml are detectable but are subject to large errors resulting from variations in the protein concentration of the test sera.

**Blocking with toxin and toxoid.** To verify the specificity of the assay, blocking curves were run with diphtheria toxin and a 40-hr toxoid preparation, using a human antiserum to diphtheria toxoid at a dilution which precipitated 87% of the $^{125}$I toxin in the absence of additional unlabeled toxin (Fig. 4). The reaction may be completely blocked with both toxin and toxoid. Over most of the curve, the toxin and the toxoid block the reaction to the same extent, which suggests that the antigenic determinants common to diphtheria toxoid and toxoid are stable during the conversion of toxin to toxoid.

**Characteristics of human immune sera.**
Selected human sera were studied with respect to their anti-diphtheria toxin level, avidity, and anti-fragment A level. Antibody levels in the sera were determined relative to standard curves, similar to those in Fig. 3, generated in the same run by serial twofold dilutions of U.S. standard diphtheria antitoxin lot A-27. Duplicate assays were done on each sample at dilutions in which 25 to 55% of the antigen precipitated, except when the antibody level at $\frac{1}{2}$ dilution precipitated less than 25% of the antigen (Table 1). The sera 1 to 10 are drawn from normal adults with an unknown previous history of diphtheria immunizations; postimmunization sera were drawn 2 weeks after inoculation with adult-type combined diphtheria-tetanus toxoid. Sera 11 to 15 were from children who had had a diphtheria-tetanus immunization series and were drawn before a booster immunization and 5 months afterwards. Over a wide range of antibody levels, no large differences in apparent avidity, as indicated by the ratio of units at 2.5 ng/ml to units at 25 ng/ml, were observed, although generally the human antisera were slightly more affected by dilution (less avid) than the hyperimmune horse standard serum. There is, however, substantial variation among human immune sera in the proportion of the antitoxin which is directed against fragment A, but after a booster there is anti-fragment A in all the responder sera studied.
Unresponsiveness to diphtheria immunization. Two individuals who have remained Schick positive after repeated diphtheria toxoid immunizations were studied. One is a 7-year-old girl who received a primary diphtheria-pertussis-tetanus immunization series and a booster injection of toxoid, after which she was Schick positive. Her anti-diphtheria toxin titer measured before and after a second booster was less than 0.0002 unit/ml. Immuno-
logical evaluation was initiated because she developed meningitis and pyarthrosis due to *Hemophilus influenzae* type b at 6 years of age. Her immunoglobulin levels are normal, and she has no apparent immunological deficiency.

A second nonresponder, a 37-year-old man, had had a primary immunization in childhood in addition to at least three subsequent booster doses of toxoid and has remained Schick positive. His anti-diphtheria toxin titer both before and after an additional booster was less than 0.0002 unit/ml. There is no suggestion of a generalized immune deficiency, and he has normal immunoglobulin levels (entry 8, Table 2). His wife has a normal level (0.02 units/ml, no recent booster), and the anti-diphtheria toxin response of his children is entirely normal (entries 11–15, Table 1).

An investigation of the frequency of unresponsiveness was made using sera drawn 2 weeks after booster immunization of young men (U.S. Marine Corps inductees). Of 214 men

<table>
<thead>
<tr>
<th>Boost</th>
<th>Anti-diphtheria toxin (units/ml)</th>
<th>Units at 25 ng per ml/(units at 25 ng/ml)</th>
<th>Anti-fragment A units/ml</th>
<th>Units anti-fragment A/units anti-toxin</th>
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<tbody>
<tr>
<td>1. First</td>
<td>2.48 (At 2.5 ng/ml)</td>
<td>0.78</td>
<td>2.74</td>
<td>1.10</td>
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<tr>
<td>2. First</td>
<td>0.044</td>
<td>1.43</td>
<td>0.077</td>
<td>1.75</td>
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<tr>
<td>3. First</td>
<td>6.10</td>
<td>0.93</td>
<td>5.74</td>
<td>0.94</td>
</tr>
<tr>
<td>4. First</td>
<td>1.13</td>
<td>0.77</td>
<td>1.30</td>
<td>0.58</td>
</tr>
<tr>
<td>5. First</td>
<td>1.23</td>
<td>0.88</td>
<td>0.40</td>
<td>0.33</td>
</tr>
<tr>
<td>6. First Second</td>
<td>0.031</td>
<td>0.031</td>
<td>1.0</td>
<td>0.014</td>
</tr>
<tr>
<td>7. First</td>
<td>0.119</td>
<td>0.95</td>
<td>0.126</td>
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<tr>
<td>8. Pre First</td>
<td>0.075</td>
<td>0.73</td>
<td>&lt;0.004</td>
<td>&lt;0.05</td>
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<tr>
<td>9. Pre First</td>
<td>1.58</td>
<td>0.67</td>
<td>0.220</td>
<td>0.14</td>
</tr>
<tr>
<td>10. Pre First</td>
<td>0.125</td>
<td>0.87</td>
<td>0.095</td>
<td>0.76</td>
</tr>
<tr>
<td>11. Pre First</td>
<td>3.36</td>
<td>0.97</td>
<td>1.32</td>
<td>0.37</td>
</tr>
<tr>
<td>12. Pre First</td>
<td>0.83</td>
<td>0.70</td>
<td>0.025</td>
<td>0.03</td>
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<tr>
<td>13. Pre First</td>
<td>27.8</td>
<td>0.59</td>
<td>6.72</td>
<td>0.24</td>
</tr>
<tr>
<td>14. Pre First</td>
<td>0.97</td>
<td>0.93</td>
<td>0.96</td>
<td>0.60</td>
</tr>
<tr>
<td>15. Pre First</td>
<td>2.84</td>
<td>0.58</td>
<td>0.60</td>
<td>0.24</td>
</tr>
<tr>
<td>16. Pre First</td>
<td>2.56</td>
<td>0.85</td>
<td>2.18</td>
<td>0.85</td>
</tr>
<tr>
<td>17. Pre First</td>
<td>3.20</td>
<td>1.0</td>
<td>0.12</td>
<td>0.02</td>
</tr>
<tr>
<td>18. Pre First</td>
<td>0.57</td>
<td>0.80</td>
<td>2.36</td>
<td>1.03</td>
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<tr>
<td>19. Pre First</td>
<td>9.88</td>
<td>0.94</td>
<td>0.049</td>
<td>0.45</td>
</tr>
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</table>

* At 2.5 ng/ml.
tested, 7 had less than 0.0002 unit/ml antitoxin (entries 1-7 in Table 2). Six of seven men were given a second booster 30 days after the first; four of these men made no immune response detectable in sera drawn 2 weeks afterwards. Thus, the frequency of unresponsiveness to one booster dose of toxoid in this population is about 3%, and the frequency of unresponsiveness to a second booster is about 2%. Absence of an immune response to diphtheria appears to be an isolated deficiency, unrelated to a generalized immune deficiency, since the immunoglobulin levels are normal (Table 2) and since all but one of these men made precipitating antibody to the tetanus toxoid with which they were concurrently immunized.

**DISCUSSION**

Anti-diphtheria toxin assays recently reviewed by Van Ramshorst (11) include bioassays and passive hemagglutination. A specialized radioimmunoassay has also been used and has demonstrated that in the sera tested the primary binding titer is proportional to the neutralization titer (9). The ammonium sulfate primary binding assay described here has substantial advantages in accuracy and ease of performance and is as sensitive as other techniques. The finding that human immune sera have an avidity similar to the standard serum independent of antibody levels is not unexpected since in all probability the individuals tested have had multiple immunizations.

Both fragment A and fragment B are immunogenic, and fragment A retains its ability to react with antibody when separated from the diphtheria toxin molecule. It has been suggested (5) that the immunological reactions of spontaneously dissociated fragments of diphtheria toxin may account for the presence of some of the multiple antigenic components previously observed in purified toxin preparations. Free fragment A is, however, unlikely to be present in the immunizing toxoid, since cross-linking resistant to SDS and mercaptoethanol occurs between fragment A and fragment B during formaldehyde treatment. Indeed, since intracellular dissociation of the fragments is apparently necessary for the toxic activity, the observed cross-linking is sufficient to account for the loss of toxicity during formaldehyde treatment. It seems likely then that the antigenic structure of the fragments is produced to the fragment A in the configuration in which it occurs in association with fragment B, and that many of the antigenic determinants are retained when fragment A is rendered enzymatically active by dissociation from fragment B.

The specific immunological unresponsiveness to diphtheria toxoid which occurs in a small proportion of otherwise normal individuals has been previously noted (3). In at least two individuals that we have studied, no antibody is detectable despite a documented history of a primary immunization series and subsequent boosters. The percentage of nonresponders after two booster immunizations found in this study was 2%. Although specific nonresponsiveness to diphtheria toxoid is a relatively rare characteristic, it may be of substantial interest should it prove to be a genetic effect similar to the genetically determined nonresponsiveness of rodents to low doses of specific protein antigens (12).

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**LITERATURE CITED**