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Heterogeneity of Group A Streptococcal Pyrogenic Exotoxin Type B

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Streptococcal pyrogenic exotoxin type B purified from culture filtrates of either the NY-5 or T-19 strain of group A streptococcus was found to be heterogeneous in charge. Three protein fractions with isoelectric points of 8.0, 8.4, and 9.0 were isolated by differential solubility in ethanol and acetate-buffered saline followed by isoelectric focusing and shown to be antigenically identical to streptococcal pyrogenic exotoxin type B. The molecular weights of all three fractions were approximately 17,500, as determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, with aggregates forming in the presence of hyaluronic acid. Only the pI 8.4 fraction showed the characteristic activities of streptococcal pyrogenic exotoxin in rabbits: pyrogenicity and ability to enhance susceptibility to lethal endotoxin shock. The pI 8.0 and pI 9.0 fractions were not pyrogenic, but could be used to immunize against pyrogenicity. These two fractions failed either to enhance lethal endotoxin shock or to immunize against enhancement activity. When the isolated fractions were electrofocused again they appeared heterogeneous, suggesting an instability of the B toxin molecular forms.

Streptococcal pyrogenic exotoxins (SPE), types A, B, and C, are extracellular proteins produced by group A streptococci (5, 12, 13, 16, 20, 21). These toxins are pyrogenic when injected intravenously into rabbits and are associated with the streptococcal erythrogenic toxins (21). Other biological properties of the SPE include the enhancement of susceptibility of rabbits, mice, and monkeys to lethal endotoxin shock (13, 18, 19), alteration of reticuloendothelial clearance function (6, 8), and alteration of the antibody response to sheep erythrocytes in rabbits and mice (6, 6a, 7, 9, 10). These biological properties have been reviewed (22).

Microheterogeneity within SPE types has been observed. SPE-A, with a molecular weight of approximately 8,000 and a pI of 4.5 to 5.5 (5), has been shown to exist in two antigenically identical forms, differing slightly in charge properties (5, 15). SPE-C, isolated from strain T18P with a molecular weight of approximately 13,000 and a pI of 6.7 (16), only occasionally shows microheterogeneous forms. However, SPE-C isolated from the NY-5 strain of group A streptococcus has a slightly higher pI of 7.0, although the toxins are antigenically identical.

SPE-B, when isolated by ion-exchange chromatography, consisted of a major protein with minor contaminating proteins with pI's ranging from 8.0 to 9.5 and a molecular weight of approximately 22,000 (5). This study was undertaken to ascertain whether the heterogeneity of protein forms contained in the SPE-B fraction (5) represented molecular variants of the same toxin or whether it was due to the presence of other streptococcal products.

MATERIALS AND METHODS

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

Bacterial strains. The NY-5 strain (type 10/12) of group A streptococcus came originally from Rebecca Lancefield of the Rockefeller Institute. The T19 strain was isolated on 29 January 1953 at the U.S. Naval Training Center, Bainbridge, Md., and designated 089704 (Fu-7). The organisms were maintained as lyophilized cultures in the presence of defibrinated fresh rabbit blood.

Production and purification of SPE. The streptococcal strains were grown in a beef heart dialysate medium (21), and the culture supernatant fluid containing the toxin was obtained as described previously (5). Toxin was partially purified by differential precipitation by ethanol and solubilization in acetate-buffered saline at pH 4.5 (13). The preparation obtained, referred to as EtOH-2, has been shown previously to be free of streptolysins O and S, nicotinamide adenine dinucleotide nucleosidase, ribonuclease, deoxyribonuclease, proteinase activities, and cell wall products (5, 13), but contained up to 60% hyaluronic acid by weight (5).

The EtOH-2 toxin preparation subsequently was subjected to thin-layer isoelectric focusing using the LKB 2117 Multiphor electrophoresis apparatus (LKB-Produkter, Stockholm, Sweden) as described previously for the isolation of SPE-C (16). An ampholyte range of either pH 3.5 to 10 or pH 7 to 9 was used.

After electrofocusing, protein bands were located (24) and then scraped from the thin-layer plate. Protein was removed from the gel by filtration through glass wool. Ampholytes were removed by dialysis (16), and the toxin preparations were stored lyophilized in the presence of 25% hyaluronic acid, which maintained toxin activity. The isoelectric points of protein preparations were estimated from the pH gradient of the plate (16).

Biochemical assays. Protein was measured by the microbiuret method of Zamenhof (25) with bovine serum albumin (Pentex, Miles Laboratories, Inc., Kankakee, Ill.) as the standard.

Thin-layer polyacrylamide gel isoelectric focusing using a pH 3.5 to 10 ampholyte range was performed with the LKB Multiphor apparatus. Gels were stained with Coomassie brilliant blue R-250, and isoelectric points were estimated (5).

The molecular weights of the proteins were determined using sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (23). Unless otherwise stated, proteins were incubated for 2 h at 37°C with SDS and 2-mercaptoethanol before electrophoresis. Molecular weight standards were bovine serum albumin, ovalbumin, pepsin, β-lactoglobulin, and lysozyme. Gels were stained with Coomassie brilliant blue R-250.

Biological assays. Solutions of SPE were prepared in sterile pyrogen-free phosphate-buffered saline (0.005 M sodium phosphate [pH 7.0] plus 0.15 M sodium chloride) for all biological assays. Pyrogenicity and enhanced susceptibility to lethal endotoxin shock were measured as described earlier (13).

Immunizations. Pyrogenic immunity to SPE preparations was developed by administering 100 μg of SPE per kg of body weight intravenously every other day for 2 weeks (13). For hyperimmunization, American Dutch rabbits were given toxin in Freund incomplete adjuvant subcutaneously according to an already described procedure (16). Rabbits were bled 1 week after booster injections, and the serum was used for Ouchterlony immunodiffusion tests.

RESULTS

Purification of SPE-B. EtOH-2 toxin from the NY-5 strain of group A streptococcus, when subjected to preparative thin-layer isoelectric focusing, showed at least seven protein bands on the stained print (Fig. 1). The horizonal banding represents artifact induced during the staining process. The bands labeled SPE-A and SPE-C were identified previously (5, 16).

Since research had shown that SPE-B was a basic protein (5), each of the three basic protein fractions shown in Fig. 1 was tested for B toxin activity. The fractions were tested for pyrogenicity in rabbits, and the minimum pyrogenic dose of active toxin per kg of body weight required to produce a fever response of 0.5°C at 4 h was determined (Table 1). Neither the fraction with a pI of 8.0 nor that with a pI of 9.0 was pyrogenic at a dose of 100 μg/kg. The fraction with a pI of 8.4 was pyrogenic, with a minimum 4-h pyrogenic dose of 3.4 μg/kg. The pI 8.0 and 9.0 fractions failed to enhance the susceptibility of rabbits to lethal endotoxin shock; none of the five rabbits died in either group. In contrast, the pI 8.4 fraction did enhance lethal endotoxin shock. Three of five rabbits died when injected

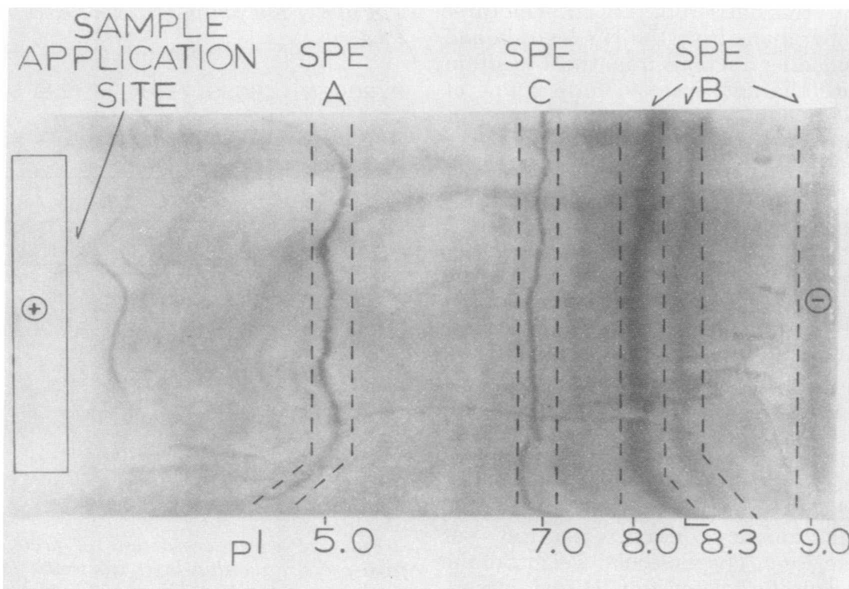

FIG. 1. *Zymogram print of strain NY-5-EtOH-2 toxin (100 mg) subjected to preparative thin-layer isoelectric focusing. Sample applied at anode end.*

TABLE 1. *Pyrogenicity of basic protein fractions obtained from strain NY-5 and their ability to enhance lethal endotoxin shock*

Fraction tested (pI)	Fever response (°C) at 4 h[a]	MPD-4 (μg/kg)[b]	Deaths due to enhancement[c]
8.0	0.3		0/5
8.4	1.3	3.4	3/5
9.0	0.2		0/5

[a] 100 μg of protein was given intravenously per kg of body weight.

[b] MPD-4, Minimum pyrogenic dose of toxin required to produce a fever response of 0.5°C 4 h after intravenous injection.

[c] Rabbits were given 25 μg of endotoxin from *Salmonella typhimurium* per kg of body weight intravenously 5 h after injection of SPE (100 μg/kg); five rabbits per group.

with 100 μg of this fraction per kg, followed by 25 μg of endotoxin per kg (Table 1).

The different basic protein fractions were found in variable combinations and amounts with different batches of EtOH-2 toxin. Only the pI 8.0 and 8.4 fractions were consistently present when the T19 strain of group A streptococcus was used to produce type B toxin. However, each of the three basic protein fractions, when present, corresponded in both pI and pyrogenic activity to the NY-5 fractions.

Identification of the basic protein fractions. The pyrogenically active basic protein fraction (pI 8.4) was used to hyperimmunize rabbits, and each of the basic proteins was then reacted with this antiserum (Fig. 2). The three protein preparations from the NY-5 strain and the corresponding fractions from the T19 strain reacted with the antiserum to form a line of identity, with only a single precipitin arc for each protein fraction.

To assess further the antigenic relatedness of the three protein fractions, tests for pyrogenic cross-immunity between protein preparations were performed. In each test the pyrogenically active type B toxin (pI 8.4) was used as the challenge toxin (Fig. 3). Animals immunized with any of the protein preparations were pyrogenically immune to challenge with the active toxin. However, only immunization with the active type B toxin conferred immunity to enhanced susceptibility to lethal endotoxin shock; none of the five rabbits died when immunized with the pI 8.4 fraction; three of five died when immunized with either the pI 8.0 or 9.0 fraction.

Physicochemical characterization of SPE-B fractions. The molecular weights of the three antigenically related type B toxin preparations were estimated by SDS-polyacrylamide gel electrophoresis (Fig. 4). The three toxin preparations migrated as homogeneous proteins and had molecular weights of approximately 17,500. When hyaluronic acid was added to the purified toxin preparations before SDS-polyacrylamide gel electrophoresis was performed, molecular weights of approximately 68,000 were observed with minor bands at 17,500 and 43,000 (Fig. 5a). Incubation of the pI 9.0 fraction overnight, rather than for 2 h, at 37°C with SDS and 2-mercaptoethanol resulted in an increased concentration of the 17,500-dalton material relative

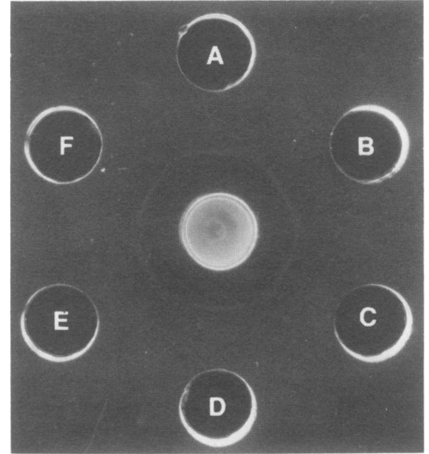

FIG. 2. *Ouchterlony immunodiffusion in Noble agar. Center well contains antiserum raised against type B toxin (pI 8.4). Outer wells, from A to F, contain: NY-5, pI 8.0; NY-5, pI 8.4; NY-5, pI 9.0; T19, pI 8.0; T19, pI 8.4; T19, pI 9.0. All toxin concentrations were 2 mg/ml.*

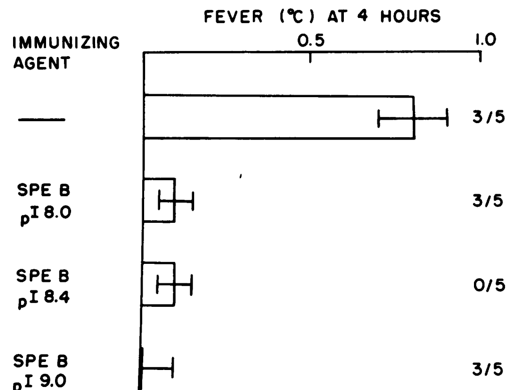

FIG. 3. *Pyrogen cross tests of protein fractions from preparative thin-layer isoelectric focusing. All immunizing doses contained 100 μg of protein per kg. Challenge doses were 50 μg of SPE-B (pI 8.4) per kg. Bars indicate ± standard error of the mean.*

to the 68,000- and 43,000-dalton material (Fig. 5b).

The amino acid compositions of the three toxin preparations were determined. Like SPE-A and -C, the B toxin preparations lacked cysteine and contained very little or no methionine. No major differences in amino acid composition were found between the fractions, with the re-

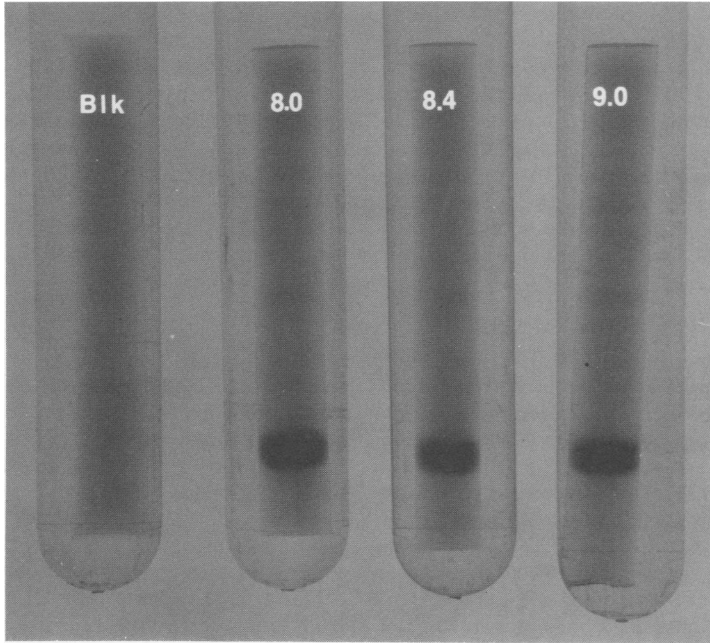

FIG. 4. *SDS-polyacrylamide gel electrophoresis of SPE-B preparations from preparative thin-layer isoelectric focusing plate. Blk, Blank; 8.0, B pI 8.0; 8.4, B pI 8.4; 9.0, B pI 9.0. Toxin concentrations were 100 μg per gel. Stained with Coomassie brilliant blue R-250.*

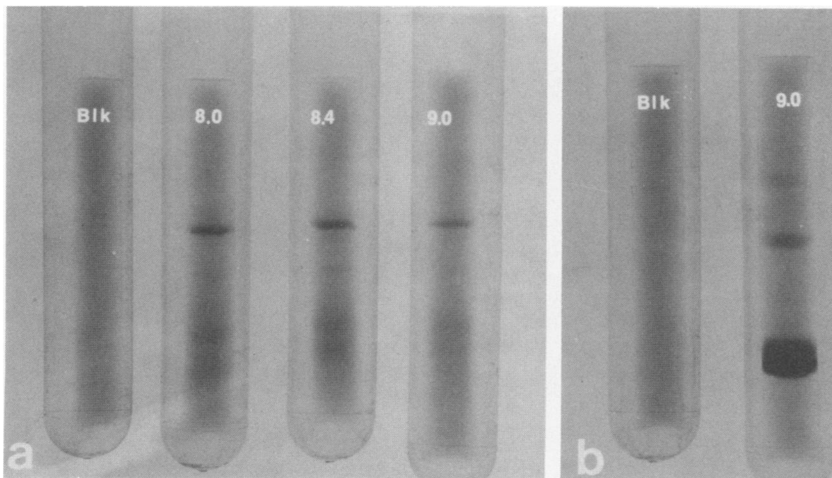

FIG. 5. *(a) SDS-polyacrylamide gel electrophoresis of B toxin preparations containing 25% hyaluronic acid. Toxins were incubated for 2 h with SDS and 2-mercaptoethanol before running. Blk, Blank; 8.0, B pI 8.0; 8.4, B pI 8.4; 9.0, B pI 9.0. Toxin protein concentrations were 50 μg per gel. Gels were stained with Coomassie brilliant blue R-250. (b) SDS-polyacrylamide gel electrophoresis of B pI 9.0 containing hyaluronic acid. Toxin (200 μg of protein per gel) was incubated for 12 h with SDS and 2-mercaptoethanol before running. Gels were stained with Coomassie brilliant blue R-250.*

sults being similar to those previously reported (5).

The stabilities of the B toxin preparations were assessed by re-electrofocusing in polyacrylamide gels (Fig. 6). The protein preparation with a pI of 8.0 and the active SPE-B both showed similar patterns of multiple bands, but with different intensities. An additional band with a pI of approximately 7.5 was present in the active type B toxin preparation. This material formed a line of partial identity with the pI 8.4 fraction when tested by immunodiffusion. The pI 9.0 fraction showed two bands at about pI 9.0, corresponding to bands present in the refocused pI 8.0 and 8.4 fractions. The more anodal bands (pI 7.5 to 8.4) were not found in the refocused pI 9.0 fraction.

DISCUSSION

In the present study, SPE-B has been shown to exist in at least three molecular forms having different charge properties. Microheterogeneity has been observed previously for SPE-A (5, 15), but the heterogeneity observed for type B toxin was considerably greater; a range of pI's from 8.0 to approximately 9.0 was obtained. Other proteins, including staphylococcal enterotoxins A, B, and C (2, 3, 4), staphylococcal exfoliative toxin (14), L-amino acid oxidase (11), and a myeloma protein (1), have also been shown to exist in multiple forms differing in charge properties. In one case at least (1), small changes in amounts of amidated dicarboxylic amino acids have been implicated in the heterogeneity. Amino acid analysis of SPE-B (5) has shown considerable amounts of glutamyl and aspartyl residues which could, by amidation, contribute to the charge differences observed with the SPE-B fractions.

The three SPE-B toxin preparations isolated in the present study appeared to be antigenically identical by Ouchterlony immunodiffusion. The fractions differed, however, in biological activities. Only the pI 8.4 fraction showed the pyrogenic and enhancing activities typical of SPE. In other studies in our laboratory, only the pI 8.4 fraction was able to suppress the antibody response to sheep erythrocytes by mouse spleen cells in culture (6a). The other two fractions (pI 8.0 and 9.0) may be missing or have altered sites responsible for these activities. Since the inactive fractions could be used to produce pyrogenic immunity but not immunity to enhancement to the active fraction, they can be considered partial natural toxoids, containing the antibody recognition site for blocking pyrogenicity. An additional reactive group must be required for immunization against the enhancement of endotoxin shock, because immunization with the pI 8.0 and 9.0 fractions failed to protect rabbits from this effect. These results suggest that different functional groups on the SPE-B molecule

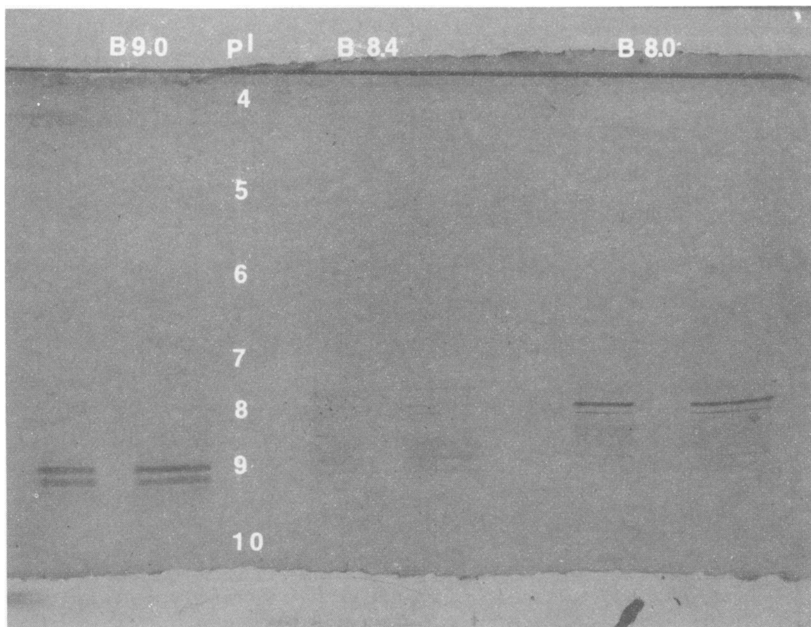

FIG. 6. *Thin-layer polyacrylamide gel isoelectric focusing of B toxin preparations. Stained with Coomassie brilliant blue R-250.*

may be required for pyrogenicity, enhancement of lethal endotoxin shock, and induction of immunity to these activities. It is not surprising that there are multiple functional groups on the toxin molecule, since previous research (5, 13, 16, 21) has identified three SPEs which share biological activities, but do not cross-protect against either pyrogenicity or enhancement. The separation of the immunizing sites for pyrogenic and enhancing activities in natural toxoids of SPE-B is consistent with other studies done in our laboratory (16a).

The minimum pyrogenic dose of type B toxin is at least four times higher than that of either SPE-A or -C (5, 16), and B toxin does not enhance lethal endotoxin shock as well as A or C toxin, suggesting that the toxin is either intrinsically less active or more rapidly degraded to biologically inactive forms. The latter hypothesis is supported by the results obtained from the thin-layer polyacrylamide gel isoelectric focusing experiment. After refocusing, with the exception of one protein band, the active B toxin did not significantly differ in types of bands present from the biologically inactive pI 8.0 fraction, suggesting that the active toxin may be degrading into the pI 8.0 material. Since the pI 9.0 fraction contained only the two most basic bands, and these were present in the other fractions as well, they may represent final degradation products. The active and inactive forms of the toxin did not differ substantially in molecular weight or amino acid composition.

The data presented in this paper suggest that the charge heterogeneity of purified SPE-B preparations may be due to instability of the toxin molecule rather than contamination by other proteins. The active toxin shares important biological properties with the other SPE, but contains antigenically identical inactive forms when isolated by isoelectric focusing.

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