INFECTION AND IMMUNITY, Dec. 1980, p. 835-846
0019-9567/80/12-0835/12$02.00/0

Vol. 30, No. 3

Immunochemical Studies of Diphtherial Toxin and Related Nontoxic Mutant Proteins

STANLEY J. CRYZ, JR.,† SUSAN L. WELKOS,‡ AND RANDALL K. HOLMES*

Uniformed Services University of the Health Sciences, Bethesda, Maryland 20014

Competitive binding radioimmunoassays were used to analyze the immunochemistry of diphtherial toxin. Rabbit antisera obtained by immunization with formolized toxoid or fragment A were used to characterize purified toxin, toxoid, fragment A, and related nontoxic mutant proteins. Antitoxoid serum had a high titer of neutralizing activity. Most of the antibodies in antitoxoid bound to toxin but not to fragment A. The anti-fragment A antibodies that were present in antitoxoid recognized determinants of fragment A that were exposed on unnicked toxin. Formaldehyde treatment partially destroyed antibody-binding sites associated with the A and B domains of toxin. Anti-fragment A serum had a low titer of neutralizing activity. The specificities of the anti-fragment A antibodies in antitoxoid and anti-fragment A sera were different. Approximately half of the anti-fragment A antibodies in anti-fragment A serum recognized determinants of fragment A that were masked in toxin. Per unit of fragment A-binding activity, anti-fragment A serum was significantly more potent than antitoxoid serum as an inhibitor of the enzymatic activity of fragment A. By analyzing the antigenic structure of several nontoxic mutant proteins (cross-reacting materials) that cross-react with toxin, we distinguished three different subgroups of antigenic determinants associated with the B domain of toxin. Furthermore, the exposed antigenic determinants of the A domain of toxin were separated into two subgroups, both of which were distinct from the masked determinants of the A domain. The radioimmunoassays described here provide rapid, sensitive, quantitative, and versatile methods for immunochemical characterization of toxin or related cross-reacting proteins encoded by corynebacteriophages.

The structure and activity of diphtherial toxin have been extensively studied (3, 22). After treatment with trypsin and reducing agents, toxin (molecular weight [MW], 62,000) can be dissociated into two polypeptides, fragment A (MW, 21,145) and fragment B (MW, ca. 38,000) (5, 6, 8). Fragment A, the amino-terminal region of the toxin molecule, is an enzyme that catalyzes transfer of the adenosine diphosphate ribose moiety from nicotinamide adenine dinucleotide to eucaryotic elongation factor 2 (EF-2), thereby inactivating EF-2 and inhibiting protein synthesis in eucaryotic cells (4, 9, 15). Fragment B, the carboxy-terminal portion of the toxin molecule is essential for the binding of toxin to specific receptors on the plasma membrane of susceptible eucaryotic cells (16, 29).

The immunochemistry of diphtherial toxin has also been studied in detail. Several investigators, notably Pope and Stevens (24-26), Relyveld and Raynaud (27), and, more recently, Pappenheimer et al. (23), provided evidence that diphtherial toxin possesses multiple antigenic determinants that are functionally distinct. Pappenheimer and co-workers prepared horse and rabbit antitoxoids absorbed with the nontoxic mutant protein CRM45 that were specific for the 17,000-MW carboxy-terminal region of toxin (23). The absorbed antisera lost the ability to inhibit enzymatic activity of purified fragment A, contained only 25 to 30% of the toxin-precipitable antibodies present before absorption, but retained all of the original neutralizing activity. These investigators concluded that antibodies directed against the carboxy-terminal region of fragment B were primarily responsible for neutralization of toxicity. Although anti-fragment A antibodies comprised a relatively small fraction of the toxin-precipitable antibodies in rabbit or horse antitoxoids, anti-fragment A represented a larger fraction of the toxin-precipitable antibodies in several rabbit antisera against the nontoxic mutant protein CRM197 (23). Furthermore, rabbit anti-A antiserum failed to precipitate toxin completely and was incapable of neu-

† Present address: Department of Microbiology and Immunology, University of Oregon Health Science Center, Portland, OR 97205.

‡ Present address: Department of Microbiology and Immunology, Eastern Virginia Medical School, Norfolk, VA 23501.

tralizing the toxicity of toxin. Based on these findings, Pappenheimer et al. concluded that most of the antigenic determinants of fragment A were masked in native diphtherial toxin and become exposed only after partial degradation or denaturation of toxin or CRM197 (23). Furthermore, they proposed that the avidity of a neutralizing antitoxin is inversely proportional to its content of nonneutralizing anti-fragment A antibodies (23).

Bazaral et al. used radioimmunoassays to analyze serum from humans immunized with diphtherial toxoid and demonstrated antibodies against both the A and B domains of toxin in such sera (2). Gill et al. also reported a radioimmunoassay for diphtherial toxin, but used it only for quantitative assays for toxin antigen (10).

Rittenburg et al. prepared mouse antisera against diphtherial toxin and against formolized toxoid. The properties of these antisera were compared by immunodiffusion, passive hemagglutination, neutralization, and fragment A enzyme inhibition tests (28). Antitoxin but not antitoxoid revealed an antigenic difference between toxin and toxoid in immunodiffusion tests. Rittenburg et al. concluded that treatment of toxin with formaldehyde to produce toxoid had destroyed one or more of the antigenic determinants of toxin. Furthermore, only antitoxin inhibited the enzymatic activity of purified fragment A and reacted strongly with fragment A in immunodiffusion tests, although both antitoxin and antitoxoid contained anti-fragment A antibodies that were detectable by passive hemagglutination. These data demonstrated that anti-fragment A antibodies could be separated into at least two functionally distinct subgroups that did or did not inhibit the enzymatic activity of fragment A.

Many questions concerning the immunochemistry of diphtherial toxin have not been resolved. The mapping of antigenic determinants associated with various regions of the toxin molecule has been initiated, but is not yet highly refined. The number of antigenic determinants associated with fragment A is unknown, and it is unclear how they are distributed between exposed and masked regions of diphtherial toxin. Furthermore, the relative roles of stabilization and destruction of antigenic determinants of toxin by treatment with formaldehyde require further clarification.

In the present study, we used competitive-binding radioimmunoassays to investigate the antigenic structure of diphtherial toxin, diphtherial toxoid, fragment A, and a series of nontoxic mutant proteins (cross-reacting materials, CRMs) that are structurally related to diphtherial toxin. The goals of this study were to examine further the distribution of antigenic determinants on the toxin molecule, to analyze the effects of formaldehyde treatment on immunogenicity and antibody-binding capacity of toxin, and to develop rapid methods for immunochemical characterization of nontoxic mutant proteins encoded by *tox* mutants of corynebacteriophages.

MATERIALS AND METHODS

Bacteria and bacteriophages. The nontoxinogenic strain *Corynebacterium diphtheriae* C7, corynebacteriophage β^{tox+}, and the lysogenic toxinogenic strain *C. diphtheriae* C7(β^{tox+}) have been designated as wild-type strains in previous studies (1, 13). The following lysogenic strains of *C. diphtheriae* harboring *tox* mutants of phage β are maintained in our laboratory and have been previously reported (12): C7(β^{tox-1}), C7(β^{tox-2}), C7(β^{tox-3}), C7(β^{tox-4}), C7(β^{tox-5}), C7(β^{tox-6}), C7(β^{tox-7}), C7(β^{tox-30}), and C7(β^{tox-45}). *C. diphtheriae* C7($\beta^{tox-197}$) was provided by T. Uchida (29). The Cowan I strain of *Staphylococcus aureus* was obtained from E. D. Rosenblum.

Media and growth conditions. PGT medium was the casein hydrolysate medium of Mueller and Miller (21) as modified by Barksdale and Pappenheimer (1). For production of diphtherial toxin and related nontoxic mutant proteins, the deferrated, maltose-supplemented PGT medium was adjusted to 0.075 μg of added Fe^{2+} per ml, and cultures were grown and harvested as described previously (12). Filter-sterilized culture supernatants were stored at 4°C and were tested within 24 h.

Diphtherial toxin, fragment A, and toxoid. Purification of an unnicked sample of diphtherial toxin (designated Jn141) was reported previously (14). A preparation of partially nicked diphtherial toxin was purchased from Connaught Laboratories (Toronto, Canada), purified according to published methods (4), and designated Dc202. Fragment A was isolated from trypsin-treated Dc202 toxin by gel filtration on Sephadex G-100 in the presence of 4 M urea and 50 mM 2-mercaptoethylamine (17). Purified toxin and fragment A were stored in aliquots at −70°C. Samples of purified diphtherial toxin Jn141 (3.4 mg/ml) and Dc202 (1.0 mg/ml) were converted to toxoids by treatment with 0.2 and 0.5% Formalin, respectively, in the presence of 0.025 M lysine at 22°C for 1 month (19).

Antisera. Rabbits were immunized by repeated intramuscular injections of Jn141 toxoid (100 μg/dose) or of purified fragment A (10 μg/dose) in complete Freund adjuvant. The R21 antitoxoid and B55 anti-fragment A sera, obtained from the terminal bleedings of rabbits R21 and B55, were used in radioimmunoassays for diphtherial toxin and for fragment A.

Radioiodination of diphtherial toxin and fragment A. Radioiodination of proteins was performed by a modification of published methods (11). To 50 μg of toxin or fragment A in 1 ml of pH 7.2 phosphate-buffered saline solution at 22°C, 1 mCi of carrier-free $Na^{125}I$ (Amersham/Searle Corp., Arlington Heights, Ill.), 1 μg of lactoperoxidase (Sigma Chemical Co., St. Louis, Mo.), and 25 μl of 0.03% H_2O_2 were added. After 5 min, 25 μl of 0.03% H_2O_2 was again added. Beginning

5 min later the reaction mixture was dialyzed for 18 h against phosphate-buffered saline containing 5 mM NaI. Monomeric ^{125}I-labeled toxin or ^{125}I-labeled fragment A was obtained by gel filtration on Sephadex G-100 columns in PBS containing 1 mg of bovine serum albumin per ml and 5 mM NaI. Freshly prepared radioiodinated toxin and fragment A typically had specific activities between 1 and 10 μCi/μg of protein and were more than 90% immunoprecipitable.

Preparation of *S. aureus*. Staphylococci were used as a particulate absorbant for immune complexes containing immunoglobulin G. A 10% suspension of the formaldehyde-treated Cowan I strain of *S. aureus* was prepared as described by Kessler (18) and was stored at −70°C. The bacterial suspension was thawed and washed according to published procedures (18).

Competitive-binding radioimmunoassays for diphtherial toxin and fragment A. Radioimmunoassays were carried out in 0.5-ml reaction mixtures at 22°C in polystyrene test tubes (12 by 75 mm). All reagents were diluted in assay buffer containing 150 mM NaCl, 50 mM tris(hydroxymethyl)aminomethane (pH 7.4), 5 mM ethylenediaminetetraacetic acid, 0.02% NaN_3, 0.05% Nonidet P-40 (Accurate Chemical and Scientific Co., Hicksville, N.Y.), 1 mg of bovine serum albumin ml, and 5 mM NaI (18). Reagents were added to the reaction mixtures in the following order: (i) assay buffer; (ii) radioiodinated antigen (ca. 10,000 cpm of ^{125}I-labeled diphtherial toxin or ^{125}I-fragment A); (iii) nonradioactive antigen (toxin, toxoid, fragment A, or sterile culture supernatant from an appropriate strain of *C. diphtheriae*); and (iv) a sufficient quantity of antiserum (R21 antitoxoid or B55 anti-fragment A) to bind ca. 50% of the radiolabeled antigen in the absence of nonradioactive competing antigen. Typical volumes of antiserum required were 0.005 μl of R21 antitoxoid or 0.20 μl of B55 anti-fragment A per assay for ^{125}I-toxin and 0.01 μl of R21 antitoxoid or 0.20 μl of B55 anti-fragment A per assay for ^{125}I-fragment A. Actual volumes varied slightly with different samples of ^{125}I-toxin or ^{125}I-fragment A, depending on specific activity, time since iodination, or other factors. The reaction mixtures were incubated for 15 min at 22°C with occasional gentle mixing. Next, a 100-μl sample of the staphylococcal suspension in assay buffer was added to each tube, and incubation was continued for 10 min. The staphylococci from each reaction mixture were collected by centrifugation at 4°C and washed three times with 1.0-ml samples of assay buffer at 4°C, and radioactivity in the absorbed immune complexes was counted in a Searle 1185 gamma counter.

All assays were performed in duplicate, and mean values were calculated. Background counts from controls lacking antiserum were subtracted. Radioactivity bound was expressed as a fraction of total radioactivity and was designated B. The data were plotted as $1/B$ versus the concentration of nonradioactive competing antigen, since this representation generated approximately linear standard curves (7).

Inhibition of nicotinamide adenine dinucleotide:EF-2 adenosine diphosphate ribosyltransferase activity by antisera. Samples of purified fragment A were assayed for enzyme activity by the procedure of Collier and Kandel (4). Partially purified wheat germ EF-2 was prepared by a method provided by R. J. Collier (personal communication) and was substituted for rabbit reticulocyte EF-2. The reactions were stopped after 15 min by adding equal volumes of cold 10% trichloroacetic acid, and the precipitates were collected on membrane filters (Millipore Corp., Bedford, Mass.). After washing and drying the filters, they were counted in toluene with Liquifluor (New England Nuclear, Boston, Mass.) in a liquid scintillation counter (LSC 9000; Beckman Instruments, Inc., Fullerton, Calif.).

Inhibition of enzyme activity by antitoxoid or anti-fragment A serum was measured after preincubating 25-ng samples of fragment A with dilutions of antiserum for 15 min at 25°C. The remaining components for the enzyme assays were then added, and the reaction mixtures were incubated and processed as described above. Enzymatic activities in the presence of R21 antitoxoid or B55 anti-fragment A serum were expressed as the percentages of activities in control assays with the same concentrations of normal rabbit serum.

Neutralization tests. Assays for neutralizing antibody were performed by intracutaneous tests in rabbits (30). Samples containing 50 ng of Dc202 toxin per ml and appropriate dilutions of antiserum were preincubated for 30 min at 22°C, and 0.2-ml portions were injected intracutaneously. Reactions were observed after 96 h to determine the volumes of antiserum required to neutralize the 10-ng test dose of toxin. Titers were calculated by comparison with assays of the United States Standard Antitoxin (lot A32, 6 AU/ml) performed at the same time.

Immunodiffusion tests. Ouchterlony-type immunodiffusion tests were performed as described previously (14).

Polyacrylamide gel electrophoresis. Proteins were boiled for 2 min in buffered sample treatment mixture containing 6 M urea, 1% sodium dodecyl sulfate, and 0.2% 2-mercaptoethanol. Electrophoresis was carried out in 13% polyacrylamide slab gels containing 0.1% sodium dodecyl sulfate in the discontinuous buffer system described by Maizel (20). After electrophoresis, gels were stained with Coomassie brilliant blue.

RESULTS

Our initial experiments were performed to characterize the reagents used in this study. Analysis of purified fragment A of diphtherial toxin, purified unnicked toxin Jn141, and purified nicked toxin Dc202 by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate and 2-mercaptoethanol revealed no contaminating polypeptides (Fig. 1A). Rabbits were immunized with formolized toxoid prepared from unnicked toxin Jn141 (serum R21) or with purified fragment A (serum B55). Ouchterlony-type immunodiffusion tests were performed to test the reactions of these sera with diphtherial toxin and with fragment A (Fig. 1B). Toxin and fragment A reacted with the R21 antitoxoid to form a line of partial identity with

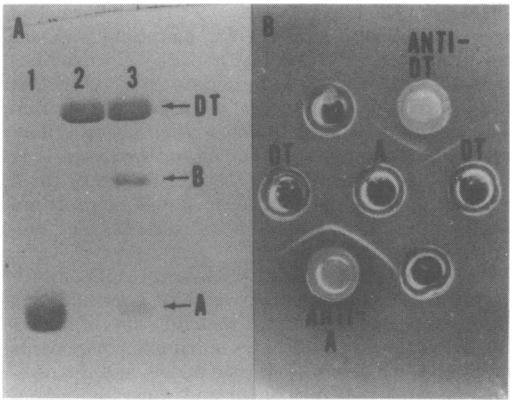

FIG. 1. *(A) Comparison of fragment A, unnicked toxin Jn141, and nicked toxin Dc202 by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Samples were as follows: (1) 5 μg of fragment A; (2) 5 μg of Jn141 toxin; and (3) 5 μg of Dc202 toxin. Arrows indicate stained bands corresponding to intact diphtherial toxin (DT), fragment B (B), and fragment A (A). (B) Comparison of antitoxoid and anti-fragment A sera by gel immunodiffusion. Wells contained R21 antitoxoid serum (anti-DT, diluted 1:5), B55 anti-fragment A serum (anti-A, undiluted), diphtherial toxin Dc202 (DT, 50 μg/ml), and fragment A (A, 25 μg/ml). Toxin and fragment A reacted with anti-DT to form a line of partial identity with a distinct spur extending toward the well containing fragment A. In contrast, anti-A reacted with toxin and fragment A to form a line of apparent identity. In some experiments with anti-A, a small indistinct spur was observed extending toward the well containing fragment A and was most apparent when the concentrations of DT and A were increased to 100 and 50 μg/ml, respectively (data not shown).*

a distinct spur extending toward the well containing fragment A. In contrast, toxin and fragment A reacted with the B55 anti-fragment A serum in this experiment to form a line of identity. In neutralization tests the titer of the R21 antitoxoid was 600 U/ml, and the titer of the B55 anti-fragment A serum was 15 U/ml.

Samples of ^{125}I-labeled toxin and ^{125}I-labeled fragment A were prepared, and sera R21 and B55 were tested to determine their ability to bind to these radiolabeled antigens (Fig. 2). Both sera formed immune complexes with ^{125}I-toxin and with ^{125}I-fragment A. We defined arbitrary units of toxin-binding and fragment A-binding activities as the amounts of serum necessary to bind 50% of the ^{125}I-toxin or ^{125}I-fragment A under the conditions of the experiment depicted in Fig. 2. The R21 antitoxoid contained 230 toxin-binding units per ml and 67 fragment A-binding units per ml (Fig. 2A), whereas the B55 anti-fragment A serum contained 4.2 toxin-binding units per ml and 5.0 fragment A-binding units per ml (Fig. 2B).

Next, we used these antisera and radiolabeled antigens to develop several competitive binding radioimmunoassays. Each reaction mixture contained approximately 10,000 cpm of radiolabeled antigen (^{125}I-toxin or ^{125}I-fragment A), sufficient antibody (serum R21 or serum B55) to bind ca. 50% of the labeled antigen in the absence of competing antigen, plus various amounts of nonradioactive competing antigen (purified toxin, purified fragment A, or toxoid). These assays for toxin and for fragment A detected nanogram quantities of the homologous antigens (Fig. 3 and 4). We used these assay systems to compare the specificities of the R21 antitoxoid and the B55 anti-fragment A sera.

With R21 antitoxoid and ^{125}I-toxin (Fig. 3A), nonradioactive toxin competed extensively and generated a linear standard curve, whereas fragment A competed poorly. We calculated that ca. 75% of the antibodies in R21 antitoxoid recognized antigenic determinants of toxin that were not present on fragment A.

In contrast, with R21 antitoxoid and ^{125}I-fragment A (Fig. 4A), both nonradioactive toxin and nonradioactive fragment A competed extensively and yielded linear competition curves, suggesting that all major antigenic determinants of fragment A that were recognized by R21 antitoxoid were also exposed on diphtherial toxin. This interpretation was supported by two additional observations. First, in Fig. 4A, the ratio of the slopes of the standard curves obtained with fragment A and with toxin was ca. 3 to 1. This is close to the ratio of the molecular weights of toxin and fragment A (2.9 to 1). Thus, on a molar basis, toxin and fragment A were comparable in competing with ^{125}I-fragment A for binding to antibodies in the R21 antitoxoid. Second, the partially nicked toxin Dc202 and the unnicked toxin Jn141 were tested for their ability to compete in this radioimmunoassay for fragment A and were found to be indistinguishable (Fig. 5). These findings made it very unlikely that the observed competition of diphtherial toxin with ^{125}I-fragment A was dependent to any significant degree on the presence of free fragment A as a contaminant in these preparations of purified diphtherial toxin.

When toxoid prepared from Dc202 toxin was tested in assays with R21 antitoxoid, nonlinear competition curves were observed both with ^{125}I-toxin (Fig. 3B) and with ^{125}I-fragment A (Fig. 4B). In addition, the initial slopes of these competition curves were much lower than the slopes of the standard curves obtained with toxin (note the different scales on the abscissa in Fig. 3A, 3B, 4A, and 4B). The lower slopes indicated that formolized toxoid had fewer antibody-binding sites than equimolar amounts of diphtherial

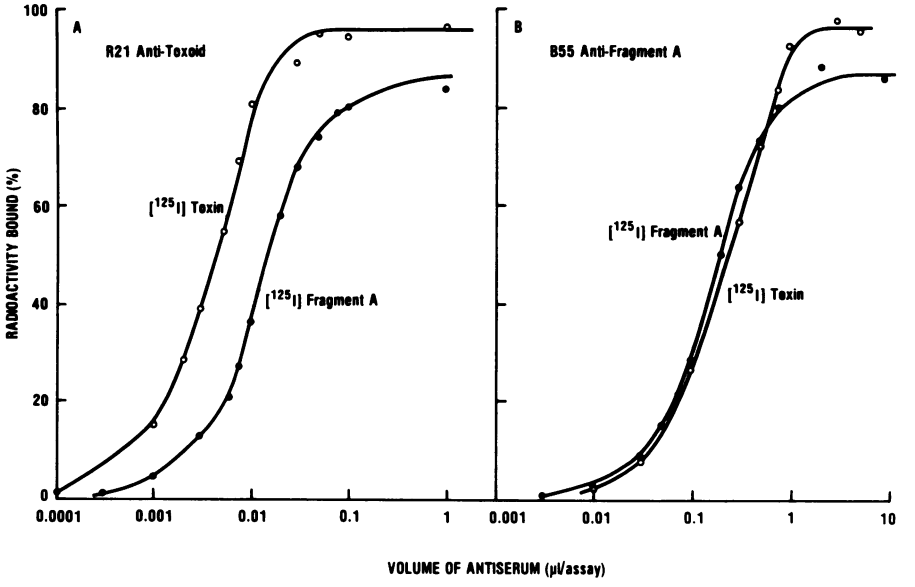

FIG. 2. *Binding and immunoprecipitation of ^{125}I-labeled diphtherial toxin and ^{125}I-labeled fragment A by R21 antitoxoid and B55 anti-fragment A sera. Reaction mixtures contained either ^{125}I-diphtherial toxin (10 ng; 9.1 µCi/µg) or ^{125}I-fragment A (20 ng; 2.3 µCi/µg) and the volumes of serum R21 or B55 indicated. The samples were processed as described in the text, and the percentage of the total radioactivity bound was plotted versus the log of the volume of the antiserum. In this experiment 96% of the ^{125}I-diphtherial toxin and 87% of the ^{125}I-fragment A were immunoprecipitable.*

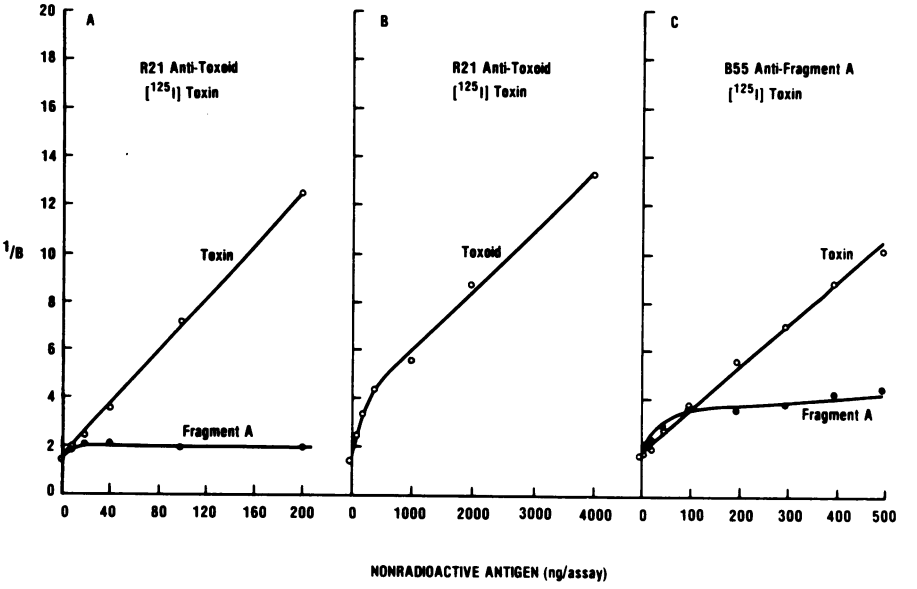

FIG. 3. *Competitive binding radioimmunoassays with ^{125}I-labeled diphtherial toxin. Standardized reaction mixtures contained ^{125}I-diphtherial toxin, non-radioactive competing antigens, and antiserum (R21 antitoxoid or B55 anti-fragment A serum). The samples were processed as described in the text. The reciprocal of the fraction of ^{125}I-diphtherial toxin bound (1/B) was plotted as a function of the concentration of the non-radioactive competing antigen.*

toxin. The nonlinear nature of the competition curves obtained with toxoid indicated that some of the antibody-binding sites of toxin were more susceptible than others to destruction by formaldehyde during preparation of toxoid.

Next we attempted to determine whether pu-

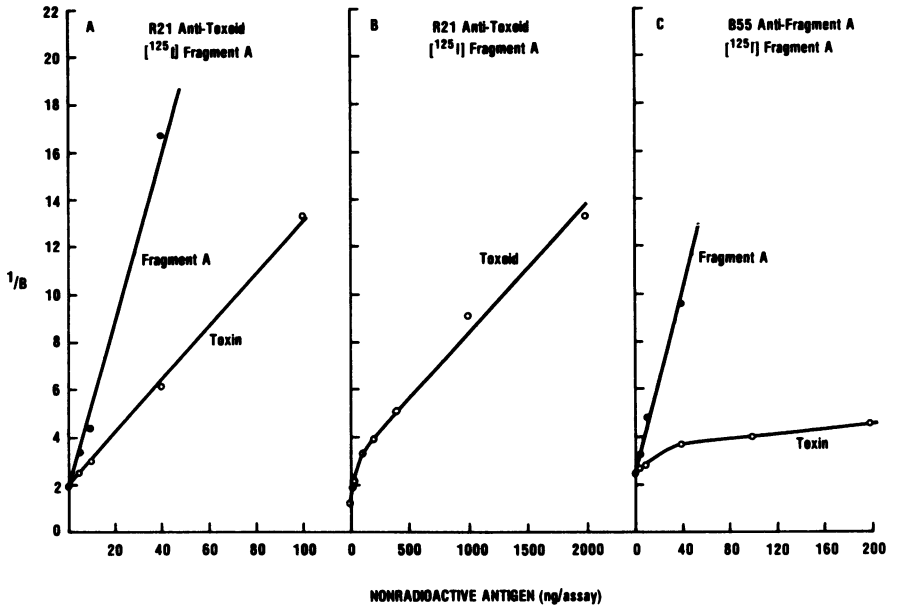

FIG. 4. *Competitive binding radioimmunoassays with ^{125}I-labeled fragment A. Standardized reaction mixtures contained ^{125}I-fragment A, non-radioactive competing antigens, and antiserum (R21 antitoxoid or B55 anti-fragment A serum). The samples were processed, and the data were plotted as described in the legend to Fig. 3.*

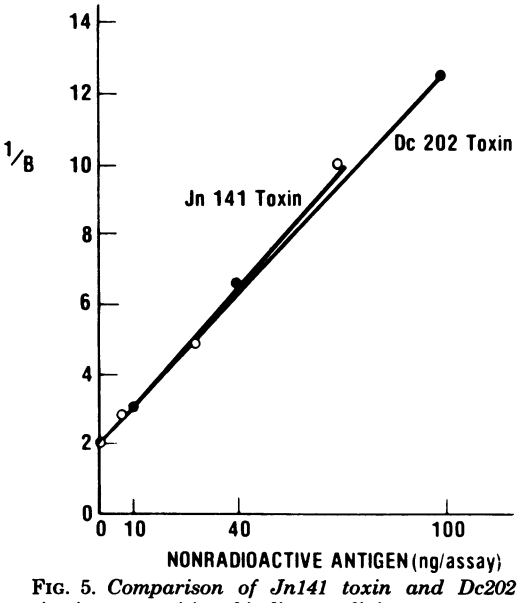

FIG. 5. *Comparison of Jn141 toxin and Dc202 toxin in competitive binding radioimmunoassays with ^{125}I-labeled fragment A and R21 antitoxoid serum. The samples were processed and the data were plotted as described in Fig. 3. Each point represents the average of three independent determinations.*

rified fragment A had major immunogenic determinants that were absent or masked in formolized toxoid. With B55 anti-fragment A and ^{125}I-fragment A (Fig. 4C), nonradioactive fragment A competed extensively and generated a linear competition curve, whereas competition by purified toxin was incomplete. From these data we estimated that approximately half of the anti-fragment A antibodies in serum B55 recognized antigenic determinants of fragment A that were not exposed on diphtherial toxin. Thus, the specificities of the anti-fragment A antibodies in the B55 anti-fragment A serum and in the R21 anti-toxoid were strikingly different.

Previous investigators reported that anti-fragment A sera had little (28) or no (23) neutralizing activity. As noted above, our B55 anti-fragment A serum had weak neutralizing activity. We demonstrated that serum B55 contained some antibodies that bound to ^{125}I-toxin and against which nonradioactive fragment A could not compete (Fig. 3C). We calculated that the ratios of toxin-neutralizing activity to ^{125}I-toxin-binding activity were similar for B55 anti-fragment A and for R21 antitoxoid. From these observations, we concluded that our purified fragment A was probably contaminated with trace quantities of detoxified toxin or fragment B that were undetected by toxicity tests and electrophoretic analysis but elicited a weak antibody response specific for determinants associated with the B domain of toxin.

To obtain additional information concerning

the differing specificities of the anti-fragment A antibodies in sera R21 and B55, we tested the abilities of these sera to inhibit the nicotinamide adenine dinucleotide:EF-2 adenosine diphosphate ribosyltransferase activity of purified fragment A of diphtherial toxin (Fig. 6). Both antisera inhibited this enzymatic activity, and the slopes of the inhibition curves were similar for the two sera. We defined an arbitrary unit of antienzyme activity as the amount of immune serum necessary to inhibit the activity of purified fragment A by 50% under the conditions used in the experiment depicted in Fig. 6. By this criterion the R21 antitoxoid serum contained 91 U of antienzyme activity per ml and the B55 anti-fragment A serum contained 28 U of anti-enzyme activity per ml. Per unit of fragment A binding activity (Fig. 2) B55 anti-fragment A serum contained 5.6 U of anti-enzyme activity and was a much more potent inhibitor than R21 antitoxoid which contained only 1.4 U of antienzyme activity.

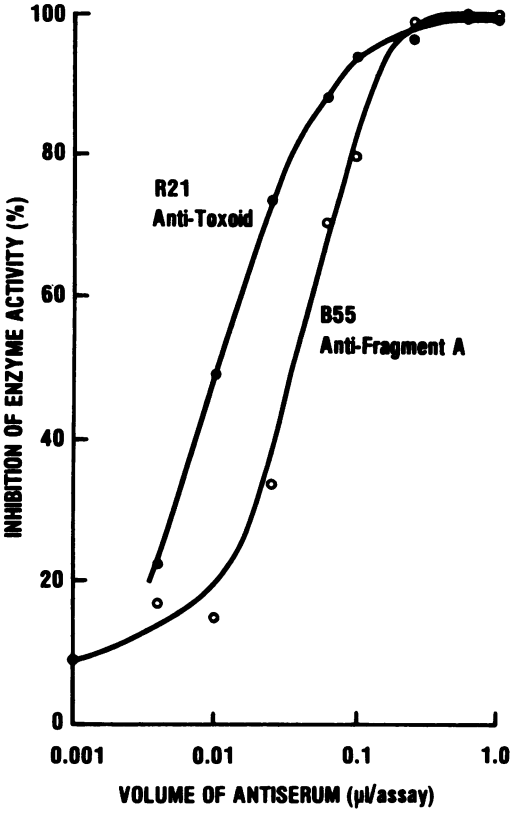

FIG. 6. *Inhibition of the adenosine diphosphate ribosyltransferase activity of fragment A by R21 antitoxoid and B55 anti-fragment A sera. Samples of fragment A (25 ng/assay) were preincubated with various concentrations of antiserum or normal rabbit serum and were then assayed for enzymatic activity as described in the text. The percent inhibition of enzymatic activity at each concentration of antiserum was calculated by comparison with the appropriate normal rabbit serum control.*

In previous studies, several mutant proteins (CRMs) encoded by the structural gene for diphtherial toxin were purified, and their immunochemical properties were analyzed by immunodiffusion and quantitative precipitin tests (23, 29). We have used the radioimmunoassay methods described above to characterize a larger series of CRMs that were studied previously by other methods (12).

Supernatants from cultures of wild-type and mutant *C. diphtheriae* strains were tested for toxin or related antigens. The results obtained in the assay with ^{125}I-diphtherial toxin and R21 antitoxoid are shown in Fig. 7. Linear competition curves were obtained with C7(β^{tox+}) and C7($\beta^{tox\text{-}197}$), strains that produce antigenically indistinguishable, 62,000-MW *tox* gene products (29). Control experiments with the nontoxinogenic strain *C. diphtheriae* C7 revealed no bacterial products that competed with diphtherial toxin (data not shown). C7($\beta^{tox\text{-}30}$) and C7($\beta^{tox\text{-}45}$) are strains harboring mutant β phages that code for nontoxic CRM proteins of 30,000 MW and 45,000 MW, respectively, corresponding to amino-terminal fragments of diphtherial toxin (29). With strains C7($\beta^{tox\text{-}30}$) and C7($\beta^{tox\text{-}45}$), partial competition was demonstrated by the hyperbolic curves that approached specific plateau values in Fig. 7. These plateau values reflected quantitatively the extent of the incomplete cross-reactions of CRM45 and CRM30 with diphtherial toxin. Slight competition similar to C7($\beta^{tox\text{-}30}$) was observed with C7($\beta^{tox\text{-}3}$), a strain that produces a CRM protein of ca. 34,000 MW (12) (data not shown). Very little competition was detected with strain C7($\beta^{tox\text{-}2}$), a mutant that produces a nontoxic CRM protein of ca. 26,000 MW. In previous studies, the multiple antigenic determinants associated with the B fragment of toxin were separated into two subgroups, those present in toxin but not in CRM45 and those present in CRM45 but not in fragment A (23). In the present study, the quantitative differences between the CRMs encoded by phages $\beta^{tox\text{-}2}$, $\beta^{tox\text{-}30}$, and $\beta^{tox\text{-}197}$ in Fig. 7 enabled us to distinguish a minimum of three subgroups of antigenic determinants associated with the B subunit of diphtherial toxin.

The antigenic determinants of fragment A were also analyzed in more detail by testing culture supernatants for their ability to compete in radioimmunoassays with ^{125}I-fragment A and R21 antitoxoid (Fig. 8 and 9). Strains C7($\beta^{tox\text{-}45}$), C7($\beta^{tox\text{-}30}$), C7($\beta^{tox\text{-}2}$), and C7($\beta^{tox\text{-}3}$), all of which produce CRM proteins with an intact fragment

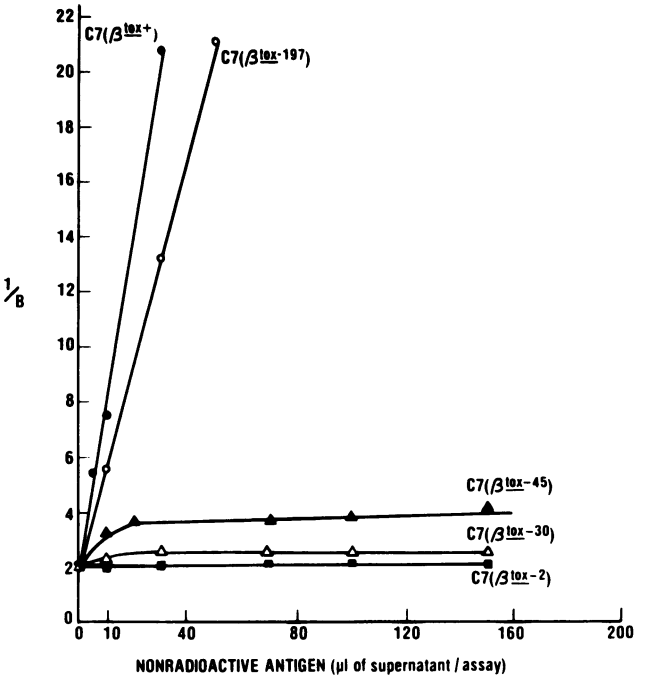

FIG. 7. *Analysis of culture supernatants from wild-type and mutant strains of C. diphtheriae in competitive binding radioimmunoassays with ^{125}I-labeled diphtherial toxin and R21 antitoxoid serum. Processing of samples and plotting of data were as described in the legend to Fig. 3. By comparison with a toxin standard curve determined simultaneously, we calculated that culture supernatants of strains C7(β^{tox+}) and C7($\beta^{tox-197}$) contained 7.7 and 4.1 µg of toxin antigen per ml, respectively. Strains C7(β^{tox-45}), C7(β^{tox-30}), and C7(β^{tox-2}) produced CRM proteins that showed partial cross-reactions with toxin and differed quantitatively in the extent of their cross-reactions.*

A region, yielded linear competition curves indistinguishable from the curve obtained with purified fragment A except for antigen concentration (Fig. 8). In contrast, supernatant from C7(β^{tox-1}), which synthesizes a CRM protein of ca. 20,000 daltons, lacking a short fragment of the carboxy-terminal portion of the fragment A region (12), yielded a nonlinear curve that appeared to approach a plateau, indicating incomplete competition with fragment A. *C. diphtheriae* C7 lysogens harboring mutant β phages previously characterized as non-toxinogenic and CRM$^-$ (12) were also tested. Strains C7(β^{tox-4}), C7(β^{tox-5}), C7(β^{tox-6}), and C7(β^{tox-7}) displayed no detectable competition with ^{125}I-diphtherial toxin (data not shown). In the radioimmunoassay with ^{125}I-fragment A (Fig. 9), the supernatant from strain C7(β^{tox-5}) showed partial competition, indicating that C7(β^{tox-5}) produced CRM smaller than fragment A that was undetected in our previous studies (12). As shown above (Fig. 4A and 5), the radioimmunoassay with ^{125}I-fragment A and R21 antitoxoid was specific for antigenic determinants of fragment A that were also exposed on intact diphtherial toxin. The partial competition observed in Fig. 8 and 9 with C7(β^{tox-1}) and C7(β^{tox-5}) demonstrated that these exposed determinants of fragment A on intact toxin could be separated into at least two subgroups. The additional antigenic determinants of fragment A that were masked on intact toxin and were demonstrated by using serum B55 (Fig. 4C) thus represented a third subgroup of specific antigenic determinants associated with fragment A.

We extended these studies by testing the culture supernatants from *C. diphtheriae* strains C7(β^{tox-1}), C7(β^{tox-4}), C7(β^{tox-5}), C7(β^{tox-6}), and C7(β^{tox-7}) by radioimmunoassays with ^{125}I-fragment A and with B55 anti-fragment A serum (data not shown). We demonstrated that C7(β^{tox-1}) and C7(β^{tox-5}) produced CRMs that competed poorly with fragment A, but these experiments did not define additional subgroups of antigenic determinants associated with fragment A.

DISCUSSION

In the present study we investigated the immunochemistry of diphtherial toxin by compar-

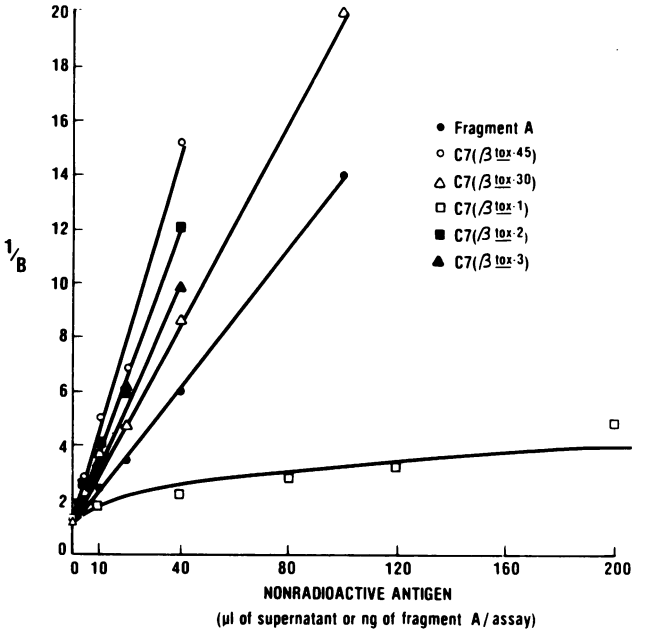

FIG. 8. *Analysis of culture supernatants from strains of C. diphtheriae C7 harboring tox⁻ CRM⁺ mutants of corynebacteriophage β by competitive binding radioimmunoassays with ^{125}I-labeled fragment A and R21 antitoxoid serum. Processing of samples and plotting of data were as described in the legend to Fig. 3. By comparison with a standard curve for fragment A determined simultaneously, we calculated that the culture supernatants contained the following concentrations of antigen expressed as fragment A equivalents: C7(β^{tox-45}), 2.6 μg/ml; C7(β^{tox-30}), 1.3 μg/ml; C7(β^{tox-2}), 2.2 μg/ml; and C7(β^{tox-3}), 1.9 μg/ml.*

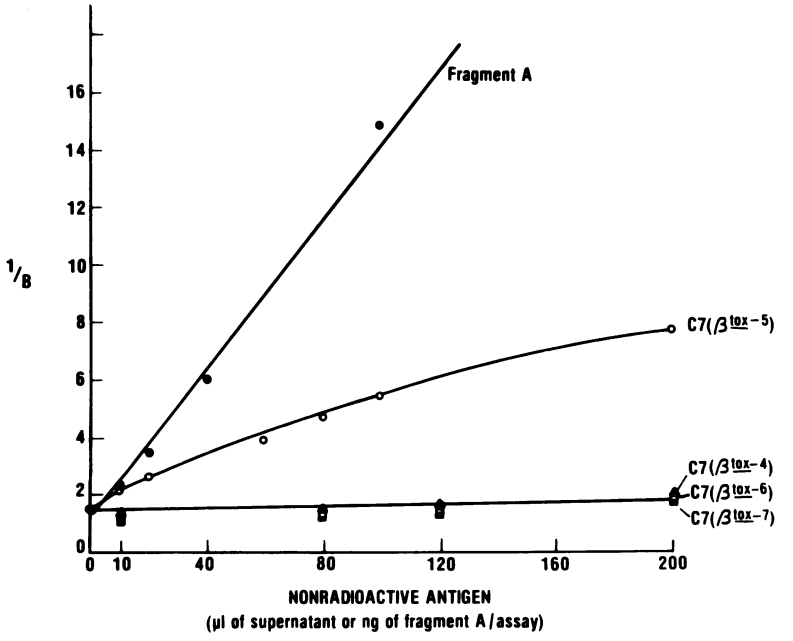

FIG. 9. *Analysis of culture supernatants from strains of C. diphtheriae C7 harboring mutants of corynebacteriophage β previously classified as tox⁻ CRM⁻ by competitive binding radioimmunoassays with ^{125}I-labeled fragment A and R21 antitoxoid serum. Processing of samples and plotting of data were as described in the legend to Fig. 3. By this procedure we established that C7(β^{tox-5}) produced a CRM that partially cross-reacted with fragment A and that was not detected in our previous studies (12).*

ing the extent to which toxin, fragment A, toxoid, and various CRM proteins competed in radioimmunoassays for diphtherial toxin and for fragment A. We also determined the titers of our R21 antitoxoid and B55 anti-fragment A sera for toxin neutralization, for inhibition of enzymatic activity of purified fragment A, and for binding to radiolabeled toxin and fragment A. We have confirmed and extended previous studies of the relationships between structure, function, and antigenicity of specific domains of the toxin molecule. We have also demonstrated the versatility of radioimmunoassays for characterizing CRMs encoded by *tox* mutants of corynebacteriophage β.

Our R21 antitoxoid was a rabbit antiserum obtained by immunization with toxoid prepared from a sample of the unnicked diphtherial toxin Jn141. In contrast, the antitoxoids used in studies reported previously were obtained by immunization with toxoids prepared from partially or completely nicked toxins or from incompletely characterized toxins. Dc202 toxoid was used as the competing toxoid antigen for our radioimmunoassays because Jn141 toxoid was no longer available when these studies were performed. Both toxoids were potent immunogens and had been used successfully to produce hyperimmune neutralizing antisera of high titer in rabbits, goats, or both. Our data demonstrated that prolonged treatment of diphtherial toxin Dc202 with Formalin plus lysine resulted in partial destruction of antibody-binding sites on both the A and B domains. This was shown in Fig. 3B and 4B both by the lower slopes of the toxoid competition curves and by the deviations of these curves from the linear responses obtained with Dc202 toxin. Since very high concentrations of toxoid competed successfully for binding of essentially all of the ^{125}I-labeled diphtherial toxin or ^{125}I-labeled fragment A to antibodies in antitoxoid, it is apparent that none of the major antibody-binding sites of toxin was quantitatively destroyed by treatment with Formalin. From these observations, we conclude that partial destruction of antibody-binding sites and stabilization of immunogenic determinants involved in eliciting neutralizing antibody responses were both consequences of treatment of toxin with formaldehyde. In contrast to our findings, Bazaral et al. did not detect any significant differences between toxin and formolized toxoid as competing antigens in a competitive binding radioimmunoassay for toxin using human serum (2). It is noteworthy, however, that the toxoid used in their experiments was treated with formaldehyde for a relatively short time (40 h).

We demonstrated directly that antibodies in our antitoxoid bound to ^{125}I-toxin and ^{125}I-fragment A (Fig. 2). We showed that samples of nicked toxin Dc202 and unnicked toxin Jn141 competed in an identical manner with ^{125}I-fragment A for binding to anti-fragment A-specific antibodies in our antitoxoid (Fig. 5). Finally, we confirmed that our preparation of toxin Jn141 remained unnicked and contained no detectable free fragment A at the time these studies were performed (Fig. 1A). We concluded that the anti-fragment A-specific antibodies in our antitoxoid were directed against exposed antigenic determinants on intact diphtherial toxin. We confirmed the existence of fragment A-specific immunogenic determinants that are masked in diphtherial toxoid but present in purified fragment A (Fig. 4C). Both antitoxoid and anti-fragment A rabbit sera inhibited the enzymatic activity of fragment A (Fig. 6), although the inhibitory activity per unit of fragment A-binding activity was significantly greater with the anti-fragment A serum. Previous studies have demonstrated that unnicked diphtherial toxin must be activated by limited proteolysis and reduction to generate the enzymatically active fragment A (4, 9). All of the data currently available are consistent with the notion that the catalytic site of fragment A is not exposed in intact toxin and may correspond to a masked antigenic determinant of toxin.

Fragment A and each different CRM protein that corresponds to an amino-terminal fragment of toxin lacks a unique polypeptide representing a complementary carboxy-terminal fragment of toxin. Fragment A and these amino-terminal CRM fragments could not compete for binding to antibodies specific for the missing carboxy-terminal regions of toxin. This incomplete cross-reactivity was reflected by the plateaus in the competition curves with fragment A and the CRM fragments (Fig. 3A and 7). Incomplete cross-reactivity with ^{125}I-fragment A was also observed with CRM fragments shorter than fragment A in supernatants of strains C7($\beta^{tox\text{-}1}$) and C7($\beta^{tox\text{-}5}$) (Fig. 8 and 9). A minimum estimate of the number of subgroups of antigenic determinants exposed on diphtherial toxin could be inferred from the quantitatively different cross-reactions observed with the different amino-terminal CRM fragments in our radioimmunoassays. In this manner we distinguished three subgroups of exposed antigenic determinants on toxin that were associated with the B domain (Fig. 7) and two subgroups of exposed antigenic determinants that were associated with the A domain (Fig. 8 and 9). The antigenic determinants of fragment A that were masked in toxoid constitute a third subgroup of fragment A-specific determinants (Fig. 4C).

The radioimmunoassays reported here have

several advantages for analyzing the immunochemistry of diphtherial toxin and related mutant proteins. The assays are quantitative and very sensitive. The specificity of the assays is determined by the purified radiolabeled antigens and the antisera. It is not necessary to purify each CRM protein before it can be tested; the amounts of antigen and antibody required are much less than for quantitative precipitin tests; and the assays directly demonstrate binding of antigen to antibody rather than a secondary phenomenon such as precipitation or agglutination. By these methods it is relatively easy to compare the CRMs from *C. diphtheriae* strains harboring many different *tox* mutants of corynebacteriophage β. Our results from toxin, toxoid, and the well-studied mutant proteins CRM197 and CRM45 confirmed the work of other investigators (2, 23, 28) and demonstrated the essential agreement between our conclusions and theirs. The additional data we obtained with the mutant phages β^{tox-1}, β^{tox-2}, β^{tox-3}, β^{tox-4}, β^{tox-5}, β^{tox-6}, β^{tox-7}, and β^{tox-30} extended previous characterizations of these phages and enabled us to discriminate with greater precision the subgroups of exposed antigenic determinants of the B and A domains and the masked antigenic determinants of the A domain of diphtherial toxin. Finally, for investigators involved in the isolation and characterization of additional *tox* mutants of corynebacteriophages, the assays described here provide a rapid, convenient, sensitive, and quantitative system for immunochemical characterization of the mutant *tox* gene products.

ACKNOWLEDGMENTS

These studies were supported in part by Public Health Service research grant 5 R22 AI14107 from the National Institute of Allergy and Infectious Diseases and by research grant R07031 from the Uniformed Services University of the Health Sciences.

We thank Irmgard Dinger for secretarial assistance.

LITERATURE CITED

1. **Barksdale, W. L., and A. M. Pappenheimer, Jr.** 1954. Phage-host relationships in nontoxigenic and toxigenic diphtheria bacilli. J. Bacteriol. **67**:220–232.
2. **Bazaral, M., P. J. Goscienski, and R. N. Hamburger.** 1973. Characteristics of human antibody to diphtheria toxin. Infect. Immun. **7**:130–136.
3. **Collier, R. J.** 1975. Diphtheria toxin: mode of action and structure. Bacteriol. Rev. **34**:54–85.
4. **Collier, R. J., and J. Kandel.** 1971. Structure and activity of diphtheria toxin. I. Thiol-dependent dissociation of a fraction of toxin into enzymatically active and inactive fragments. J. Biol. Chem. **246**:1496–1503.
5. **DeLange, R. J., R. E. Drazin, and R. J. Collier.** 1976. Amino-acid sequence of fragment A, an enzymatically active fragment from diphtheria toxin. Proc. Natl. Acad. Sci. U.S.A. **73**:69–72.
6. **Drazin, R., J. Kandel, and R. J. Collier.** 1971. Structure and activity of diphtheria toxin. II. Attack by trypsin at a specific site within the intact molecule. J. Biol. Chem. **246**:1504–1510.
7. **Ekins, R. P.** 1974. Radioimmunoassay and saturation analysis. Basic principles and theory. Br. Med. Bull. **30**: 3–11.
8. **Gill, D. M., and L. L. Dinius.** 1971. Observations on the structure of diphtheria toxin. J. Biol. Chem. **246**:1485–1491.
9. **Gill, D. M., and A. M. Pappenheimer, Jr.** 1971. Structure-activity relationships in diphtheria toxin. J. Biol. Chem. **246**:1492–1495.
10. **Gill, D. M., T. Uchida, and R. A. Singer.** 1972. Expression of diphtheria toxin genes carried by integrated and non-integrated phage beta. Virology **50**:664–668.
11. **Haustein, D.** 1975. Effective radioiodination by lactoperoxidase and solubilization of cell-surface proteins of cultured murine T lymphoma cells. J. Immunol. Methods **7**:25–38.
12. **Holmes, R. K.** 1976. Characterization and genetic mapping of nontoxinogenic (*tox*) mutants of corynebacteriophage β. J. Virol. **19**:195–207.
13. **Holmes, R. K., and L. Barksdale.** 1969. Genetic analysis of tox^+ and tox^- bacteriophages of *Corynebacterium diphtheriae*. J. Virol. **3**:586–598.
14. **Holmes, R. K., and R. B. Perlow.** 1975. Quantitative assay of diphtherial toxin and of immunologically cross-reacting proteins by reversed passive hemagglutination. Infect. Immun. **12**:1392–1400.
15. **Honjo, T., Y. Nishizuka, O. Hayaishi, and I. Kato.** 1968. Diphtheria toxin-dependent adenosine diphosphate ribosylation of aminoacyl transferase II and inhibition of protein synthesis by diphtheria toxin. J. Biol. Chem. **243**:3553–3555.
16. **Ittelson, T. R., and D. M. Gill.** 1973. Diphtheria toxin: specific competition for cell receptors. Nature (London) **242**:330–332.
17. **Kandel, J., R. J. Collier, and D. W. Chung.** 1974. Interaction of fragment A from diphtheria toxin with nicotinamide adenine dinucleotide. J. Biol. Chem. **249**: 2088–2097.
18. **Kessler, S. W.** 1975. Rapid isolation of antigens from cells with a staphylococcal protein A-antibody adsorbent: parameters of the interaction of antibody-antigen complexes with protein A. J. Immunol. **115**:1617–1624.
19. **Linggood, F. V., M. F. Stevens, A. J. Fulthorpe, A. J. Woiwod, and C. G. Pope.** 1963. The toxoiding of purified diphtheria toxin. Br. J. Exp. Pathol. **44**:177–188.
20. **Maizel, J. V., Jr.** 1971. Polyacrylamide gel electrophoresis of viral protein, p. 179–246. *In* K. Maramorosch and H. Koprowski (ed.), Methods in virology, vol. 5. Academic Press, Inc., New York.
21. **Mueller, J. H., and P. A. Miller.** 1941. Production of diphtheria toxin of high potency (100Lf) on a reproducible medium. J. Immunol. **40**:21–32.
22. **Pappenheimer, A. M., Jr.** 1977. Diphtheria toxin. Annu. Rev. Biochem. **46**:69–94.
23. **Pappenheimer, A. M., Jr., T. Uchida, and A. A. Harper.** 1972. An immunological study of the diphtheria toxin molecule. Immunochemistry **9**:891–906.
24. **Pope, C. G., and M. F. Stevens.** 1958. The properties of crystalline diphtheria toxin-protein: its antigenic composition as determined by gel-diffusion methods. Br. J. Exp. Pathol. **39**:150–157.
25. **Pope, C. G., and M. F. Stevens.** 1958. The properties of crystalline diphtheria toxin-protein: observations on the phosphate-stable antigens. Br. J. Exp. Pathol. **39**:386–392.
26. **Pope, C. G., and M. F. Stevens.** 1958. The properties of crystalline diphtheria toxin-protein: observations on the pepsin and trypsin stable antigens. Br. J. Exp. Pathol. **39**:490–496.
27. **Relyveld, E. H., and M. Raynaud.** 1959. Étude quantitative de la réaction entre la toxine diphtérique et divers

serums antidiphtériques de faible pouvoir neutralisant. Ann. Inst. Pasteur (Paris) **96**:537-547.

28. **Rittenburg, M. B., C. T. Pinney, Jr., and B. H. Iglewski.** 1976. Antigenic relationships on the diphtheria toxin molecule: antitoxin versus antitoxoid. Infect. Immun. **14**:122-128.
29. **Uchida, T., A. M. Pappenheimer, Jr., and R. J. Greany.** 1973. Diphtheria toxin and related proteins. I. Isolation and properties of mutant proteins serologically related to diphtheria toxin. J. Biol. Chem. **248**:3838-3844.
30. **Wilson, G. S., and A. Miles.** 1975. Topley and Wilson's principles of bacteriology and immunity, 6th ed., vol. 2, p. 1830-1834. The Williams & Wilkins Co., Baltimore.