# Mitogenicity of Formalinized Toxoids of Staphylococcal Enterotoxin B

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Staphylococcal enterotoxin B is a potent mitogen for mouse and human lymphocytes. Mitogenic activity was retained after detoxification of the enterotoxin by formaldehyde at pH 5.0, 7.5, or 9.5. The most active toxoid (pH 7.5) was separated into a monomeric, a dimeric, and a polymeric fraction ($1 \times 10^5$ to $3 \times 10^5$ molecular weight) by gel filtration, and although each fraction demonstrated mitogenic activity, the polymeric fraction was clearly the most efficacious. These data show that mitogenicity of staphylococcal enterotoxin B does not depend on toxicity. This suggests that the mitogenic and toxic activities are effected by different sites on the molecule.

The staphylococcal enterotoxins are simple proteins elaborated by certain strains of *Staphylococcus aureus*. Several antigenic variants have been recognized but all induce the same biological effect, namely, emesis and diarrhea in a limited number of mammalian species (1). Peavy et al. (7) demonstrated that enterotoxin B (SEB) was a potent mitogen for mouse and human lymphocytes, and Greaves et al. (5) showed that human T cells from tonsil tissue responded well to SEB whereas B cells responded weakly. It has recently been found that enterotoxins A and $C_1$ were as effective mitogens as SEB (8a), demonstrating that the mitogenic activity of the staphylococcal enterotoxins is independent of their antigenic determinants. It has not been established, however, whether there is a relation between the toxic and the mitogenic activities. This study was undertaken to determine whether there are common conformational elements in the sites on these proteins that induce these two biological responses. Since SEB is a well-characterized low-molecular-weight protein, it seems possible that the specific part of the molecule responsible for its mitogenic properties could be determined. We report here on the effect of formaldehyde inactivation of the toxin on its mitogenic activity. It was found that detoxification does not destroy the mitogenic site of the molecule.

## MATERIALS AND METHODS

The isolation of SEB (8) and the preparation of formalinized toxoids (9) from it have been described. Toxoids were prepared at three pH values, 5.0, 7.5, and 9.5, and the products are designated, respectively, 5.0-fSEB, 7.5-fSEB, and 9.5-fSEB. For use in lymphocyte stimulation tests, the preparations were dialyzed exhaustively against phosphate-buffered saline, centrifuged for 5 min at $500 \times g$ to remove gross aggregates, filtered through 0.2-$\mu$m filters (Sybron Corp., Rochester, N.Y.), and concentrated to approximately 3 mg/ml (Kjeldahl N) by ultrafiltration over a UM-2 membrane (Amicon Corp., Lexington, Mass.). Concanavalin A was obtained from Pharmacia Fine Chemicals, Piscataway, N.J.

Stimulation of mouse spleen cells (strain C57BL/6) was tested in microtiter plates by the method of Holmgren et al. (6) except that $10^6$ cells in a final volume of 250 $\mu$l were incubated for 44 h under 5% $CO_2$-95% air and then pulsed with 1 $\mu$Ci of [$^3$H]thymidine (specific activity, 1.9 Ci/mmol; Schwarz/Mann, Orangeburg, N.Y.) for 4 h. Cell viability was evaluated by the trypan blue exclusion test. Blast transformation was determined by light microscopy of May-Grünwald-Giemsa-stained cell populations (3). In this series of experiments $2 \times 10^6$ spleen cells in a final volume of 1.0 ml were incubated in sealed polypropylene tubes in air.

## RESULTS AND DISCUSSION

All three formalinized SEB preparations have been demonstrated to be nontoxic in monkeys (9). No emesis or diarrhea was observed when 5 mg of each toxoid, equivalent to at least $10^4$ times the intravenous mean effective dose, was injected subcutaneously into rhesus monkeys. Their mitogenic efficacy for mouse spleen cell stimulation is shown in Fig. 1. Although the toxoid preparations were less active than native SEB, all were mitogenic. The order of their mitogenic activity was 7.5-fSEB > 5.0-

fSEB > 9.5-fSEB; however, only rarely did the 7.5-fSEB stimulate thymidine uptake to the same extent as SEB. In most instances the optimal concentration of 7.5-fSEB gave about two-thirds the level of mitogenic activity of native SEB, and considerably greater amounts of the toxoid were required to reach this level. The results shown in Table 1, from comparative experiments using SEB, 7.5-fSEB, and concanavalin A, demonstrate that the degree of blast transformation and the viability of the cells in culture were essentially equivalent for the two forms of the enterotoxin, whereas concanavalin A appeared to stimulate blast transformation sooner and to a greater degree.

It has been shown that the formalinization of SEB at pH 7.5 brings about extensive polymerization (9). However, membrane filtration used as a final step in the preparation of the toxoids



FIG. 1. *Mitogenic activity of SEB and formalinized SEB toxoids. Each point is the mean of six replicates. Symbols:* ●, *SEB;* ○, *7.5-fSEB;* □, *5.0-fSEB;* ▲, *9.5-fSEB.*

removed the very high-molecular-weight aggregates, and indeed, it has now been found that 7.5-fSEB passed completely through an XM-300 Amicon filter, indicating that all the particles in the preparation were less than $3 \times 10^5$ molecular weight. The 7.5-fSEB was further fractionated by gel filtration through Sephadex G-100 to determine the effect of molecular size upon mitogenicity. Three fractions were obtained corresponding to a monomer, a dimer, and a broad peak at the void volume containing particles $>1 \times 10^5$ molecular weight. These molecular sizes were verified by polyacrylamide gel electrophoresis in sodium dodecyl sulfate. All three fractions when assayed for in vitro cell stimulation were mitogenic with the largest molecules showing the greatest mitogenicity. The effect of varying the time of treatment with formaldehyde from 3 days to 4 weeks upon the distribution and mitogenicity of the three fractions was also investigated. Elution patterns up to 2 weeks were virtually identical, but 4-week preparations contained significantly less monomeric and dimeric material. Again in each case the polymeric fraction was the most active and, when the monomer and dimer represented a significant proportion of the total mass, it was more potent than the parent toxoid. A typical dose response curve for a polymeric fraction is shown in Fig. 2. The activity of these fractions was unchanged for at least 1 month.

The inferior mitogenic activity of the monomeric and dimeric components of 7.5-fSEB was associated with a total lack of activity of both in the quantitative precipitin test against rabbit anti-SEB serum. This is consistent with the mitogenic and serological activity of the toxoid prepared at pH 9.5 which is almost completely monomeric (9). As described here, 9.5-fSEB is the least mitogenic of the three toxoid preparations; it was previously demonstrated to be com-
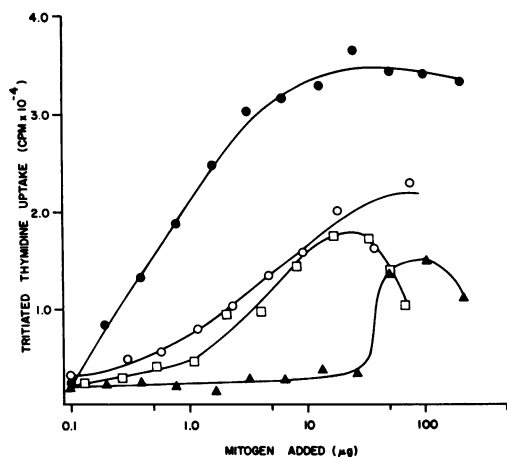
TABLE 1. *Effects of SEB, 7.5-fSEB, and concanavalin A (Con A) on mouse spleen cells*

| Determination[a] | Mitogen | | | |
|---|---|---|---|---|
| | None | SEB (11 μg) | 7.5-fSEB (86 μg) | Con A (6 μg) |
| [³H]thymidine incorporated[b] | 1,144 ± 230 | 20,172 ± 1,552 | 12,475 ± 3,140 | 25,256 ± 4,362 |
| % Viability at 48 h | 72 | 74 | 67 | 75 |
| % Blast transformation | | | | |
| At 48 h | 11 | 27 | 26 | 65 |
| At 72 h | 13 | 63 | 64 | 90 |

[a] Each value is based on four replicate samples. The results shown represent the peak mitogenic dose response for each mitogen.

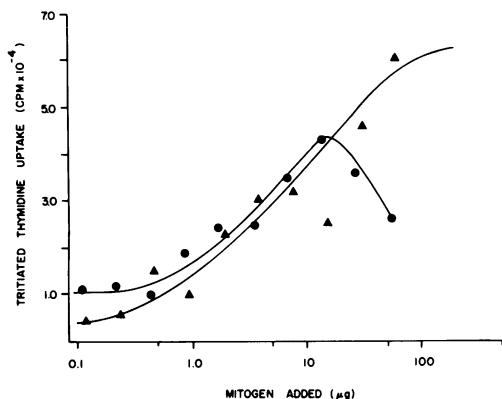[b] Expressed as mean counts per minute ± standard error of the mean.

FIG. 2. *Mitogenic activity of SEB and the polymeric fraction of a 2-week preparation of 7.5-fSEB isolated by gel filtration. Symbols:* ●, *SEB;* ▲, *polymeric fraction of 7.5-fSEB.*

pletely devoid of serological activity (9). It is likely that the intramolecular cross-links that predominate in the monomeric species of toxoid are highly disruptive of surface structure and responsible for these phenomena.

A significant finding in this study concerns the possible relationship between toxicity and mitogenicity of SEB. Despite extensive chemical changes and complete loss of toxic activity, all three toxoid preparations interact with mouse lymphoid cells and induce mitogenesis. Therefore, the mitogenic site of SEB must represent a surface element distinct from those conformational features that are responsible for its primary toxic biological activity. However, although not involved in toxicity per se, the mitogenic site may nevertheless play a role in toxicity. It could be that the generation of toxicity is a two-step process: interaction with a membrane receptor followed by translocation either within the membrane or across the membrane into the cytoplasm to affect the appropriate enzyme system(s). An example of the first possibility is provided by cholera toxin, which after binding to a ganglioside purportedly moves to and then activates membrane adenylcyclase (2). The second possibility is illustrated by the action of diphtheria toxin in which one fragment of the toxin binds to the cell and the other then passes through the membrane and inactivates an elongation factor (4).

A further important consideration in studies of this nature is the problem of comparing an in vitro effect of a substance with its in vivo activity. There is always the possibility that the biological activity of an altered protein may be due to different distribution, metabolism, or destruction in the organism rather than to a different interaction with the biologically active effector cell type. We believe nevertheless that loss of emetic activity of the toxoids of SEB is indeed a result of the destruction of toxic sites. It is significant that both polymeric and monomeric components in the 7.5-fSEB preparation are nontoxic. Furthermore this interpretation is supported by a large body of evidence on the inactivation of toxins (and viruses) by formaldehyde.

Further studies are underway to determine whether monomeric toxoid can effectively inhibit the mitogenic action of native SEB and whether polypeptide fragments of the enterotoxin are mitogenic. In this way it may be possible to outline in more detail the parts of the SEB molecule that are necessary for mitogenesis and what the mitogenic mechanism entails.

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