INFECTION AND IMMUNITY, Jan. 1989, p. 23–28
0019-9567/89/010023-06$02.00/0
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Vol. 57, No. 1

Biological and Immunological Properties of the Carboxyl Terminus of Staphylococcal Enterotoxin C1

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Received 24 June 1988/Accepted 19 September 1988

Comparisons of recently published primary sequences of staphylococcal and streptococcal pyrogenic toxins prompted an evaluation of biological and immunological properties of the C terminus of staphylococcal enterotoxin C1. The 59 N-terminal amino acids were deleted from the toxin by digestion with trypsin. The resulting fragment (M_r, 20,659) contained the remaining 180 C-terminal residues. This fragment (Trp F1) consisted of two polypeptide chains (Trp F1a and Trp F1b) linked by cysteine residues. Trp F1 was mitogenic, pyrogenic, and enhanced susceptibility of rabbits to lethal endotoxin shock. In addition, this fragment contained at least one antigenic epitope that cross-reacted with enterotoxin B.

Staphylococcal enterotoxin (Ent) C1 belongs to a family of related pyrogenic toxins (PTs) produced by *Staphylococcus aureus* and *Streptococcus pyogenes*. Included in this family of toxins are staphylococcal Ents A through E (3), pyrogenic exotoxins A and B (37), toxic shock syndrome toxin 1 (4), and streptococcal pyrogenic exotoxins (SPEs) A through C (1).

All of the PTs are pyrogenic and immunosuppressive as a result of their abilities to induce nonspecific T-lymphocyte mitogenicity and enhance host susceptibility to lethal endotoxin shock (2, 13, 24, 32, 37, 40, 46). At least one human illness, toxic shock syndrome, results from these shared toxic activities; several PTs including toxic shock syndrome toxin 1, Ent B, Ent C1, and SPEs have been implicated as causative agents (14, 15, 18, 35, 38). The Ents are distinguished from other PTs in this family by their additional ability to induce vomiting and diarrhea after oral administration and are the agents responsible for staphylococcal food poisoning (16). Likewise, SPEs are unique in their capacity to induce heart damage (39).

To date, many of the PT structural genes have been cloned and sequenced (5–7, 9, 11, 12, 19, 23–25, 28, 34, 48). This work suggests that the shared immunobiological properties of PTs result from conserved regions on the toxins. For instance, mature Ent C1 contains 239 amino acids with a calculated molecular weight of 27,496 and shares considerable amino acid homology with Ent B and SPE A (68 and 46%, respectively) (12). All three toxins are highly conserved in their carboxyl ends and in the regions adjacent to the centrally located Ent cysteine loop. In contrast, the N-terminal region of SPE A displays little similarity to either Ent. Although other PTs are less homologous, some uniformly conserved carboxyl regions were also found by computer alignment of these three toxins with Ent A and SPE C (6, 20).

This present study was undertaken to evaluate the toxic and immunological properties of the conserved C terminus of Ent C1. Since other investigators partially mapped the trypsin-sensitive bonds in Ent C1 (41), we used this enzyme to delete the N terminus of the toxin. We purified the 20,659-dalton C-terminal fragment and found that it retains several biological properties of the native Ent C1 and also contains a cross-reactive antigenic epitope.

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MATERIALS AND METHODS

Toxin purification. Ent C1 and Ent B were prepared from cultures of *S. aureus* MNDON (11) and *S. aureus* MNLO (35), respectively. *S. pyogenes* 594 was used to prepare SPE A. Cultures were grown with aeration (37°C) in pyrogen-free dialyzable beef heart medium (38), and toxins were purified from ethanol precipitates by preparative thin-layer isoelectric focusing (IEF) (8, 38).

Preparation and purification of Ent C1 tryptic fragments. Trypsin type XI was purchased from Sigma Chemical Co., St. Louis, Mo., and was used according to standard conditions (50). Ent C1 (4 mg) and trypsin (80 μg) were mixed and incubated (37°C) in 1 ml of 0.046 M Tris buffer (pH 8.1) containing 0.0115 M $CaCl_2$. After 30 min, the tryptic digestion products were separated by preparative IEF in a pH gradient of 3 to 10 with 5 M urea. Protein bands were visualized by zymogram printing.

In some experiments an attempt was made to separate tryptic fragments that were covalently linked through an internal disulfide bridge. Because alkylation of Ent C1 fragments with iodoacetamide has been shown to result in biologically inactive fragments and solubility difficulties (43), we chose to use *S*-pyridylethylation with 4-vinylpyridine (17). After trypsin digestion, the peptides (4 mg) were incubated with 2-mercaptoethanol (5%, vol/vol), guanidine hydrochloride (6 M), and 0.1 M EDTA in 0.25 M Tris buffer (pH 8.5). After 2 h, the thiol-modifying agent 4-vinylpyridine (4%, vol/vol) was added, and the incubation was continued for an additional 2 h at room temperature. The reaction mixture was dialyzed against water, and protein bands were resolved by IEF as described above.

SDS-PAGE. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed in 12.5% polyacrylamide vertical gel slabs with the discontinuous buffer system of Laemmli (29). Samples were dissolved in a sample buffer (0.02 M Tris, 2.3% SDS, and 10% glycerol [pH 6.8]) with or without 2-mercaptoethanol (5%, vol/vol). In some experiments, proteins resolved by SDS-PAGE were tested for reactivity with polyclonal antibodies or monoclonal antibodies (MAbs) by using a standard immunoblot assay (11).

Amino acid sequencing. The N-terminal amino acid se-

quences of IEF-purified tryptic fragments were determined by the automated Edman degradation technique of Hewick et al. (21). Instrumentation included a model 470A gas-phase protein sequencer interfaced to a model 120A PTH amino acid analyzer (Applied Biosystems, Foster City, Calif.).

Antiserum preparation. Hyperimmune antisera were prepared by immunization of American Dutch-belted rabbits with purified PTs or tryptic fragments (36).

MAb techniques. Splenocytes from immune BALB/cWAT mice were fused with X63-Ag8.653 murine myeloma cells (26). Detailed methods for immunization, fusion, and screening have been described previously (10). The relative titer of each MAb was measured against Ent C1, Ent B, and SPE A by using an enzyme-linked immunosorbent assay (ELISA) (8). MAb typing was also done by ELISA with light chain- and isotype-specific antibody conjugates (Southern Biotechnology Associates, Inc., Birmingham, Ala.).

Biological assays. IEF-purified Ent C1 and its tryptic digestion fragments were used for biological assays. Mitogenicity was assessed by using rabbit splenocytes as indicators of proliferation in a 4-day assay (33). [^{3}H]thymidine was obtained from Amersham Corp., Arlington Heights, Ill. Pyrogenicity and enhancement of host susceptibility to lethal endotoxin shock were determined in rabbits (1.0 to 2.0 kg) as described previously (27). Endotoxin was prepared from *Salmonella typhimurium* by the method of Westphal and Jann (49) and was administered intravenously 4 h after the animals received Ent C1 or toxin fragments.

Mitogenicity neutralization assay. Anti-Ent C1 tryptic fragment MAbs were partially purified from ascites by precipitation with $(NH_4)_2SO_4$ (33%, wt/vol). The antibodies were dissolved, dialyzed exhaustively against 0.1 M Na_2HCO_3–0.5 M NaCl, and adjusted to contain a protein concentration of 800 μg/ml. X63-Ag8.653 control ascites was treated identically. Ent C1 (1.0 μg in RPMI 1640 medium) was mixed with 5 μl of MAb solution in a final volume of 50 μl and incubated for 2 h (37°C). Residual mitogenic activity of the toxin was measured after the addition of murine (BALB/cWAT) splenocytes in 200 μl of RPMI 1640 medium supplemented with 2% fetal bovine serum, 2 mM L-glutamine, penicillin (100 U/ml), and streptomycin (100 μg/ml).

Statistics. Mitogenesis and mitogenicity neutralization data were analyzed by Student's *t* test of unpaired, normally distributed data.

RESULTS AND DISCUSSION

Production and purification of Ent C1 tryptic fragments. This study was undertaken to evaluate the biological and immunological properties of the C terminus of a representative PT, Ent C1. Recently published sequences of staphylococcal and streptococcal PTs have demonstrated that there are several stretches of C-terminal amino acids uniformly conserved throughout the toxin family. Although some toxins also share N-terminal homology, this region is not consistently conserved. We were therefore interested in determining whether shared biological properties and cross-reactive antigenic epitopes could be localized to the C terminus of Ent C1. Trypsin treatment was used to generate C-terminal fragments of Ent C1. Maximum digestion of Ent C1 by trypsin occurred within 10 min (results not shown). Less than 5% of the toxin was completely resistant to proteolysis, even after 12 h. Figure 1 shows a zymogram print from a typical IEF run for purification of Ent C1 tryptic fragments. Approximately 50% of the protein focused as two bands (Trp F1 and Trp F2) in the anodal half of the gel bed.

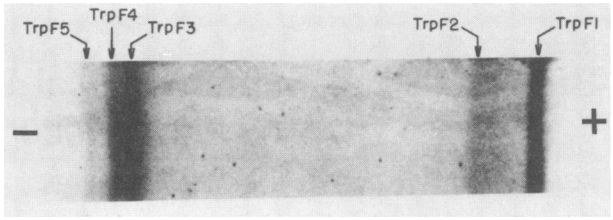

FIG. 1. Resolution of Ent C1 tryptic digestion products by IEF. After IEF in a pH gradient of 3.0 to 10 in the presence of 6 M urea, protein bands (Trps F1 through F5) were visualized by zymogram printing and staining with Coomassie blue.

Three additional protein bands (Trp F3, Trp F4, and Trp F5) migrated cathodally to the region corresponding to the isoelectric point (pI 8.6) of undigested Ent C1. In the absence of urea, the anodal bands were not observed.

The composition of each of the five IEF bands was assessed by SDS-PAGE in the presence and absence of 2-mercaptoethanol. Nonreduced Trp F1 migrated as a homogenous band with an M_r of approximately 19,000 (Fig. 2). Under reducing conditions, Trp F1 resolved into two smaller bands (Trp F1a and Trp F1b). The other four IEF bands, Trps F2 through F5, had compositions similar to Trp F1 but also contained various amounts of intact or singly cleaved toxin and were not used in subsequent experiments.

Sequence analysis and mapping of Trp F1. Trp F1 appears to be analogous to the 22,000-dalton C-terminal fragment obtained by Spero et al. (41). Except for solubility properties, both fragments are similar physicochemically. Based upon the published primary sequence of Ent C1 (12), Trp F1 has a calculated molecular weight of 20,659. The lower-than-expected value (M_r, 19,000) obtained by SDS-PAGE analysis in this study is probably due to strong noncovalent forces inherent in the molecule. This property was reported previously (41, 43) and is also evident from the high concentration of urea required to separate Trp F1 from larger-molecular-weight (M_r, 27,000) cationic material that contained particles of native and nicked toxin (Trps F2 through F5). We were unable to isolate the 59-amino acid (M_r, 6,773) N-terminal fragment by IEF. This fragment has a higher content (>20%) of basic amino acids than the cationic (pI 8.6) native toxin and probably migrated into the cathode. Six cycles of N-terminal amino acid sequencing were performed on Trp F1. The analysis produced two signals per cycle, indicating equal molar amounts of two free N termini. Comparison of the dual sequence with the previously published primary sequence of Ent C1 (12) identified the two N termini as residues 60 through 65 and 104 through 109 (Fig. 3). Residues 60 (asparagine) and 104 (valine) are each preceded by lysine, and their locations are similar to those of two trypsin-sensitive bonds previously reported for Ent C1 (41). Based upon the findings that Trp F1 has two N termini and separates into two smaller fragments under reducing conditions in the presence of SDS, the composition shown in Fig. 3 was predicted for the fragment.

The molecular weight of Trp F1b, determined from its migration in SDS-PAGE, was approximately 15,000. This is consistent with a value of 15,663 calculated for the molecular weight of the 136 amino acids in the C terminus of Ent C1. The molecular weight of Trp F1a was not determined by the gel system used in this study. However, to form a disulfide linkage with Trp F1b, at least residues 60 through cysteine 93 and the next lysine residue 98 must be retained. Attempts to isolate Trp F1a and Trp F1b in IEF gels by prior reduction

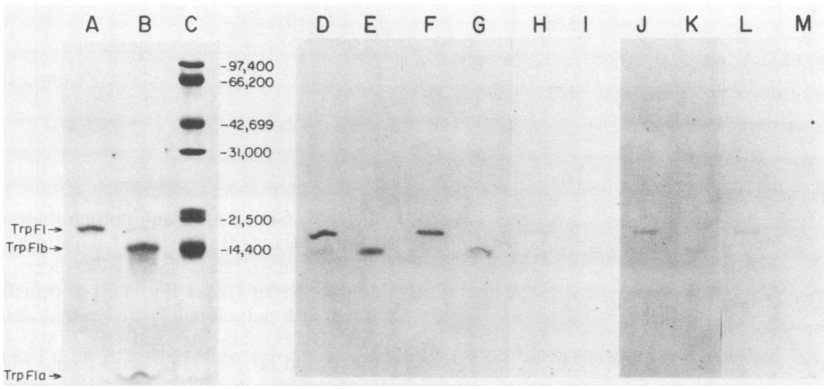

FIG. 2. SDS-PAGE and immunoblot analysis of IEF-purified Trp F1. The fragment (10 μg) was electrophoresed without (lanes A, D, F, H, J, and L) or with (lanes B, E, G, I, K, and M) prior reduction with 2-mercaptoethanol. Molecular masses of protein standards (lane C) are given in daltons. After electrophoresis, proteins were stained with Coomassie blue (lanes A through C) or transferred to nitrocellulose and probed with anti-Trp F1 MAbs 4B2H5 (lanes D and E), 2F8G5 (lanes F and G), 3C12D10 (lanes H and I), 1E11B6 (lanes J and K), and 3B8C9 (lanes L and M).

and *S*-pyridylethylation of cysteine residues were unsuccessful.

Biological activities of Trp F1. The mitogenic capacity of Trp F1 was compared with that of Ent C1 at three doses (Fig. 4). Trp F1 was mitogenic at all three doses tested. There was no statistically significant difference in the incorporation of [^{3}H]thymidine into splenocyte DNA after exposure to 1.0 or 0.1 μg of either stimulant, although at a lower dose (0.01 μg) Trp F1 was less mitogenic than the native toxin ($P < 0.01$). Spero and Morlock (43) reported previously that the M_r 22,000 C-terminal toxin fragment was not mitogenic. This discrepancy may be the result of insolubility of the fragment in that study. IEF-purified Trp F1 used in this present study was highly soluble in aqueous solutions and in media used for biological assays. Spero and Morlock found that the complementary N-terminal polypeptide possessed a low level of mitogenic activity at high doses (10 to 100 μg) (43). Our results do not exclude the possibility that Ent C1 possesses this additional weakly mitogenic domain, since we could not isolate the N-terminal fragment for evaluation in our system.

Rabbits were used to evaluate the biological activity of Trp F1 in vivo (Fig. 5). The animals were given an intravenous injection containing Ent C1 (5.0 μg of body weight) or an equal molar amount (3.8 μg/kg of body weight) of Trp F1.

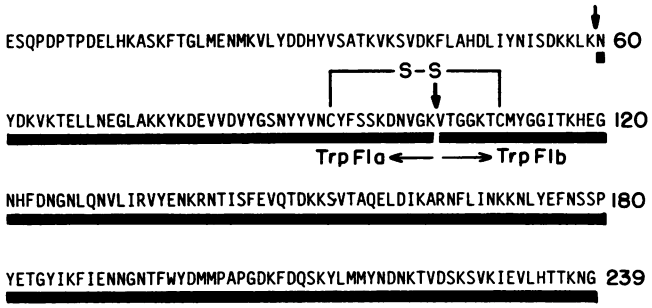

FIG. 3. Predicted chemical composition of Trp F1. The complete primary sequence of Ent C1 (12) is shown. Trypsin-sensitive bonds are indicated by arrows, and the proposed amino acid sequence composition of Trp F1 is underlined. Reduction of the intramolecular disulfide bond (-S-S-) results in the separation of Trp F1 into Trp F1a and Trp F1b.

Ent C1 and Trp F1 were equally pyrogenic and also enhanced susceptibility of rabbits to lethal endotoxin shock. All three rabbits in each group succumbed after receiving a standard endotoxin challenge (1/50 50% lethal dose) at the 4-h time point.

Mitogenicity neutralization by MAbs. Five anti-Trp F1 MAbs were isolated and characterized. Table 1 shows the results of isotyping and ELISA titers against Ent C1. In an initial experiment, all five MAbs reacted with varied degrees of intensity with nonreduced Trp F1 in immunoblot assays (Fig. 2). In addition, two MAbs (4B2H4 and 2F8G5) reacted with Trp F1b after reduction of Trp F1.

To rule out the possibility that mitogenesis induced by Trp F1 was due to an undetectable contaminating fragment and to localize activity to Trp F1a or Trp F1b, the MAbs were tested for the ability to neutralize undigested Ent C1. The toxin was preincubated with each MAb, and the residual mitogenic capacity was assessed. Four of the MAbs signifi-

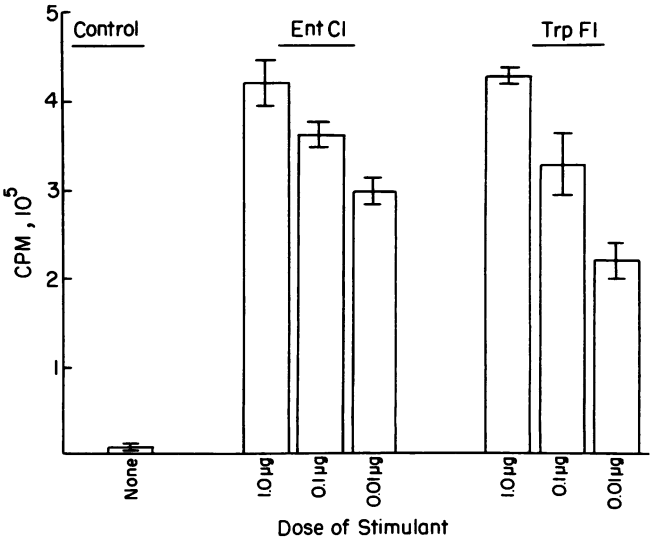

FIG. 4. Comparison of the mitogenic capacity of Ent C1 and Trp F1 for rabbit splenocytes. Control cells received no stimulant. Incorporation of [^{3}H]thymidine is expressed as the mean ± standard error of the mean of four tests.

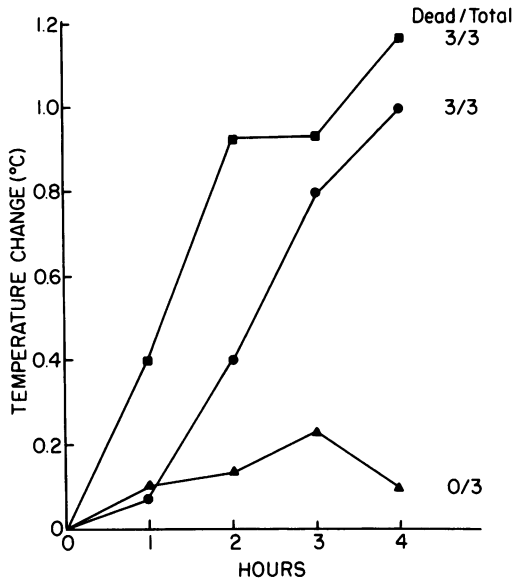

FIG. 5. Pyrogenicity and enhancement of rabbit susceptibility to lethal endotoxin shock. The mean rectal temperature rise of rabbits (three per group) was monitored after intravenous administration of Ent C1 (5 μg/kg) (■), an equal molar amount of Trp F1 (3.8 μg/kg) (●), or the same volume of diluent (▲). After 4 h, endotoxin (10 μg/kg) was administered intravenously, and the animals were observed for 72 h for death.

cantly reduced Ent C1-induced mitogenesis ($P < 0.05$). One MAb (2F8G5) that reacted with the 15,663-dalton fragment in immunoblot assays also partially neutralized mitogenesis induced by native Ent C1. This suggests that Trp F1b contributes to the mitogenic activity. It is unclear why complete neutralization was not achieved, since in a previous report we found that many MAbs produced against the native toxin could completely neutralize mitogenesis (10). Anti-Trp F1 neutralizing MAbs probably recognize an epitope that has been modified as a result of trypsin digestion. Such antibodies would thus be expected to react weakly with the native toxin and also be less effective at neutralizing biological activity. Similarly, anti-Ent C1 MAbs reacted only weakly with Trp F1 in immunoblots (data not shown).

Immunological properties of Trp F1. Several assays were performed to assess cross-reactive antigenic epitopes on Trp F1. No significant cross-reactivity was observed between anti-Trp F1 polyclonal antibodies or MAbs and SPE A, SPE C, or toxic shock syndrome toxin 1 (data not shown). However, rabbit anti-Trp F1 antiserum reacted strongly with Ent C1 and Ent B in immunoblots (Fig. 6). Three anti-Trp F1 MAbs cross-reacted with Ent B in the ELISA (Table 1). Some cross-reactivity was also observed in immunoblot assays (Fig. 6). One MAb (4B2H5), which reacted strongly with Ent B and Ent C1 in the ELISA, also reacted with the same two toxins in immunoblots. All other MAbs reacted with Ent C1 only.

TABLE 1. Effect of anti-Trp F1 MAbs on the mitogenicity of Ent C1[a]

MAb	Isotype[b]	ELISA titer[c] against:		Incorporation of [^{3}H]thymidine, 10^3 cpm ± SEM (*P*)
		Ent C1	Ent B	
2F8G5	IgG1	512,000	<500	87 ± 12 (<0.003)
4B2H5	IgG1	64,000	32,000	134 ± 13 (−)[d]
3C12D10	IgA	8,000	8,000	121 ± 7 (<0.05)
1E11B6	IgM	1,000	<500	122 ± 7 (<0.05)
3B8C9	IgM	32,000	32,000	117 ± 4 (<0.02)
Control[e]		<500	500	146 ± 16

[a] MAbs were partially purified from ascites by precipitation with $(NH_4)_2SO_4$, dissolved, and adjusted to an equivalent protein concentration (800 μg/ml). *P* indicates the statistical significance of mitogenesis inhibition by MAb compared with that after preincubation in control ascites. Analysis was by Student's *t* test. Results are the means of four tests. Ent C1 was added at 1.0 μg.
[b] Immunoglobulin class.
[c] Titer in mouse ascitic fluid.
[d] No statistically significant inhibition ($P > 0.1$).
[e] Control was prepared from pooled ascites induced by X63-Ag8.653 cells.

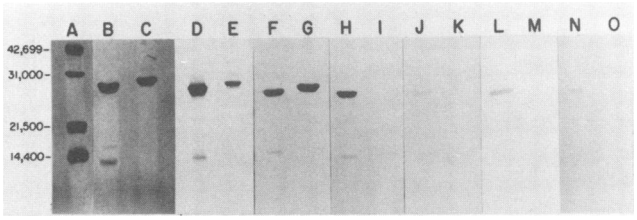

FIG. 6. SDS-PAGE and immunoblot analysis of Ent C1 and Ent B. Ent C1 (lanes B, D, F, H, J, L, and N) and Ent B (lanes C, E, G, I, K, M, and O) (20 μg/lane) were electrophoresed and stained with Coomassie blue (lanes B and C) or transferred to nitrocellulose and probed with polyclonal Trp F1 rabbit antiserum (lanes D and E) or MAbs 4B2H5 (lanes F and G), 2F8G5 (lanes H and I), 3C12D10 (lanes J and K), 1E11B6 (lanes L and M), and 3B8C9 (lanes N and O). Molecular masses of protein standards (lane A) are given in daltons.

Several other investigators have demonstrated immunological cross-reactivity between Ent C1 and Ent B by using a variety of methods (10, 22, 30, 44, 45, 47). It was previously proposed that the N-terminal regions of Ent B and Ent C1 contain the antigenic determinants primarily responsible for reactivity with heterologous antibodies, whereas the C termini induce the formation of homologous antibodies (44). However, Thompson et al. (47) produced MAbs that cross-reacted with Ent B and Ent C1; the cross-reactive antibodies did not bind to N-terminal toxin fragments but did react with C-terminal polypeptides. The results of this present study are in agreement with the latter report, since Trp F1 possesses at least one cross-reactive epitope. Only one anti-Trp F1 MAb cross-reacted strongly with Ent B in immunoblot assays. The lack of reactivity by some of the other MAbs was probably due to denaturation of the epitope during SDS-PAGE, since two additional antibodies cross-reacted with Ent B in the ELISAs.

The biological activities demonstrated for the C-terminal Trp F1 in this study include pyrogenicity, mitogenicity, and enhancement of endotoxin shock. These three activities are among those possessed by all staphylococcal and streptococcal PTs. Ent A and Ent B also retain biological activity after limited digestion with trypsin, although these activities have not been conclusively mapped to specific peptides (31, 42). Taken together with sequence homology studies, these combined results suggest that in studies of shared biological activities and, in some cases, immunological cross-reactivity, the conserved domains in the C-terminal regions of other PTs should be examined.

ACKNOWLEDGMENTS

This work was supported by Public Health Service grant HL 36611 from the National Institutes of Health and by the Kimberly-Clark Corp. G.A.B. was supported by a fellowship from the American Heart Association, Minnesota Affiliate.

We thank Lisa Jensen for typing the manuscript and Tim Leonard for art and photographic work. The N-terminal amino acid sequence

analysis was performed by Selene Swanson at the University of Minnesota Microchemical Facility.

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