Tetanus Toxin and Antigenic Derivatives

I. Purification of the Biologically Active Monomer

SAMUEL G. MURPHY AND KENT D. MILLER

Division of Laboratories and Research, New York State Department of Health, and Department of Microbiology, Albany Medical College, Albany, New York 12201

Received for publication 17 June 1967

A procedure for the isolation of pure tetanus toxin in a lethal monomeric form was developed based on the extraction of whole cells and chromatographic techniques. A crude extract of toxin was obtained by hypertonic extraction of cells from a 72-hr culture of Clostridium tetani Massachusetts strain. The extract was precipitated with ammonium sulfate and further purified by sequential use of ion-exchange chromatography and gel filtration. The degree of purification obtained by the fractionation procedures was monitored by polyacrylamide gel electrophoresis. The pure toxin has an average specific activity of 150 × 10^6 mouse MLD per mg of N and 3,000 Lf per mg of N. Immunological purity was demonstrated by a single line on both immunoelectrophoresis and agar double diffusion. One band was obtained on polyacrylamide electrophoresis, as was a single symmetrical peak in the ultracentrifuge and on Sephadex G-100 chromatography. The pure protein has an absorbancy ratio (280/260 μm) of 2.1 in phosphate buffer (pH 7.5).

Tetanospasmin, the neurotoxin produced by Clostridium tetani, has been subjected to many fractionation procedures. Prior to 1959, precipitation techniques were the most widely employed procedures. [Reports on their use were reviewed by Turpin and Raynaud (18)]. Recently, several chromatographic purifications have been reported. For these procedures, either ion-exchange chromatography (1, 8, 15, 17) or gel filtration (7, 15) has been used. None of the preparations obtained by these methods has been shown to be a single component with respect to both immunological and chemical criteria of purity.

In an attempt to produce an immunogen free from other protein contaminants, culture filtrates from autolyzed cells were precipitated or ultrafiltered prior to purification on a technical-scale Sephadex G-100 column (20 × 120 cm). These procedures were abandoned as inoperable. It was necessary to explore other methods that would allow smaller working volumes and the application of additional parameters of separation to obtain pure tetanus toxin.

MATERIALS AND METHODS

Chromatographic materials. Microgranular diethylaminoethyl (DEAE) cellulose, Whatman DE-52, obtained from H. Reeve Angel and Co., Inc., Clifton, N.J., was cycled in 0.5 N HCl and 0.5 N NaOH prior to equilibration in 0.05 M tris(hydroxymethyl)amino-
methane (Tris) chloride buffer (pH 7.5). Sephadex G-50, fine, and Sephadex G-100 were obtained from Pharmacia Fine Chemicals, Inc., Piscataway, N.J.

The Sephadex G-50 was used as supplied. Sephadex G-100 was sieved on a Tyler Portable Sieve Shaker (Fisher Scientific Co., Pittsburgh, Pa.) fitted with U.S. Standard Sieve Series, 63- and 88-μm screens. The 63- to 88-μm particles were used in both preparative and analytical Sephadex G-100 columns.

Preparation of crude cell extract. C. tetani Massachusetts strain was obtained from W. C. Latham, Massachusetts Department of Public Health, Institute of Laboratories, Boston, Mass. A modified Mueller medium (6), 8 liters in 9-liter bottles, was inoculated and incubated at 33 C for 72 hr. In preliminary experiments 30-liter cultures were employed; in later experiments, 100-liter lots were used. Bacteria were harvested by continuous centrifugation at room temperature in a DeLaval Gyrotester (The DeLavel Separator Co., Poughkeepsie, N.Y.). The cell paste was removed after each centrifugation of 50 liters of medium.

Figure 1 is a flow diagram of the purification procedure. The cell paste was suspended in an extraction fluid consisting of 1.0 M NaCl and 0.1 M Na citrate according to the elegant method of Raynaud (13). The cells were extracted for 5 days at 4 C, and the resulting extract (CEX) was separated from cell debris by centrifugation at 10,000 × g for 20 min at −5 C in a Sorvall RC-2 centrifuge with a GSA head.

The cell extract (CEX) was precipitated by the addition of 470 g of solid (NH₄)₂SO₄ per liter of CEX. The pH was adjusted to 5.0 with HCl. The precipitate was allowed to settle overnight at 4 C. It was collected by centrifugation in a Sorvall RC-2 as described above and dissolved in 0.05 M Tris chloride buffer (pH 7.5). This concentrated CEX was desalted on Sephadex G-50 (7.5 × 90 cm) in 0.025 M Tris chloride buffer (pH 7.5). All chromatography was performed at 4 C.
Mouse minimal lethal dose (MLD) was determined by intramuscular injection of 0.5 ml of the appropriate dilution into the thigh of each of five 16- to 20-g white Swiss mice (11).

Flocculating parameters, Lf and Kf, were determined by the method of Dean and Webb (2). In all tests, the concentration of the antibody solution was 50 Lf per ml as standardized against reference tetanus

Chromatography of CEX. The desalted CEX was adsorbed on a DEAE cellulose column previously washed until the effluent was equal to the influent buffer with respect to pH and conductivity. The toxin was eluted with a linear gradient of 0.05 M Tris chloride (pH 7.5) to 0.05 M Tris buffer (pH 7.5), 0.1 M NaCl.

Pooled selected fractions from the DEAE cellulose column were precipitated by adding solid (NH₄)₂SO₄ to 0.5 saturation. The precipitate was redissolved in 0.1 M phosphate buffer (pH 7.5) and chromatographed on Sephadex G-100, 63 to 88 ml (5.0 × 235 cm), equilibrated in 0.1 M phosphate buffer (pH 7.5).

All columns were eluted at a constant flow rate. A modified Micro bilateral roller pump (The Holter Co., Bridgeport, Pa.) was used as a metering pump to slow the flow rate.

Analytic procedures. Spectrophotometric readings of column effluents at 260 and 280 mÅ were made on a Gilford model 1200 spectrophotometer attached to a Beckman DU spectrophotometer. Conductivity of the DEAE cellulose elution was measured with a conductivity bridge (model RC 1682; Industrial Instrument, Cedar Grove, N.J.).

Polyacrylamide gel electrophoresis was performed by a modification of the Hjertén procedure (3). The basic buffer system was Tris-phosphate (pH 8.6) with a final molarity of 0.07 M. The samples were applied in 20% sucrose overlaid on the gel. A loading current of 2 ma/tube was applied for 15 min, and the current was then increased to 5 ma/tube for 1 hr.
antitoxin furnished by the State Serum Institute of Copenhagen. The final volume in all tubes was adjusted to 1.5 ml with saline. The tubes were incubated at 50°C, and the first flocculation was chosen as the Lf. Immunological purity was assayed by agar double diffusion (10) and by immunoelectrophoresis (16).

Sedimentation velocity was determined with a Spinco Model E ultracentrifuge. Kjeldahl nitrogen determinations were performed according to methods described by Kabat (4).

RESULTS

Preliminary experiments were carried out on more than twenty 30-liter preparations with only slight variations in yield and purity noted. Chromatography of the desalted CEX on DEAE cellulose is illustrated in Fig. 2. Tetanus toxin is eluted in this chromatogram at a conductance of approximately 2.5 mho, 4°C. In Fig. 3, the chromatography of purified toxin on Sephadex G-100 is compared with that of the crude CEX. The point of tetanus toxin elution is the only point where the absorbance at 280 mμ exceeds that at 260 mμ in the elution of CEX.
Since the basic chromatographic pattern for DEAE cellulose was established in preliminary experiments, the elution of the 100-liter preparations was terminated at conductance of 5.0 mmmhos, 4 C. In a typical 100-liter preparation (Fig. 4), fractions 80 to 160 were pooled, precipitated with solid (NH₄)₂SO₄, and redissolved in buffer to a volume of 50 ml. This solution contained approximately 825 optical density (OD) units (at 280 mµ). It was applied to the Sephadex G-100 column (Fig. 5). Polyacrylamide electrophoresis of fractions taken at intervals during the DEAE cellulose and Sephadex G-100 chromatograms is shown in Fig. 6 and 7.

Fractions 33 to 49 from the Sephadex G-100 column were pooled and precipitated with solid (NH₄)₂SO₄ at 0.5 saturation. The precipitate was redissolved in phosphate buffer (ionic strength = 0.2, pH 6.0) to a protein concentration of 1.5% and desalted in the same buffer on Sephadex G-50. Pure toxin did not denature and precipitate out of solution when stored at a protein concentration of 1% in the phosphate buffer.

Immunological purity was demonstrated by both immunoelectrophoresis and agar double diffusion. Figure 8 demonstrates that the pure protein yields one line of precipitation. No additional lines were obtained for the pure toxin when the concentration of antibody or toxin was varied between 50 and 1,000 Lf and the constant component was 200 Lf.

The pure protein has an absorbancy ratio (280/260 mµ) of 2.1 at 1.0 OD at 280 mµ, with Beers law followed between 1.5 and 0.3 OD at 280 mµ. Figure 9 is a plot of OD versus N used to convert MLD and Lf specific activity values from OD at 280 mµ to milligrams of N.

The ultracentrifuge patterns in Fig. 10 demonstrate a single component with an observed S value of 6.4. Within the limits of all assays employed, the final product described above is a pure, homogeneous protein.

The yield and properties of this separation procedure are demonstrated in Table 1.

**DISCUSSION**

Large volumes of tetanus culture filtrates have been precipitated in this laboratory with methanol and ammonium sulfate but, because of the diffi-
Table 1. Yield and properties of tetanus toxin from a 100-liter culture

<table>
<thead>
<tr>
<th>Purification step</th>
<th>Vol</th>
<th>OD/ml 260 μm</th>
<th>OD/ml 280 μm</th>
<th>Lf/OD 280</th>
<th>MLD/Lf</th>
<th>Lf (% recovery)</th>
</tr>
</thead>
<tbody>
<tr>
<td>72-hr culture</td>
<td>100 liters</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CEX</td>
<td>10 liters</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Concentrated CEX</td>
<td>900 ml</td>
<td>24.0</td>
<td>17.80</td>
<td>29.2</td>
<td>30,000</td>
<td>90</td>
</tr>
<tr>
<td>Desalted CEX</td>
<td>1,000 ml</td>
<td>16.0</td>
<td>11.70</td>
<td>34.0</td>
<td>80</td>
<td></td>
</tr>
<tr>
<td>DEAE cellulose tubes 80-160</td>
<td>800 ml</td>
<td>0.50</td>
<td>1.05</td>
<td>360.0</td>
<td></td>
<td>60</td>
</tr>
<tr>
<td>Sephadex G-100 tubes 33-49</td>
<td>195 ml</td>
<td>1.80</td>
<td>3.75</td>
<td>360.0</td>
<td>50,000</td>
<td>53</td>
</tr>
</tbody>
</table>

difficulty in collecting the precipitate compared with harvesting whole bacteria, the extraction process appears far superior. The advantages of using a cell extract rather than a culture filtrate were described by Raynaud and co-workers (13, 18). The data confirm that extraction of cells facilitates the purification of tetanus toxin in three important ways: (i) culture media and extracellular products are removed from the bacteria during centrifugation; (ii) fewer antigens are present in the CEX than in culture filtrates; and (iii) the 10-fold reduction in volume facilitates processing. These procedures have been used to process 200 liter lots, and larger culture volumes could probably be handled with the same equipment.

Gel filtration is superior to dialysis for desalting and exchanging buffers prior to chromatography. The denaturation of toxin is minimized, presumably due to the shorter exposure to surfaces required in gel filtration to obtain equivalent desalting.

The product from the DEAE cellulose column is approximately 95% pure. Most of the remaining contaminating material is removed by the succeeding chromatography on Sephadex G-100. However, both the leading and trailing edges from the Sephadex chromatogram are frequently still contaminated and must be discarded or reprocessed. For this reason, the leading edge, center, and trailing edge of all toxin peaks must be monitored before fractions are pooled. The polyacrylamide electrophoresis is a particularly sensitive tool for general monitoring during toxin separations. Its superiority in this case is exemplified by comparison of the desalted CEX as analyzed by polyacrylamide electrophoresis (Fig. 5) and by agar double diffusion (Fig. 7).

Another important test for the presence of trace contaminants, especially nucleic acids or denatured products is deviation of the 280/260 μm ratio from the 2.1 range. At protein concentrations greater than 2 mg/ml there is a decrease in ratio to approximately 2.0, presumably owing to light scattering. However, material with a ratio less than 2.0 has been found impure when examined by polyacrylamide electrophoresis.

Gel filtration, excellent for the final purification of toxin, did not yield sufficiently pure material when used as the only chromatographic step, even on longer columns than reported here. The first half of the peak always contained nucleic acids and other proteins, whereas the last part of the peak was contaminated with the peptidase originally described by Miller et al. (9). These data do not confirm the work of Salenstedt and Tirunarayanan (15), who stated that gel filtration yielded a greater purification than did ion-exchange chromatography.

The properties of tetanus toxin isolated by our procedure are similar to those reported by other investigators (1, 18). It is difficult to compare Lf values due to the different reference standards employed by other investigators. In addition, the quantitation of the test is limited by replicate dilutions necessary to obtain readable flocculations. The MLD assay is even less precise because of the influence of mouse weights and environment. We have not found the MLD to vary by greater than 20% from 150 × 10^6 MLD per mg of N for freshly isolated material. With storage time, there is a reduction in MLD but no observed physical change in the molecule. It seems possible that the great differences in MLD values observed by Turpin and Raynaud (18) and Dawson and Mauritzen (1) were differences in the degree of denaturation during the isolation procedure.

The protein molecule itself can become denatured and precipitate (1), as we have also observed. This denaturation is especially true when dilute toxin is in contact with glass, cellulose, or collodion membrane. The latter fact may have caused some of the minor peaks in the ultracentrifuge that were observed by Dawson and Mauritzen (1).

Pillemer (12) and Largier (5) were the first investigators to report values for the sedimentation coefficient of the toxin. They reported values of 4.5 and 3.9S, respectively, for the biologically
active component. In addition, they reported S values of 7.0 and 7.6 for an atoxic dimer. The dimer was reported as formed spontaneously within a few days at 4°C, but this has not been confirmed. Our values for the toxin, observed S = 6.4, agree with those obtained by Raynaud et al. (14) and Dawson and Mauritten (1), even on material stored for 3 weeks at 4°C.

Because of the consistency of the isolated material in both the biological and chemical assays, we propose that the material is the biologically active monomer. We have been unable to isolate a smaller or larger lethal fraction from three different starting materials: ultrafiltered or precipitated culture filtrates and CEX.

The pure tetanus toxin is obtained in good yield and in adequate quantity to permit investigation of its use as a reagent-grade immunogen. Its use as an immunogen and its physical characterization are under investigation and will be reported later.

ACKNOWLEDGMENTS

This investigation was supported by research grant HE-09902 and general research support grant 5-01-FR-5559-03 from the National Institutes of Health. The technical assistance of Patricia Apple and Arthur Marin is gratefully acknowledged.

LITERATURE CITED