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Purification and Characterization of Group A Streptococcal Pyrogenic Exotoxin Type C

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Group A streptococcal pyrogenic exotoxin (SPE) type C was partially purified by differential solubility in ethanol and acetate-buffered saline. Toxin prepared in this way consisted of protein and hyaluronic acid. After removal of hyaluronic acid, the toxin remained pyrogenic, enhanced susceptibility of rabbits to lethal endotoxin shock, was stable when treated with acid, base, or pepsin, but was inactivated by heat. Toxin further purified by thin-layer isoelectric focusing was pyrogenic and enhanced the susceptibility of rabbits to lethal endotoxin shock. Purified type C toxin appeared homogeneous when tested by Ouchterlony immunodiffusion and migrated as a single protein band in isoelectric focusing polyacrylamide gels (isoelectric point, 6.7) and sodium dodecyl sulfate-polyacrylamide gels (molecular weight, 13,200). The purified toxin was antigenically distinct from A and B SPE, and antisera raised against the purified toxin neutralized pyrogenic activity. The amino acid composition was determined.

Group A streptococci produce a variety of extracellular products, including streptococcal pyrogenic exotoxins (SPE). Three antigenically distinct SPE have been described: A (9), B (9, 17), and C (18). The SPE are pyrogenic when injected intravenously into rabbits (15, 18) and are associated with the streptococcal erythrogenic toxins (18). Other biological properties of the SPE include enhancement of susceptibility of rabbits, mice, and monkeys to lethal endotoxin shock (12), cytotoxicity for splenic macrophages (12), blockage of reticuloendothelial clearance of colloidal carbon in rabbits (6), and alteration of the antibody response to sheep erythrocytes in rabbits and mice (5, 7, 8). These biological activities of SPE have been reviewed (19).

SPE types A and B were previously purified using differential solubility in ethanol and acetate-buffered saline (12) followed by ion-exchange chromatography (3). SPE type A was shown to consist of protein and 4 to 8% hyaluronic acid (3). The molecular weight of A toxin was shown to be approximately 8,000, and it had a pI of 4.5 to 5.5 (3). SPE type B also consisted of protein and a small amount of hyaluronic acid. However, SPE type B was larger, and its molecular weight was reported to be approximately 21,900 (3). The pI of B toxin was between 8.5 and 9.5.

In contrast to types A and B SPE, little work has been done to characterize chemically the type C SPE. Previous researchers studying C toxin have used only streptococcal lesion extracts (18) or culture filtrates (16) as the source of SPE. This study describes the purification and physicochemical characterization of type C SPE.

MATERIALS AND METHODS

All reagents and glassware used for toxin purification and biological assays were maintained pyrogen-free.

Production of SPE type C. Type 18 *Streptococcus pyogenes* (T18P strain) used to produce the SPE was isolated at NMRU-4, Great Lakes, Ill. The streptococci were grown in a beef heart dialysate medium (18), and the culture supernatant fluid containing the toxin was obtained as described previously (3). Toxin was partially purified by differential solubilization in acetate-buffered saline and precipitation by ethanol (12). The SPE preparation obtained was referred to as EtOH-3.

The EtOH-3 toxin preparation (60 to 100 mg [dry weight]) subsequently was treated with hyaluronidase (Worthington Biochemicals Corp., Freehold, N.J.). The enzyme concentration used was one-tenth that of EtOH-3 material and was added to the toxin in 2.85 ml of sterile pyrogen-free distilled water. After 2 h of incubation at 37°C, 0.15 ml of ampholyte (Ampholine, LKB-Produkter, Stockholm, Sweden) was added, and thin-layer isoelectric focusing was then performed using the LKB 2117 Multiphor electrophoresis apparatus according to the manufacturer's specifications (21). Initially, the ampholyte range used was pH 3.5 to 10. In later experiments the ampholyte range was narrowed to pH 6 to 8.

After 16 h of electrofocusing, protein bands were located using a zymogram print method (21). Subsequently, protein bands were scraped from the thin-layer plate, and protein was removed from the gel by

filtration through glass wool. The isoelectric point of protein bands was estimated by comparing the position of stained protein bands to the pH gradient, which was determined by measuring the pH of eluted portions of the gel. Each fraction was concentrated to 5 ml by pervaporation and tested for pyrogenic activity.

Pyrogenically active toxin fractions were dialyzed for 24 h against 0.05 M potassium phosphate buffer (pH 7.0) containing 0.2 M sodium chloride (2 liters and one buffer change) to remove ampholytes. The toxin was then dialyzed for 6 to 8 h against pyrogen-free distilled water and lyophilized.

Biochemical assays. Hyaluronic acid content of the toxin was determined by measuring ability to form turbidity with an acid albumin solution according to the procedure described in the Worthington Enzyme Manual (Worthington Biochemicals Corp., Freehold, N.J., 1972, p. 112-113) based on the method of Kass and Seastone (11). Hyaluronic acid was obtained from Seikagaku Fine Chemicals, Tokyo, Japan, and hyaluronidase was obtained from Worthington Biochemicals Corp.

Protein was measured by the method of Lowry et al. (13). Bovine serum albumin (Pentex, Miles Laboratories, Inc., Kankakee, Ill.) served as the standard.

Thin-layer polyacrylamide gel isoelectric focusing using a pH 3.5 to 10 ampholyte solution was performed with the LKB 2117 Multiphor apparatus. Isoelectric points were estimated by comparing the position of stained protein bands to the pH gradient determined by measuring the pH of eluted portions of gel. Gels were stained with Coomassie brilliant blue R-250.

The molecular weight of the toxin was determined using sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (20). Molecular weight standards consisted of: bovine serum albumin, ovalbumin, pepsin, β-lactoglobulin, ribonuclease, and bromophenol blue. Gels were stained with Coomassie brilliant blue R-250.

The amino acid composition of the toxin was determined using a Beckman 120B automatic analyzer. Toxin was hydrolyzed (6 N HCl at 100°C) for 24 h in vials sealed under high vacuum. Tryptophan was quantified by the method of Edelhoch (4).

Biological assays. Solutions of SPE were prepared in sterile pyrogen-free phosphate-buffered saline (PBS; 0.005 M potassium phosphate, pH 7.0, plus 0.15 M sodium chloride) for all biological assays. Determinations of pyrogenicity and enhanced susceptibility to lethal endotoxin shock were done as described previously (12).

The effect of pepsin digestion of SPE on pyrogenicity was determined by the following procedure. A 2-mg amount of EtOH-3 material was placed in each of the two vials. To each vial 0.5 ml of acetate-buffered saline (pH 4.5) and 200 μg of hyaluronidase were added. After 2 h of incubation at 37°C, 0.5 ml of pepsin (120 μg/ml in acetate-buffered saline) was added to each vial. Pepsin was purchased from Nutritional Biochemicals Corp., Cleveland, Ohio, and its activity was measured using a modification of the method of Anson (1) as described in the Worthington Enzyme Manual (Worthington Biochemicals Corp., Freehold, N.J., 1972, p. 122-123). Additionally, 0.1 ml of an antibiotic solution (1,000 U of penicillin and 1,000 μg of streptomycin per ml) was added to each vial. Both vials were then incubated at 37°C for 18 h. Subsequently, the reactions were stopped by adding 1 M NaOH (final pH 6.8). To the solutions, 8.9 ml of sterile PBS was added, and pyrogenicity was measured (12). The final toxin concentration was 20 times the minimum pyrogenic dose at 4 h/ml (20 MPD-4/ml).

The effect of basic conditions on pyrogenicity of the toxin was determined. Hyaluronidase-treated EtOH-3 toxin (2 mg of toxin in 1 ml of sterile pyrogen-free distilled water) was incubated with 20 μl of 2.5 M NaOH (final pH 11.3) at 4°C for 12 h. The contents of a control vial were treated similarly except that 20 μl of sterile pyrogen-free distilled water was added rather than NaOH. The base-treated preparation was neutralized using 1 M HCl, and the volume of both vials was brought up to 10 ml (20 MPD-4/ml). Pyrogenicity of the preparations was tested (12).

The effect of heat treatment on toxin pyrogenicity was determined using the following procedure. Two milligrams of EtOH-3 toxin was placed in each of two graduated centrifuge tubes, and 200 μg of hyaluronidase and 1 ml of pyrogen-free distilled water were added to each. After 2 h of incubation at 37°C, the volume of each tube was adjusted to 5 ml with pyrogen-free distilled water. One sample was placed in a boiling-water bath for 30 min. The second sample was placed at 4°C. The volume of each tube was then adjusted to 10 ml (20 MPD-4/ml), and the pyrogenicity of each was determined (12).

The ability of antibody raised against purified type C toxin (fraction 6) to neutralize pyrogenicity was tested. A 250-μg portion of fraction 6 material was mixed with 1 ml of PBS. To each of two vials, 0.3 ml of toxin was added. To one vial, 0.7 ml of hyperimmune serum was subsequently added. The other vial received 0.7 ml of normal rabbit serum. Both vials were incubated at 37°C for 1 h and then at 4°C overnight. Each vial was diluted to 10 ml with PBS (10 MPD-4/ml), and pyrogenicity was determined (12).

Immunizations. Pyrogenic immunity to EtOH-3 was developed as described previously (12). For hyperimmunization, American Dutch rabbits were given a mixture of 0.3 ml of SPE type C (4 mg of EtOH-3 per ml; 0.8 mg of fraction 6 per ml) and 0.3 ml of Freund incomplete adjuvant subcutaneously on days 1, 14, 28, 42, and every 4 weeks thereafter. Rabbits were bled 1 week after booster injections, and the serum was used for Ouchterlony immunodiffusion.

RESULTS

Composition and activity of partially purified SPE. Partially purified SPE was prepared from culture filtrates of the T18P strain of group A streptococcus by differential solubility in ethanol and acetate-buffered saline (EtOH-3). This material (1 to 2.5 g per 20 liters of

culture filtrate) was shown to contain protein and approximately 60% hyaluronic acid by weight. Because of the viscous nature of EtOH-3 in solution, it was necessary to pretreat the material with hyaluronidase before undertaking further experiments. Decreasing the viscosity of EtOH-3 did not significantly alter the pyrogenic activity of the preparation (Fig. 1) as previously observed for SPE prepared from the NY-5 strain of group A streptococcus (3). The MPD-4 was 2 to 3 μg/kg of body weight either using untreated or hyaluronidase-treated EtOH-3 toxin. Ninety-eight percent of the hyaluronic acid was removed from the toxin by hyaluronidase treatment.

A poorly understood but biologically important property of SPE is the capacity to enhance the susceptibility of rabbits to lethal endotoxin shock (12). Untreated or hyaluronidase-treated EtOH-3 material also enhanced the susceptibility of rabbits to lethal endotoxin shock; four of five rabbits died when either toxin preparation was administered before endotoxin (Fig. 1).

Preliminary characterization of the toxin was undertaken by testing the effect of chemical and physical agents on the pyrogenicity of EtOH-3 which was pretreated with hyaluronidase. Toxin treated with either pepsin or base retained pyrogenic activity and capacity to enhance susceptibility to lethal endotoxin shock (Fig. 2). However, treatment with heat for 30 min effectively destroyed pyrogenic activity and ability to enhance lethal endotoxin shock. The toxin was considered acid stable since treatment of the ethanol-precipitated toxin with acetate-buffered saline (pH 4.5) during purification (12) did not destroy pyrogenic activity.

Purification of SPE using thin-layer isoelectric focusing. The SPE contained in EtOH-3 was further purified using thin-layer isoelectric focusing. When hyaluronidase-treated EtOH-3 was electrofocused by using an ampholyte range of pH 3.5 to 10, a maximum of eight protein bands was obtained.

After elution of the proteins from the thin-layer plate, each was concentrated and tested for reactivity by Ouchterlony immunodiffusion with hyperimmune serum raised against EtOH-3. Only fraction 2 (pI 4.5 to 5.0) and fraction 6 (pI 6.5 to 7.0) reacted with the antisera. Both fractions were subsequently tested for pyrogenicity in rabbits and capacity to enhance susceptibility to lethal endotoxin shock (Fig. 3). Fraction 2 material was neither pyrogenic nor capable of enhancing lethal endotoxin shock. Fraction 6, however, was very pyrogenic, with rabbits showing an average fever of 2.0°C at 4 h. In addition, four of five rabbits

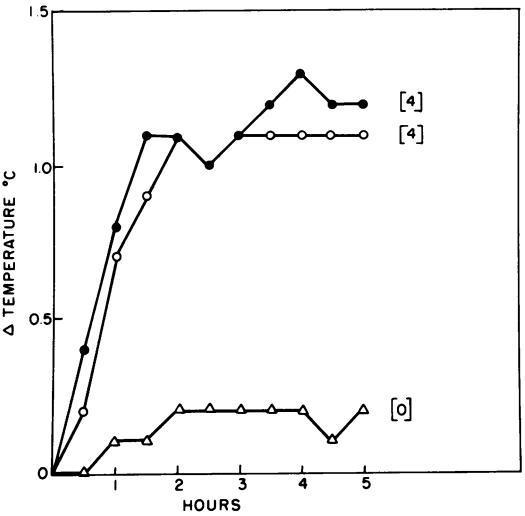

FIG. 1. *Effect of hyaluronidase treatment on pyrogenicity of T18P EtOH-3. T18P EtOH-3* (○) *contained 20 μg of toxin per ml; T18P EtOH-3 plus hyaluronidase* (●) *contained 20 μg of toxin and 2 μg of hyaluronidase per ml; hyaluronidase* (△) *contained 2 μg of hyaluronidase per ml. All solutions were incubated at 37°C for 2 h and subsequently injected intravenously into rabbits. Five rabbits in each group received 1 ml/kg at zero time. Numbers in brackets indicate the number of rabbits in each group that succumbed to lethal endotoxin shock when endotoxin (25 μg/kg) was administered intravenously at 5 h.*

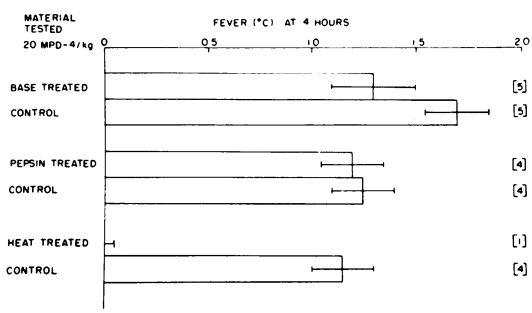

FIG. 2. *Effect of physical and chemical agents on pyrogenicity of hyaluronidase-treated T18P EtOH-3. All challenge doses of EtOH-3 SPE were 20 MPD-4/kg; a temperature change of 0.5°C was considered significant. Bars indicate ± 1 standard error. Numbers in brackets are number of the five rabbits in each group that succumbed when given endotoxin (25 μg/kg) 5 h after EtOH-3.*

succumbed to endotoxin shock. As a control for pyrogenicity of ampholytes, a 2% solution of ampholytes was given to rabbits, and the rabbits did not show significant fevers. In subsequent isoelectric focusing experiments, the ampholyte range was narrowed to pH 6 to 8. Characteristically, a major protein band with a pI of

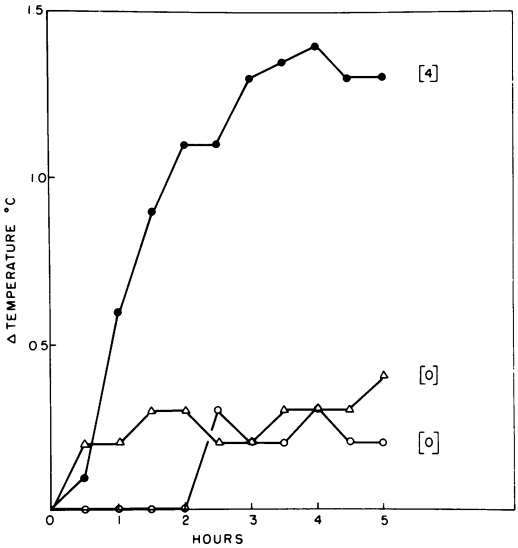

FIG. 3. *Pyrogenicity of fractions 2 and 6 from thin-layer isoelectric focusing. Fraction 2 (○); fraction 6 (●); 2% ampholyte solution (△). A temperature change of 0.5°C was considered significant. Numbers in brackets indicate the number, out of five rabbits in each group, that succumbed to lethal endotoxin shock when endotoxin (25 μg/kg) was administered intravenously at 5 h.*

6.7 was obtained, and it corresponds to fraction 6 of previous electrofocusing experiments.

Purity of SPE fraction 6. After elution from thin-layer plates and subsequent removal of ampholytes, fraction 6 contained protein (10 to 20 mg/60 mg of EtOH-3) as determined by the method of Lowry et al. (13). Hyaluronic acid was no longer detectable. Fraction 6 material appeared homogeneous when subjected to isoelectric focusing in polyacrylamide gels (Fig. 4). The isoelectric point was shown to be 6.7 to 6.8 by this procedure. The toxin protein also migrated as a homogeneous band when subjected to SDS-polyacrylamide gel electrophoresis, and the molecular weight of the toxin was estimated to be 13,200 when compared with standards.

To ascertain whether or not the SPE contained in fraction 6 exhibited increased pyrogenic activity, the MPD-4 of fraction 6 was determined and compared with EtOH-3 (Fig. 5). The MPD-4 of fraction 6 was 0.7 μg/kg compared with 3 μg/kg for EtOH-3. Greater than a fourfold increase in activity was obtained for fraction 6. Fraction 6 also enhanced susceptibility of rabbits to lethal endotoxin shock, and nine of ten rabbits died. Rabbits succumbed in less than 6 h using fraction 6 material compared with 48 h using EtOH-3 material.

Fraction 6 material was used to hyperimmunize rabbits. The antiserum was then reacted with EtOH-3 material in Ouchterlony immunodiffusion plates (Fig. 6). A single precipitin band was obtained. The antiserum did not react with either purified A or B SPE, which were obtained from the NY-5 strain of group A streptococcus. Antisera raised against

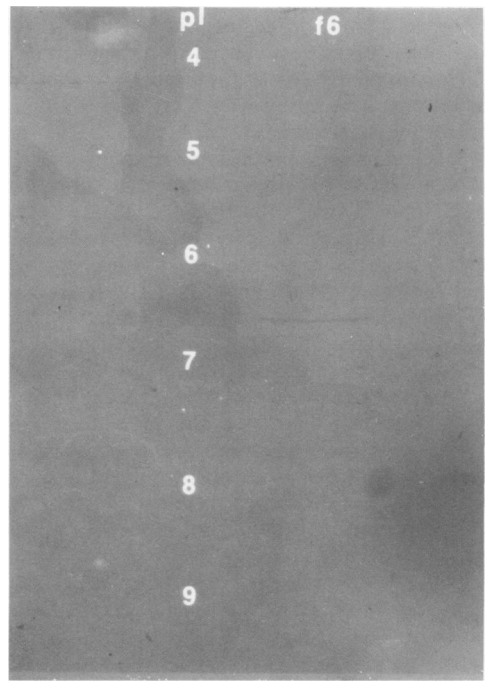

FIG. 4. *Thin-layer polyacrylamide gel isoelectric focusing of strain T18P fraction 6 material. Gel stained with Coomassie brilliant blue R-250.*

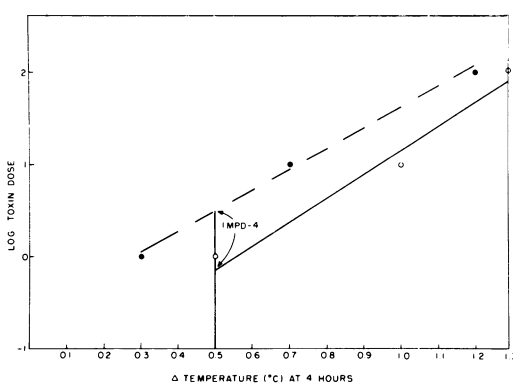

FIG. 5. *Determination of MPD-4/kg EtOH-3 (●) and fraction 6 (○) SPE; a temperature change of 0.5°C was considered the minimum significant fever. Points on graph represent the average temperature of five rabbits.*

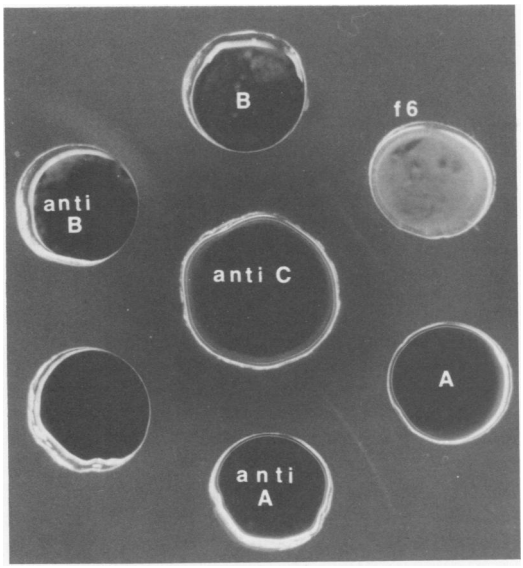

FIG. 6. *Ouchterlony immunodiffusion in Noble agar. Center well contains antiserum (anti C) raised against fraction 6. Outer wells contain A, B, and C (fraction 6) SPE purified by thin-layer isoelectric focusing, and antisera raised against A and B SPE (anti A, anti B).*

A and B SPE reacted only with the A and B SPE, respectively. A single precipitin line also was obtained when fraction 6 was tested against antiserum raised against EtOH-3 material (Fig. 7).

Antibody neutralization of SPE fraction 6 pyrogenicity. To show that antibody was protective against toxin pyrogenicity, two experiments were performed. Initially, rabbits were immunized with 20 MPD-4 of EtOH-3 SPE and subsequently tested for pyrogenic immunity. After the immunization period, rabbits were pyrogenically immune (Fig. 8), demonstrating that animals could be protected against the toxin. To ascertain whether the protection was antibody mediated, purified fraction 6 toxin was then mixed with antiserum raised against fraction 6, and the ability of the antiserum to neutralize pyrogenicity was measured. The pyrogenic activity of the antiserum-treated toxin was significantly reduced (Fig. 9). However, two of five rabbits died due to endotoxin enhancement, suggesting that the toxin was not completely neutralized.

Amino acid composition of type C toxin. The amino acid composition of fraction 6 was determined (Table 1). The toxin contained significantly more glutamine-glutamic acid, asparagine-aspartic acid, and serine than other amino acids. Using the molecular-weight determination by SDS-polyacrylamide gel electrophoresis as an estimate, a molecular weight of 12,645 based on amino acid content was obtained (14). As anticipated from the amino acid analysis, the toxin had an ultraviolet absorbance maximum of 227 nm. The low absorbance in the region of 280 nm is consistent with the absence of tyrosine, tryptophan, and cysteine in the toxin.

DISCUSSION

Group A streptococcal pyrogenic exotoxin type C was purified in this study by alcohol precipitation followed by thin-layer isoelectric focusing. These purification procedures resulted in relatively high yields of biologically active toxin, approximately 200 mg per 20 liters of culture filtrate. Pretreatment of ethanol-precipitated toxin with hyaluronidase significantly increased the amount of EtOH-3 material that could be used for further purification procedures by decreasing viscosity due to hyaluronic acid; this treatment did not reduce the biological activity of the toxin.

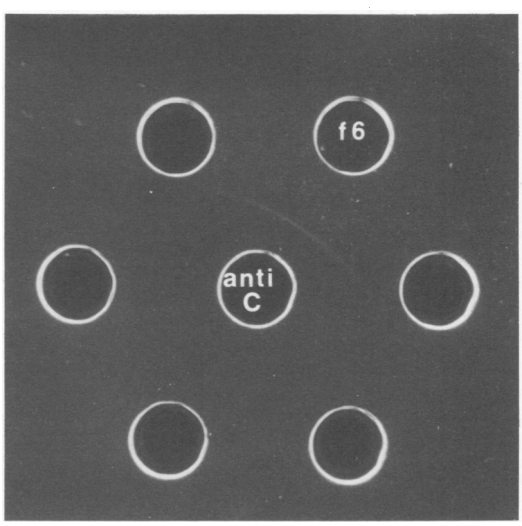

FIG. 7. *Ouchterlony immunodiffusion in Noble agar. Center well contains antiserum raised against EtOH-3 (anti C); outer well contains fraction 6 (f6).*

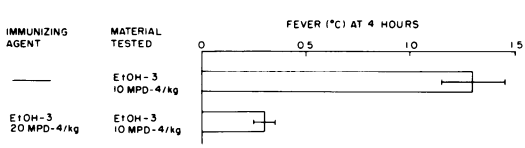

FIG. 8. *Development of pyrogenic immunity to EtOH-3 SPE. Immunizing dose of EtOH-3 was 20 MPD-4/kg. Challenge dose of EtOH-3 was 10 MPD-4/kg. A temperature change of 0.5°C was considered significant. Five rabbits in each group. Bars indicate ± 1 standard error.*

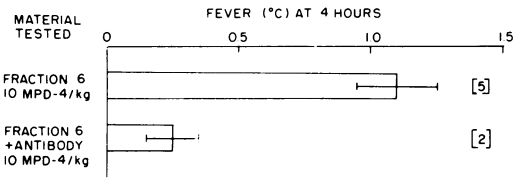

FIG. 9. *Ability of hyperimmune antiserum raised against fraction 6 to protect against pyrogenicity of fraction 6. All animals (five per group) challenged with 10 MPD-4/kg of fraction 6 material. A temperature change of 0.5°C was considered significant. Numbers in brackets indicate the number of rabbits in each group that succumbed to lethal endotoxin shock when endotoxin (25 μg/kg) was administered intravenously at 5 h.*

The SPE produced by the T18P strain was identified previously as type C (18). Pyrogenic immunity to both type A and B SPE did not confer pyrogenic cross immunity to the SPE produced by the T18P strain. The type C toxin preparation used for the studies was a streptococcal lesion extract (18), but the toxin was not sufficiently pure to study additional biological or physicochemical properties. In a later study it was shown that strain C203 U also produced type C toxin (16). No attempts were made to purify the toxin from the supernatant fluid of the culture. In the present study it was also shown that the T18P strain produces type C SPE. The C toxin, purified by isoelectric focusing, was antigenically distinct from purified preparations of both type A and B toxins using Ouchterlony immunodiffusion. In addition, C toxin was shown to differ from A and B toxins in both molecular weight and isoelectric point. Previously, it was reported that A toxin obtained from the NY-5 strain of group A streptococcus had a molecular weight of approximately 8,000 and a pI of 4.5 to 5.5 (3). Type B toxin, also obtained from NY-5 strain had a molecular weight of 21,900 and a pI of 8.5 to 9.5 (3). In contrast, type C toxin was shown to have a molecular weight of approximately 13,000 with a pI of 6.7.

The amino acid composition of type C toxin is also distinct from types A and B toxins. Type C toxin contained high concentrations of serine, glutamine-glutamic acid, and asparagine-aspartic acid. Type A toxin contained additional amino acids, and the serine concentration was not elevated (3). Similarly, type B toxin contained additional amino acids, and glycine, phenylalanine, and asparagine-aspartic acid concentrations were elevated (3). Like type A and B toxins (3), type C toxin contains little cysteine, suggesting the absence of disulfide bridges. The lack of aromatic amino acids in type C SPE suggests that the protein concentration as measured by the method of Lowry et al. (13) may have been low.

Previous research has shown that type A toxin appeared microheterogeneous (3). This phenomenon was not observed with type C toxin.

SPE type C, when purified according to procedures used in this study, retained its pyrogenicity and ability to enhance the lethality of endotoxin in rabbits. Since these biological activities are shared also by types A and B toxin, it is possible that all three toxin types share a common catalytic site. However, should the toxins share a catalytic site, that part of the molecule may lack antigenicity, since pyrogenic cross immunity between toxin types was not obtained (3, 18). The active site may be a substituent of a larger antigenic determinant, heterogeneous between toxin types. Consistent with this is the observation that antibody to each toxin will confer pyrogenic immunity to that toxin only (3, 12, 18). If the active site of the three SPE types is a common component, this site may be identified most easily using type C toxin since it contains the least number of different amino acids. Research to locate the active site of this toxin is presently being done in our laboratory.

It has been shown that the pyrogenicity of the C203 U filtrate containing SPE type C was thermoresistant (16). Heating at 96°C for 30

TABLE 1. *Amino acid composition of SPE type C[a] (fraction 6)*

Amino acid residue	No./molecule
Aspartyl[b]	15
Threonine	11
Serine	24
Glutamyl[c]	17
Proline	6
Glycine	24
Alanine	13
Cysteine	
Valine	6
Methionine	
Isoleucine	6
Leucine	6
Tyrosine	
Phenylalanine	
Ornithine	6
Lysine	
Histidine	
Arginine	
Tryptophan[d]	

[a] Molecular weight, 12,645 as determined by the method of Mahowald et al. (14).
[b] Includes aspartic acid and asparagine.
[c] Includes glutamic acid and glutamine.
[d] Determined by the method of Edelhoch (4).

min did not inactivate pyrogenicity. This thermostable component may not be type C SPE, however, since it was shown in this study that C toxin was heat labile. Alternatively, hyaluronic acid coupled to the C203 U toxin may have stabilized the toxin, making it resistant to heat.

Resistance to extremes of pH is a known property of SPE. The SPE of NY-5 strain was shown to be stable over a pH range of 1.1 to 11.0 (2). Also, Schuh et al. (16) obtained comparable results using both T18P and C203 U filtrates. Their observations were confirmed by this study, in which it was shown that type C SPE from the T18P strain was resistant to inactivation over a pH range of 4.5 to 11.3.

The resistance of SPE to proteolytic enzyme digestion was studied previously using the NY-5 strain (9, 10, 16) and several B toxin-producing strains (16, 17). It was observed that the SPE from NY-5 strain was resistant to trypsin, pepsin, and papain (16). Type B toxin was easily disgested by trypsin (17). The resistance of the pyrogens of T18P and C203U strains to proteolytic digestion was also studied (16). From these studies it was concluded that type C SPE was resistant to papain but was inactivated by pepsin and trypsin. In contrast to these previous observations, it was shown in this study that type C toxin was resistant to pepsin digestion. Further, since type C toxin lacked lysine and arginine, it would be expected that the toxin would also be resistant to trypsin. It is presently unclear what accounts for the differences in the susceptibility of type C toxin to proteolytic digestion, but the difference in purity of preparations used may have contributed significantly.

This study has shown that the type C SPE has the characteristic biological activities of other SPE. The C toxin is, however, quite different physicochemically from types A and B SPE.

ACKNOWLEDGMENTS

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