Purification and Characterization of Staphylococcal Pyrogenic Exotoxin Type B†

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ABSTRACT: Staphylococcal pyrogenic exotoxin (PE) type B was purified and characterized biochemically and biologically. The exotoxin was purified from cell-free culture supernatant fluids by using differential precipitation with ethanol and resolubilization in pyrogen-free distilled water followed by preparative thin-layer isoelectric focusing. A final purification of 153-fold was achieved on the basis of the capacity of the exotoxin to produce fever. The toxin migrated as a homogeneous protein with a molecular weight of ~18 000 when tested with sodium dodecyl sulfate–polyacrylamide gel electrophoresis. Hyperimmune antisera raised against the purified exotoxin reacted with partially purified toxin in an immunodiffusion assay to form a single precipitin line. The isoelectric point of the PE was estimated to be 8.5. Alanine was identified as the N-terminal amino acid. The exotoxin contained significant amounts of lysine but few aromatic amino acids. The PE was pyrogenic and enhanced host susceptibility to lethal shock and myocardial damage by endotoxin. In addition, the exotoxin was a potent nonspecific lymphocyte mitogen and suppressed immunoglobulin M synthesis against sheep erythrocytes.

Recently, a *Staphylococcus aureus* pyrogenic exotoxin (PE)[1] was purified and characterized biochemically and biologically (Schlievert et al., 1979b). The exotoxin was shown to be distinct from other staphylococcal toxins and the closely related group A streptococcal PE's.

The staphylococcal PE, designated type A, is a low molecular weight glycoprotein (~12,000) with an isoelectric point estimated to be 5.3 (Schlievert et al., 1979b). The toxin contains few aromatic amino acids and absorbs light weakly at a wavelength of 280 nm, but the PE contains significant amounts of lysine (10.4%) despite having an acidic isoelectric point (Schlievert et al., 1979b).

The exotoxin activity is defined in terms of capacity to produce fever and enhance host susceptibility to injury by endotoxin (Schlievert et al., 1979b). The capacity of the PE to enhance susceptibility to lethal shock and myocardial damage by endotoxin may result from inactivation of RNA synthesis in liver cells (unpublished data), thus inactivating reticuloendothelial clearance function as described previously for group A streptococcal PE's (Cunningham & Watson, 1978a; Schlievert & Watson, 1979; Schlievert et al., 1980). Other biological properties of the staphylococcal exotoxin include suppression of the IgM response to other antigens (Schlievert, 1980) and nonspecific T lymphocyte mitogenicity (Schlievert et al., 1979c). This study was undertaken to purify and characterize a second, antigenically distinct type of PE produced by *S. aureus*.

Experimental Section

Materials

The Harrisburg strain of *S. aureus* (Schlievert et al., 1979b) was used for production of staphylococcal PE types A and B. Group A streptococcal strains 594, NY-5, and T18P (Schlievert et al., 1979a) were used to prepare streptococcal PE types A, B, and C. Stock cultures were maintained lyophilized in the presence of whole defibrinated fresh rabbit blood.

American Dutch-belted or darkly pigmented rabbits were obtained from a local source and weighed from 1 to 1.5 kg. BALB/c mice weighing ~25 g were purchased from Simonsen Laboratories, Inc., Gilroy, CA.

Ampholytes (Ampholine) were purchased from LKB-Produkter, Stockholm, Sweden. Endotoxin (*Salmonella minnesota* Re595) was a generous gift from D. W. Watson, Department of Microbiology, University of Minnesota. Mammalian cell culture media were purchased from GIBCO (Grand Island Biological Co., New York, NY). SE were obtained from Mission Laboratory Supply, Inc., Rosemead, CA. Reagents for solvent systems to be used for thin-layer chromatography were purchased from J. T. Baker Chemical Co., Phillipsburg, NJ. All other reagents were obtained from Sigma Chemical Co., St. Louis, MO. Reagents and glassware required for toxin production and biological assays were maintained pyrogen-free.

Methods

Preparation of PE's. Staphylococci were cultured in 10 L of a dialyzable beef-heart medium for 10–12 h at 37 °C (Kim & Watson, 1970). Subsequently, cells were removed by continuous flow centrifugation (27000*g*, Sorvall RC-5b refrigerated centrifuge with continuous flow attachment, Du Pont Instruments, Wilmington, DE). PE's were purified from culture supernatant fluids by using differential precipitation with 4 °C ethanol and resolubilization in distilled water, followed by preparative thin-layer isoelectric focusing (Schlievert et al., 1979b). Purified toxins were stored lyophilized. Group A streptococcal PE's were purified according to procedures described previously (Kim & Watson, 1970; Schlievert et al., 1977).

Biochemical Assays. Protein was determined by the microbiuret assay (Zamenhof, 1947) with bovine serum albumin serving as the standard. RNA was quantified by using the orcinol method (Ashwell, 1957) with yeast RNA as the standard. DNA was measured by using the diphenylamine

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[1] Abbreviations used: PE, pyrogenic exotoxin; IgM, immunoglobulin M; SE, sheep erythrocytes; $NaDodSO_4$, sodium dodecyl sulfate; MPD-4, minimum pyrogenic dose per kilogram of rabbit body weight after 4 h; iv, intravenous or intravenously; Cl_3AcOH, trichloroacetic acid; CPM ± SEM, counts per minute ± standard error of the mean; RNA, ribonucleic acid; DNA, deoxyribonucleic acid.

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reagent (Dische, 1955) with calf thymus DNA as the standard. Total carbohydrate was estimated by the phenol–sulfuric acid method (Dubois et al., 1956) with glucose serving as the standard.

Molecular Weight Determination. The molecular weight of the toxin was estimated by use of $NaDodSO_4$–polyacrylamide gel electrophoresis (Weber & Osborn, 1969). The molecular weight under both reducing and nonreducing conditions was determined from plots of mobility vs. the log of the molecular weight for a standard mixture of proteins which contained lysozyme, β-lactoglobulin, pepsin, ovalbumin, bovine serum albumin. Gels were stained with Coomassie brilliant blue R250.

Reisoelectric Focusing. The PE was electrofocused in polyacrylamide gels according to the manufacturer's specifications (Winter et al., 1975). The isoelectric point was estimated by comparing the position of the stained protein to the pH gradient determined by measuring the pH of eluted portions of gel. Gels were stained with Coomassie brilliant blue R250.

Amino Acid Composition. The amino acid composition of the exotoxin was determined by using a Beckman Model 121 analyzer, Beckman Instruments, Inc., Irvine, CA. Tryptophan was measured by using the method of Edelhoch (1967).

N-Terminal amino acid analysis was performed by using the dansyl chloride method (Narita et al., 1970). Dansylated amino acids were resolved by using thin-layer chromatography on silica gel (Eastman Kodak Co., Rochester, NY). Two solvent systems were employed: chloroform–ethanol–acetic acid, 38:4:3; benzene–pyridine–acetic acid, 16:4:1. Amino acids at the same approximate molar concentration of toxin were treated similarly and were used as controls.

Assays for Pyrogenicity and Capacity To Enhance Susceptibility to Endotoxin. Pyrogenic activity and capacity to enhance host susceptibility to lethal shock and myocardial damage were measured as described by Kim & Watson (1970). Briefly, rabbits (five per group) were given PE iv, and then fever responses were recorded for a 4-h period.

Routinely, three doses of exotoxin were used for estimation of MPD-4 which was obtained from a plot of the log of exotoxin dose vs. the average change in body temperature after 4 h (straight-line plot). One MPD-4 (unit) was defined as the dose of PE required to produce a 0.5 °C change in rabbit body temperature per kilogram after 4 h.

In assays to assess the capacity to enhance host susceptibility to endotoxin, animals were given endotoxin (1 μg/kg) iv 4 h after receiving PE. Deaths were recorded over a 24-h period. Survivors were examined after 3–4 days for gross myocardial necrosis. The LD_{50} of endotoxin alone in rabbits was ~500 μg/kg determined by using the method of Reed & Muench (1938). Animals were hyperimmunized with exotoxin emulsified in Freund's incomplete adjuvant (Schlievert et al., 1977).

Immunological Assays. Nonspecific mitogenicity was determined by using human cord-blood lymphocytes and rabbit and mouse splenocytes in a 4-day assay (Barsumian et al., 1978b). The capacity to suppress the direct plaque-forming cell (IgM) response to SE was evaluated by using the in vitro culture method of Mishell & Dutton (1967). Plaque-forming cells were measured by using the Jerne plaque assay as described by Cunningham & Watson (1978b).

Results

Purification of PE. The methods used previously to purify staphylococcal PE type A (Schlievert et al., 1979b) were applicable also to purify type B toxin. Partially purified, ethanol-precipitated staphylococcal PE type B was pyrogenic (1

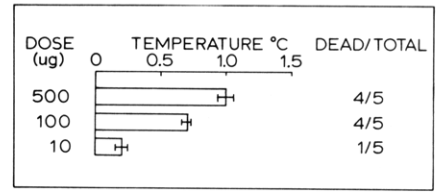

FIGURE 1: Pyrogenicity and capacity to enhance host susceptibility to lethal endotoxin (1 μg/kg) shock in rabbits of ethanol-precipitated staphylococcal PE type B; bars indicate ± SEM. The animals were previously immunized against the pyrogenicity of staphylococcal PE type A. Immunity to type A toxin was developed by giving 100 times the minimum pyrogenic dose (MPD-4) of toxin iv every other day for five injections. 1 MPD-4 was that dose of toxin required to produce an average fever response in the 5 rabbits/group 4 h after iv administration. B toxin was given iv at 0 h, and endotoxin was then given after 4 h. Deaths were recorded over a 24-h period.

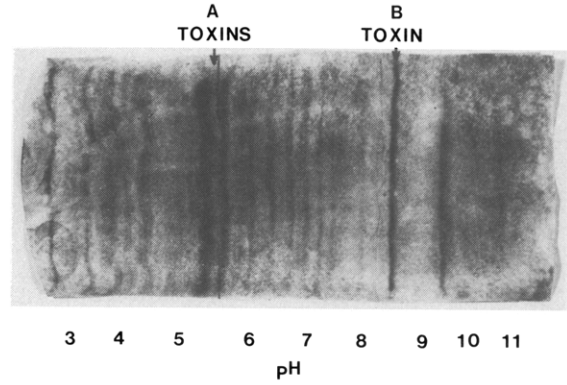

FIGURE 2: Zymogram print (Winter et al., 1975) of staphylococcal PE's electrofocused preparatively in a pH gradient of 3.5–9.5. Protein bands were located after staining with Coomassie brilliant blue R250.

MPD-4, 42 μg of protein/kg) in rabbits previously immunized against A toxin to eliminate pyrogenic activity due to that protein (Figure 1). In addition, the animals showed enhanced susceptibility to lethal endotoxin shock. The rabbits that survived the exotoxin–endotoxin challenge showed significant myocardial and limited liver necrosis 3 days after receiving toxins.

Final purification of staphylococcal PE type B employed preparative thin-layer isoelectric focusing. A representative zymogram print (Winter et al., 1975) is shown in Figure 2. B toxin migrated as a homogeneous protein with an isoelectric point of ~8.5. Staphylococcal PE type A was recovered as two antigenically identical (by double immunodiffusion) protein bands. The total numbers of proteins seen from each electrofocusing experiment varied and depended on the batch of toxin used. Also, batches of ethanol-precipitated toxin prepared from 6- to 8-h cultures contained significant amounts of A toxin but low levels of B toxin (Schlievert et al., 1979b). In contrast, significant amounts of both toxin types were obtained from 10- to 12-h cultures, suggesting B toxin was produced later during growth than A toxin.

Staphylococcal PE B, purified by using isoelectric focusing, was pyrogenic in rabbits previously immunized against type A toxin and produced typical exotoxin fever responses with peaks near 4 h (Figure 3). The MPD-4 of the purified toxin was ~4 μg of protein/kg. The toxin preparation also enhanced host susceptibility to lethal shock by endotoxin. Survivors again showed extensive myocardial and limited liver necrosis 3 days later.

Normal rabbits immunized every other day for five injections with purified B toxin were immune to challege with 20 MPD-4 homologous exotoxin (Figure 4). In contrast, nonimmune animals showed typical exotoxin fever responses. The

Table I: Purification of Staphylococcal Pyrogenic Exotoxin Type B[a]

fraction	protein (mg)	total act. (units)[b]	sp act. (units/mg of protein)	purification (x-fold)	yield of act. (%)
supernatant fluid	4×10^4	1.7×10^5	1.6		100
EtOH	2.5×10^3	6.0×10^4	24.0	15	35
IEF[c]-purified	49	1.2×10^4	245	153	7

[a] Assays for pyrogenicity were performed with animals previously immunized against type A toxin. [b] One unit (u) equals 1 MPD-4. [c] Isoelectric focusing.

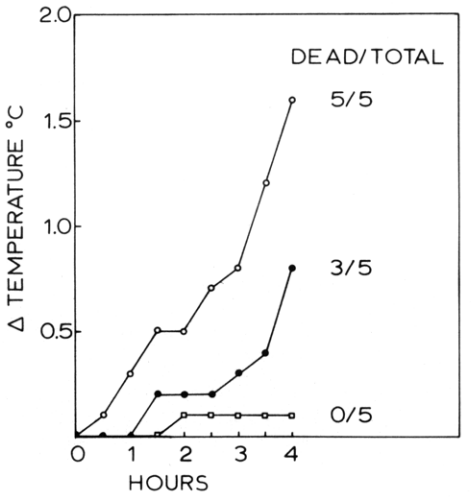

FIGURE 3: Pyrogenicity and capacity to enhance host susceptibility to lethal endotoxin shock in rabbits (5/group) of staphylococcal PE type B purified by isoelectric focusing. Exotoxin doses given at 0 h: 1 μg/kg (□); 10 μg/kg (●); 100 μg/kg (○). Endotoxin dose given at 4 h: 1 μg/kg. Deaths were recorded over a 24-h period.

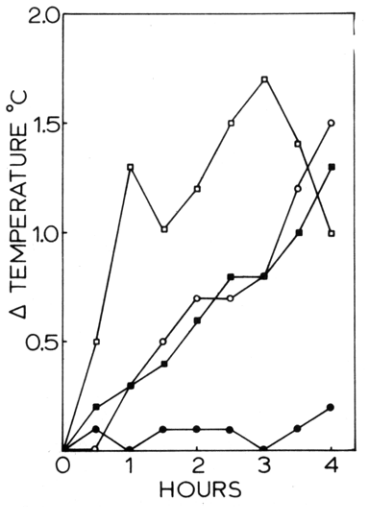

FIGURE 4: Development of pyrogenic immunity in rabbits (5/group) against staphylococcal PE type B. Animals were immunized against PE every other day for 5 injections by using 100 times the minimum pyrogenic dose (MPD-4) of exotoxin. 1 MPD-4 equals that amount of toxin required to produce an average fever response of 0.5 °C 4 h after iv injection. Nonimmune rabbits given 20 MPD-4 type B toxin (○); rabbits immunized against staphylococcal PE type B challenged with 20 MPD-4 homologous toxin (●); rabbits immune to staphylococcal PE type B challenged with 20 MPD-4 type A toxin (■), 100 MPD-3 endotoxin (□).

animals immune to B toxin were not imune to challenge with either staphylococcal PE A (20 MPD-4) or endotoxin (100 MPD-3, 0.5 μg/kg). The recovery of pyrogenic activity and degree of toxin purification are shown in Table I.

Criteria for Purity. When tested by reelectrofocusing in polyacrylamide gels, the exotoxin migrated as a homogeneous protein with an isoelectric point of 8.5 (Figure 5). The toxin

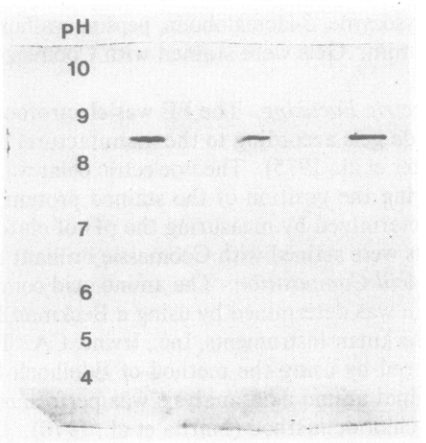

FIGURE 5: Staphylococcal PE type B (100 μg/track) reelectrofocused in polyacrylamide gel. pH gradient used was 3.5–9.5. All tracks contained the same concentration of material.

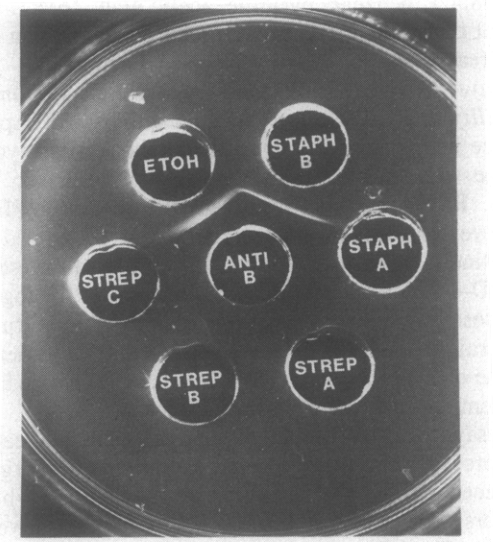

FIGURE 6: Ouchterlony immunodiffusion in Noble agar of ethanol-precipitated (10 mg of protein/mL, EtOH) and isoelectric focusing purified staphylococcal PE type B (2 mg/mL, Staph B) reacted against hyperimmune antisera raised against type B toxin purified by isoelectric focusing. Other outer wells contain the remaining known PE types (2 mg/mL). All wells contain 40 μL of material.

also migrated as a homogeneous protein with a molecular weight of ~18 000 when tested with $NaDodSO_4$–polyacrylamide gel electrophoresis under reducing and nonreducing conditions. In addition, a single N-terminal amino acid (alanine) was identified when compared with standard amino acids. Ethanol-precipitated exotoxin reacted with hyperimmune antisera raised against the purified toxin to produce a single precipitin arc when tested by Ouchterlony immunodiffusion (Figure 6). The antisera also reacted with purified staphylococcal PE B but did not react with either type A or the known group A streptococcal PE's. B toxin contained 3–5% carbohydrate, but neither DNA nor RNA was detected (<1% by weight).

Table II: Amino Acid Composition of Staphylococcal Pyrogenic Exotoxin Type B

residue	mol/mol of toxin[a]	residue	mol/mol of toxin[a]
aspartyl[b]	11.7	isoleucine	4.9
threonine	7.8	norleucine	3.4
serine	9.9	leucine	7.4
glutamyl[c]	17.2	tyrosine	3.2
proline	6.6	phenylalanine	2.2
glycine	15.6	lysine	25.3
alanine	13.2	histidine	1.1
cysteine	1.0	arginine	6.0
methionine	1.25	tryptophan[d]	1.0
valine	7.6		

[a] Based on molecular weight estimate of 18 000. [b] Aspartyl includes aspartic acid and asparagine. [c] Glutamyl includes glutamic acid and glutamine. [d] Determined by the method of Edelhoch (1967).

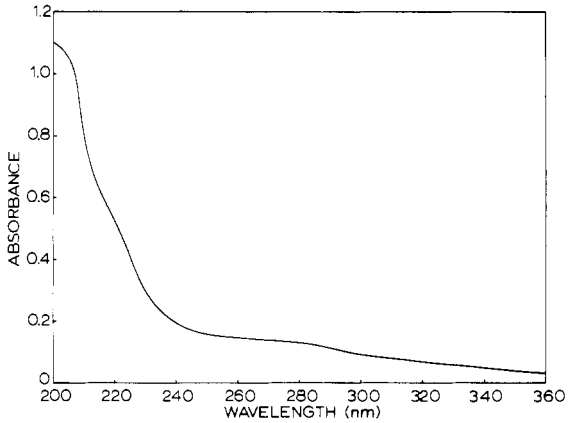

FIGURE 7: Ultraviolet absorbance spectrum of staphylococcal PE type B (100 μg/mL, 1-cm light path) determined with a Beckman Acta C III spectrophotometer (Beckman, Fellerton, CA).

The amino acid composition (Table II) indicated lysine was the major residue present, consistent with the basicity of the glycoprotein. Aromatic and sulfur-containing amino acids were present in low concentrations. The ultraviolet absorbance spectrum of staphylococcal PE B is shown in Figure 7. The toxin weakly absorbs at 280 nm, consistent with the low content of cysteine, tryptophan, and tyrosine. The toxin did not have detectable hemolysin (Bernheimer & Schwartz, 1963), enterotoxin (Matheson & Thatcher, 1955), or exofoliatin (Melish et al., 1972) activities.

Additional Properties of PE. The exotoxin was a potent nonspecific lymphocyte mitogen (Figure 8). Data obtained by using the optimal stimulatory dose of toxin are shown. Toxin doses as low as 10^{-3} μg/well also stimulated lymphocytes significantly above background. Comparable to other PE types (Barsumian et al., 1978b; Schlievert et al., 1979b), the peak responsiveness to B toxin in the human system shown occurred after 3–4 days rather than 2–3 characteristic of concanavalin A used as a positive control for cell responsiveness. Similar data were obtained when B toxin was tested for capacity to stimulate murine and rabbit splenocytes.

The exotoxin also suppressed IgM synthesis against SE (Table III). The suppressive effect was dose dependent with high and low doses failing to inhibit the direct plaque-forming cell response. The suppressive doses were comparable to those reported for other PE's (Schlievert, 1980).

Discussion

A second antigenically distinct type of staphylococcal PE was isolated and characterized in this study. Like staphylococcal PE A (Schlievert et al., 1979b) and group A streptococcal PE's (Kim & Watson, 1970; Schlievert et al., 1977; Barsumian et al., 1978a), this exotoxin type, designated B, was pyrogenic and enhanced host susceptibility to lethal shock and myocardial damage by endotoxin. Additional properties of the exotoxin shared with other PE's include nonspecific lymphocyte mitogenicity and suppression of IgM synthesis.

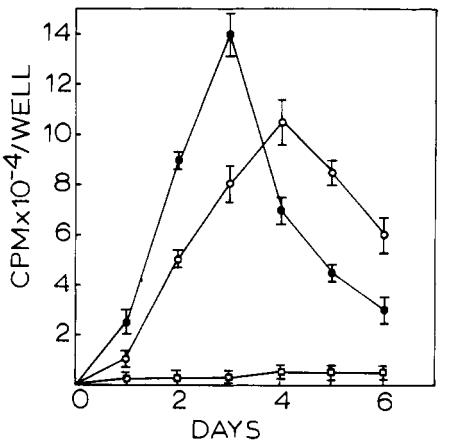

FIGURE 8: Kinetics of stimulation of human cord-blood lymphocytes (2×10^5/200 μL per well in quadruplicate) by staphylococcal PE (1 μg/20 μL per well, ○) or concanavalin A (1 μg/20 μL per well, ●). Negative control (□). 1 μCi/20 μL [*methyl*-^{3}H]thymidine (Amersham Corp., Arlington Heights, IL, 25 Ci/mmol) was added to each well 24 h before harvesting. Cells were harvested with a MASH-II (Microbiological Associates, Los Angeles, CA) and were counted with a Beckman LS 3133T scintillation counter (Beckman Instruments, Inc., Fullerton, CA). Bars indicate ± SEM.

Table III: Suppression of Direct PFC Response by Staphylococcal PE Type B

toxin dose (ng)	direct PFC[a]/ culture ± SEM	toxin dose (ng)	direct PFC[a]/ culture ± SEM
1000	680 ± 32	0.1	423 ± 11
100	700 ± 41	0.01	733 ± 21
10	257 ± 3	0.001	807 ± 42
1	352 ± 53	0	900 ± 43

[a] Direct PFC, plaque-forming cells, making IgM against SE.

On the basis of charge, molecular weight, and specific activity, staphylococcal PE B resembled closely streptococcal PE type B (Barsumian et al., 1978a). However, no antigenic cross-reactivity between the toxins was observed. Further, no evidence was obtained to indicate that staphylococcal PE B exhibited heterogeneity described previously for the streptococcal toxin (Barsumian et al., 1978a). Additionally, the amino acid compositions of the two PE's are dissimilar. Like staphylococcal PE A but unlike the streptococcal PE's, staphylococcal PE B contained significant amounts of lysine. Comparable to the other PE types, aromatic and sulfur-containing amino acids were present in low concentrations. It is of interest to note that staphylococcal PE type A was separated into two bands by isoelectric focusing, comparable to that observed for group A streptococcal PE A (Barsumian et al., 1978a).

The exotoxin described in this study lacked hemolytic, enterotoxin, and exfoliative toxin activities and was distinguished from hyaluronidase (Abramson & Friedman, 1968), leukocidin (Woodin, 1959), protein A (Forsgren & Sjoquist, 1969), DNase (Abramson & Friedman, 1968), RNase (Gladstone & Yoshida, 1967), and staphylokinase (Astrup & Mullertz, 1952) by differences in biochemical properties. However, B toxin may be the nonspecific lymphocyte mitogen described by Kreger et al. (1972). The toxin and mitogen were purified comparably and share isoelectric point and molecular weight.

However, in this study several additional properties were described.

In a previous investigation, Schlievert et al. (1980) proposed that studies to identify the etiologic agent of Kawasaki disease should include staphylococcal PE A. The studies should be extended now to evaluate also the role of type B toxin in the production of the syndrome. Further, studies should also assess the role of staphylococcal PE's in the production of a closely related syndrome, toxic shock (Todd et al., 1978).

Acknowledgments

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