Mitogenic Stimulation of Murine Spleen Cells by Brief Exposure to \textit{Staphylococcus aureus} Enterotoxin B

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Staphylococcal enterotoxin B rapidly and firmly attached to external membrane surfaces of C57BL/6 spleen cells and, unless neutralized by specific antitoxin, committed the spleen cells to strong blastogenic responses.

\textit{Staphylococcus aureus} enterotoxins are polyclonal T-cell mitogens for murine and human lymphoid cells (2, 10). To obtain information as to the rate at which staphylococcal enterotoxin B (SEB) interacts with splenocytes, C57BL/6 spleen cells were exposed for 15 min or for 1, 2, 3, and 6 h to culture medium containing SEB, followed by incubation in medium without added SEB for an additional 50, 49, 48, 47, or 44 h, respectively. Also, splenocytes incubated for 6 h with added SEB were treated by specific antitoxin to determine whether biologically active toxin is localized to spleen cell surfaces or is internalized by the cells. Blastogenic responses at 50 h were measured as increases in the rate of deoxyribonucleic acid (DNA) synthesis.

\textbf{MATERIALS AND METHODS}

SEB and specific antitoxin. Highly purified SEB (11) was stored as a salt-free, lyophilized powder at 4°C. Concentrated stock solutions of SEB were freshly prepared before each experiment. Specific immune serum (antitoxin) obtained by intradermal injection of SEB in phosphate-buffered saline (pH 7.2) into New Zealand White rabbits was clarified by centrifugation at 415 \( \times g \), decomplemented by heating at 56°C for 40 min, and sterile filtered across a 0.22-\( \mu \)m Millex disk (Millipore Corp., Bedford, Mass.).

\textbf{Spleen cell cultures.} Spleen cells obtained from 7- to 14-week-old C57BL/6 male mice were suspended to a density of \( 2.5 \times 10^{9} \) ml in RPMI-1640 medium containing 25 mM \( N \)-2-hydroxyethyl piperazine-\( N' \)-2-ethanesulfonic acid (HEPES) buffer, penicillin (100 U/ml), and streptomycin (100 \( \mu \)g/ml) (Grand Island Biological Co., Grand Island, N.Y.). Culture replicates were established by adding 80 \( \mu \)l of variably diluted SEB stock concentrate or RPMI-1640 medium without added SEB, 20 \( \mu \)l of heat-inactivated fetal calf serum, and 900 \( \mu \)l of the spleen cell suspension to polypropylene tubes (12 by 75 mm; Falcon Plastics, Oxnard, Calif.). The tubes were tightly sealed and maintained at 37°C in a humidified incubator. Some of the cultures were interrupted at various times during the first 6 h of incubation by centrifugation at 415 \( \times g \). The supernatant solutions were then discarded, and the cell pellets were drained of remaining medium by blotting the mouths of the inverted polypropylene tubes on absorbent tissue and were suspended by gentle vortexing in the culture medium described for each of the reported experiments. All cultures were pulsed with 1.0 \( \mu \)Ci of tritium-labeled thymidine (2.0 Ci/mmol; New England Nuclear Corp., Boston, Mass.) after 46 h of incubation and terminated at 50 h by an isotonic saline wash. Radioactive spleen cell DNA was precipitated with a 10% trichloroacetic acid solution at 4°C overnight, and the acid-insoluble precipitates were trapped on glass-fiber filters (no. 25; Schleicher & Schuell, Inc., Keene, N.H.) positioned with a vacuum. The filters were washed with chilled phosphate-buffered saline and transferred to scintillation vials, and the precipitates were digested at room temperature (22 to 24°C) for 16 h with NCS solubilizer (Amer sham/Searle, Arlington Heights, Ill.). Toluene containing 2,5-diphenyloxazole (5 g/liter) and 1,4-bis-[2-(5-phenyloxazolyl)]benzene (0.5 g/liter) was added to the vials, and radioactivity was determined as counts per minute in a liquid scintillation spectrometer. Mean values and standard errors of the mean counts per minute were calculated for replicate spleen cell cultures, and the stimulation index was calculated as the ratio mean counts per minute of cultures incubated with SEB/mean counts per minute of cultures incubated without SEB. The dead-cell count of 50-h cultures determined by the trypan blue exclusion method was normally below 20%.

\textbf{RESULTS AND DISCUSSION}

C57BL/6 mouse spleen cells suspended in culture medium containing a mitogenic dose of SEB demonstrate increased DNA synthesis only after the first 24 h of incubation (9). To determine whether the delayed induction of DNA synthesis represented a requirement for prolonged contact of spleen cells with SEB in culture medium, the cells were removed at varying times from medium containing SEB and placed into fresh medium without added SEB. All cultures were terminated after 50 h of incubation. A 6-h exposure of spleen cells to medium containing 1 or 30 \( \mu \)g of SEB per ml stimulated DNA synthesis to the same extent as the entire 50-h exposure to SEB-containing culture medium (Fig. 1). Each pellet obtained from spleen cells incubated in medium containing SEB was...
carefully drained before suspension in medium without added SEB, and the amount of added SEB remaining with each pellet by carry-over was thereby diluted at least 100-fold. The stimulation of C57BL/6 spleen cells by SEB is strongly dose dependent in this toxin concentration range (Fig. 1) (11); thus, the stimulation of spleen cells exposed to 1 or 30 μg of SEB per ml for 6 h cannot be explained by the small amount of carry-over SEB. Furthermore, repeated washing (three to eight times) of the splenocytes by RPMI-1640 medium after a 6-h incubation with 30 μg of SEB per ml did not diminish blastogenic responses at 50 h by more than 40%. Incubation in culture medium containing SEB for periods shorter than 6 h had progressively less effect on spleen cell DNA synthesis (Fig. 1). Nevertheless, significant stimulation of DNA synthesis was noted for spleen cells exposed to SEB-containing medium for only 15 min at either 37 or 4°C (Table 1). It is clear, therefore, that SEB added to in vitro cultures of C57BL/6 splenocytes reacts rapidly with the cells and that the delayed induction in DNA synthesis must be due to factors other than slow interaction of toxin with spleen cells.

Short treatment of C57BL/6 spleen cells with SEB antitoxin alone after a 6-h incubation with added SEB reduced stimulation of DNA synthesis by 94% (Table 2). In contrast, spleen cells washed five times with RPMI-1640 medium after a 6-h incubation with added SEB gave strong blastogenic responses after further incubation without added toxin. Spleen cell cultures treated with antitoxin (Table 2) and subsequently incubated with 45 μg of SEB per ml demonstrated a stimulation index of 7.4 relative to the antitoxin control. Furthermore, previous work in this laboratory has established that a 48-h incubation of C57BL/6 spleen cells with normal rabbit serum at a dilution inhibitory with specific SEB antitoxin has no effect on SEB-stimulated lymphoid cell DNA synthesis (11). Consequently, the blocking action of SEB antitoxin in this experiment cannot be attributed to nonspecific inhibitory effects of antitoxin treatment on SEB-responsive cells and/or cell-bound SEB. Since antibodies do not penetrate intact mammalian cells (5), it appears that SEB is localized predominantly to the external membrane surfaces of C57BL/6 spleen cells during

### Table 1. Stimulation of C57BL/6 spleen cells by a 15-min exposure to SEB

<table>
<thead>
<tr>
<th>Treatment</th>
<th>[³H]thymidine incorporation&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
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<tbody>
<tr>
<td>Time</td>
<td>Temp (°C)</td>
</tr>
<tr>
<td>15 min&lt;sup&gt;b&lt;/sup&gt;</td>
<td>37</td>
</tr>
<tr>
<td>37</td>
<td>10,721 ± 287</td>
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<tr>
<td>50 h&lt;sup&gt;c&lt;/sup&gt;</td>
<td>37</td>
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<sup>a</sup> Mean counts per minute ± standard error, based on four replicate cultures. Values for the stimulation index are given in parentheses.

<sup>b</sup> Cultures were incubated at the indicated temperatures for 15 min with or without added SEB and then suspended in RPMI-1640 medium supplemented with 2% fetal calf serum but without added SEB for a 50-h incubation at 37°C.

<sup>c</sup> Cultures were incubated for 50 h with or without added SEB.

### Table 2. Inhibition of SEB stimulation of C57BL/6 spleen cells by specific antitoxin

<table>
<thead>
<tr>
<th>Treatment</th>
<th>[³H]thymidine incorporation&lt;sup&gt;a&lt;/sup&gt;</th>
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<tbody>
<tr>
<td>No antitoxin&lt;sup&gt;d&lt;/sup&gt;</td>
<td>Control</td>
</tr>
<tr>
<td>7,444 ± 2,330</td>
<td>47,312 ± 7,089 (6.4)</td>
</tr>
<tr>
<td>Antitoxin&lt;sup&gt;d&lt;/sup&gt;</td>
<td>9,736 ± 2,173</td>
</tr>
<tr>
<td>Repeated washing&lt;sup&gt;e&lt;/sup&gt;</td>
<td>10,289 ± 733</td>
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<sup>a</sup> Cultures were incubated for 6 h with or without SEB and centrifuged at 415 × g, and the cell pellets were treated at 6 to 8 h of incubation as indicated. The cultures were again centrifuged at 415 × g, and each pellet was suspended in 1.0 ml of RPMI-1640 medium supplemented with 2% fetal calf serum but without added SEB and without specific antitoxin for incubation at 37°C.

<sup>b</sup> Mean counts per minute ± standard error, based on four replicate cultures. Values for the stimulation index are given in parentheses.

<sup>d</sup> Each culture was suspended in 1.0 ml of RPMI-1640 medium at 6 to 8 h.

<sup>e</sup> Each culture was washed with 200 μl of SEB antitoxin at 6 to 8 h.
the first 6 h of incubation and is therefore accessible for neutralization by specific antitoxin. There was no evidence for stimulation of SEB-treated spleen cells by antigen-antibody complexes over the 3-log concentration range of SEB antitoxin studied (Fig. 2). The failure of SEB-antitoxin complexes to stimulate nonimmune lymphocytes to produce the lymphokine macrophage migration inhibition factor has also been demonstrated by Kaplan (8). In addition, the absence of lymphocyte stimulation by antigen-antibody complexes formed with concanavalin A or Phaseolus vulgaris phytohemagglutinin has been reported (6, 13). It appears, therefore, that antigen-antibody complexes of polyclonal mitogens do not activate nonimmune populations of lymphocytes.

Staphylococcal enterotoxin has been shown to stimulate the secretion of macrophage migration inhibition factor by guinea pig lymph node cells (8), to increase the release of prostaglandin type E by C57BL/6 spleen cells (1), and to suppress humoral antibody responses of C57BL/6 spleen cells to heterologous erythrocytes (10). All of these in vitro activities (1, 8, 10) could participate in immunologically induced inflammatory responses. Enterotoxicogenic staphylo-

coci commonly produce purulent infection in humans (3, 4, 7, 12). The brief exposure times to SEB in ambient medium required for in vitro spleen cell activation indicate that mitogenic enterotoxin released into interstitial tissue fluid could rapidly localize to the plasma membranes of inflammatory mononuclear cells and thereby modulate immune responses to toxigenic staphylococci at sites of infection.

LITERATURE CITED