Enterotoxin Lacks Superantigenic Activity but Induces an Interleukin-6 Response from Human Peripheral Blood Mononuclear Cells

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We investigated the potential superantigenic properties of Clostridium perfringens enterotoxin (CPE) on human peripheral blood mononuclear cells (PBMC). In contrast to the findings of a previous report (P. Bowness, P. A. H. Moss, H. Tranter, J. I. Bell, and A. J. McMichael, J. Exp. Med. 176:893–896, 1992), two different, biologically active preparations of CPE had no mitogenic effects on PBMC. Furthermore, PBMC incubated with various concentrations of CPE did not elicit interleukin-1, interleukin-2, gamma interferon, or tumor necrosis factor alpha or beta, which are cytokines commonly associated with superantigenic stimulation. However, CPE did cause a dose-related release of interleukin-6 from PBMC cultures.

Clostridium perfringens enterotoxin (CPE) is traditionally recognized as a virulence factor responsible for the diarrheal and cramping symptoms associated with C. perfringens type A food poisoning (21, 22). Recent studies (20–22, 30) indicate that CPE-induced food poisoning involves the following sequence of events: (i) CPE is produced in the small intestine by sporulating C. perfringens and then initiates a series of biochemical events that alter the normal permeability of brush border membranes in small intestinal epithelial cells; (ii) these CPE-induced permeability changes become cytotoxic and cause localized tissue damage; (iii) this leads to a breakdown in normal fluid and electrolyte transport properties and hence to diarrhea (20, 21).

Recently, CPE has been associated with a number of other human and veterinary diseases in addition to food poisoning (20, 21). For example, it has been suggested (19, 26) that CPE may be associated with some cases of sudden infant death syndrome (SIDS). While little is known about the triggering mechanism(s) for SIDS-related death (25), some investigators have advocated that bacterial toxins, especially those with superantigenic properties, and toxin-induced cytokines may play a role in at least some cases of SIDS (18, 19, 29, 31). In this respect, it is noteworthy that CPE is highly lethal in animals when given intravenously (31).

Potential insights into CPE-induced death were revealed in a recent study suggesting that CPE has superantigenic activity (1). If this is true, CPE should induce lymphocyte proliferation (3, 4, 12, 14, 16, 34) and increase the levels of lymphocyte-specific cytokines in vitro (5, 7, 14, 16, 24, 34). To test this prediction, we used CPE purified from C. perfringens (23) and recombinant Escherichia coli DH5α that carry the full-length cpe gene cloned into a pTrcHis vector (10). The purity and biological activity of both CPE preparations were confirmed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and a Vero cell cytotoxicity assay, as described previously (23). Additionally, the full-length recombinant CPE species used in this study has recently been characterized in detail (10) and shown to possess, on a molar basis, the same toxicity as native CPE purified from C. perfringens. Further, it was also demonstrated (10) that under non-denaturing conditions, polyclonal anti-CPE immunoglobulin G (IgG) shows equivalent reactivity with this full-length recombinant CPE species as with native CPE purified from C. perfringens, strongly suggesting that this recombinant toxin maintains a conformation very similar to native CPE.

Various concentrations of CPE (10, 100, or 1,000 ng/ml) purified from either C. perfringens or E. coli (as specified) were incubated for 48 or 120 h with human peripheral blood mononuclear cells (PBMC) that had been purified by Ficoll-Hypaque (Sigma Chemical Co., St. Louis, Mo.) density gradient centrifugation. PBMC (2 × 10^6/ml) were pulsed with [3H]thymidine (5 μCi/ml) during the last 5 h of CPE treatment and harvested, and the incorporated radioactivity was determined by liquid scintillation and represented as counts per minute ± standard error of the mean (SEM).

CPE purified from C. perfringens failed to stimulate the proliferation of PBMC after 48 h (Table 1) or 120 h (data not shown), although a pronounced mitogenic effect was observed when the same cells were similarly incubated with known bacterial superantigens like Staphylococcus aureus toxic shock syndrome toxin 1 (TSST-1) or staphylococcal enterotoxin B (SEB; Toxin Technologies, Sarasota, Fla.). The results shown in Table 1 were confirmed in two independent laboratories (T.K. and B.F.) with eight human donors. Furthermore, experiments also revealed that recombinant CPE did not induce PBMC proliferation (data not shown). The inability of CPE-treated PBMC to incorporate [3H]thymidine was not due to CPE-induced cell death, as evidenced by exclusion of trypan blue dye at the end of the proliferation experiments. Additionally, the failure of CPE to stimulate PBMC could not be explained by the presence of an inhibitor in our preparation of CPE purified from C. perfringens, since a polyclonal mitogen like

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phagocyte proliferation in the experiments presented in Table 1 was an unexpected result. Although Bowness et al. reported that a number of Vβ-specific lymphocytes were putatively stimulated by CPE, the most reactive were lymphocytes bearing Vβ6.9 and Vβ22 (1). Since Vβ6.9- and Vβ22-expressing lymphocytes commonly occur in the general population (1, 6), it is unlikely that the different conclusions about CPE superantigenicity could be explained by the absence of reactive lymphocytes in any of the eight donors of our PBMC preparations. However, experiments were performed to confirm the presence of these putatively reactive lymphocytes in our PBMC preparations. Flow cytometry analysis of lymphocytes from two randomly chosen donors from Table 1 plus another individual not involved in our proliferation study revealed that lymphocytes expressing Vβ22 represented 2.6 ± 0.7% of the total PBMC population among all three donors. These experiments were performed with purified human PBMC (3 × 10^6 per sample) incubated for 4°C for 30 min with anti-Vβ22 IgG1 (ImmunoTech, Westbrook, Maine) diluted in Hanks’ balanced salt solution containing 2% fetal calf serum plus 0.02% sodium azide. The cells were then washed twice in diluent and incubated at 4°C for 30 min with goat anti-mouse IgG conjugated to fluorescein isothiocyanate (Becton Dickinson, San Jose, Calif.). After being washed, the relative proportion of Vβ22 expressing cells among the total PBMC population was determined with a FACSan instrument (Becton Dickinson). A background level of each PBMC preparation was established by substituting a nonspecific mouse IgG1 as the first antibody. The relative percentage of Vβ22-positive cells in our PBMC populations was determined by subtracting the background (<10% of signal) from the signal readings. Although similar analysis for Vβ6.9 could not be performed because a specific immunoreagent is not commercially available, confirmation of cells expressing Vβ22 in our PBMC samples was sufficient to establish that the lack of superantigenicity from our CPE preparations was not simply due to the absence of reportedly reactive lymphocytes (1).

To confirm our conclusion from the proliferation experiments that CPE is not a superantigen, one of us (T.K.) also examined the cytokine levels in 20-h culture supernatants from five different PBMC (10^6/ml) preparations that had been incubated with different concentrations of CPE (1 ng to 1 μg/ml) purified from C. perfringens. No significant levels of cytokines such as tumor necrosis factor alpha (TNF-α), interleukin-1 (IL-1) and IL-2, or gamma interferon (IFN-γ), which are typically associated with superantigen stimulation of PBMC (5, 7, 16, 24, 34), were detected in the supernatants of CPE-treated PBMC cultures. In these experiments, levels of IL-1, TNF-α, TNF-β, IL-4, IL-6, and IL-10 were measured by an enzyme-linked immunosorbent assay (5, 15, 16, 32, 33, 37), while IFN-γ and IL-2 concentrations were determined by cell assays (13, 32). Each cytokine assay had a detection limit of 20 pg/ml with recombinant cytokines used as standards. In contrast to our results with CPE, supernatants from PBMC cultures similarly incubated with the bacterial superantigen S. aureus TSST-1 (100 ng/ml) contained significant levels of TNF-α (1,022 pg/ml), IL-1 (121 pg/ml), IL-2 (14 U/ml), and IFN-γ (1,583 U/ml) (16). Another laboratory (D.L.S.), which incubated PBMC from two additional donors for longer periods (72 h) with our CPE preparation (0.1, 1, or 10 μg/ml) purified from C. perfringens, independently determined that CPE did not stimulate TNF-β or IL-2 production under these conditions. Furthermore, it was also shown that IL-4 and IL-10, which downregulate IL-1, TNF, and IFN-γ production (28), were not present in 20-h PBMC cultures incubated with this CPE preparation purified from C. perfringens. However, when PBMC cultures were similarly incubated with PHA (2 μg/ml), significant concentrations of IL-4 (200 pg/ml) and IL-10 (1,400 pg/ml) were evident, confirming the ability of our assays to detect these cytokines in supernatants from stimulated PBMC.

Although various cytokines commonly associated with well-characterized bacterial superantigens were not elicited by CPE purified from C. perfringens, this enterotoxin produced increased levels of IL-6 from PBMC, even at a low concentration (1 ng/ml) (Fig. 1). The higher concentrations of IL-6 did not result from endotoxin contamination of our purified CPE preparation, since IL-1 and TNF-α, which are induced by endotoxin levels as low as 10 pg/ml (2, 11, 35), were absent from our PBMC culture supernatants. Furthermore, 1 μg of purified CPE prepared from C. perfringens contained only 28 pg of endotoxin, as determined by a Limulus amebocyte lysate assay (BioWhittaker, Walkersville, Md.).

The finding that CPE can induce IL-6 production in vitro could be physiologically important for understanding the lethal effects of CPE administered intravenously or intraperitoneally, since elevated levels of this cytokine in serum have a predictive value for fatal septic shock in mice (9, 17) and humans (36). Our current finding that CPE can induce IL-6 in vitro is also interesting in light of reports that intestinal epithelial cells also

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**TABLE 1. Proliferation assays of human PBMC stimulated by CPE**

<table>
<thead>
<tr>
<th>Addition</th>
<th>Dose (ng/ml)</th>
<th>Activity (cpm)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td></td>
<td>230 ± 87</td>
</tr>
<tr>
<td>CPE</td>
<td>10</td>
<td>199 ± 54</td>
</tr>
<tr>
<td>CPE</td>
<td>100</td>
<td>405 ± 69</td>
</tr>
<tr>
<td>CPE</td>
<td>1,000</td>
<td>239 ± 20</td>
</tr>
<tr>
<td>PHA</td>
<td>4,000</td>
<td>19,531 ± 2,378</td>
</tr>
<tr>
<td>CPE + PHA</td>
<td>10 + 4,000</td>
<td>19,946 ± 299</td>
</tr>
<tr>
<td>CPE + PHA</td>
<td>100 + 4,000</td>
<td>19,287 ± 1,060</td>
</tr>
<tr>
<td>CPE + PHA</td>
<td>1,000 + 4,000</td>
<td>19,478 ± 2,614</td>
</tr>
<tr>
<td>TSST-1</td>
<td>100</td>
<td>21,506 ± 5,021</td>
</tr>
<tr>
<td>SEB</td>
<td>100</td>
<td>22,713 ± 3,155</td>
</tr>
</tbody>
</table>

* Values represent the mean and SEM of triplicate samples from one donor and are representative of experiments (48 h) done with PBMC from eight different individuals. These data were generated with CPE purified from C. perfringens.

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**FIG. 1. Production of IL-6 from PBMC incubated with different concentrations of CPE purified from C. perfringens.** Results represent the mean values and SEM of three experiments with PBMC from three donors.
produce IL-6 (27). Thus, production of proinflammatory cytokines such as IL-6 by intestinal epithelial cells and monocytes-macrophages may be stimulated by CPE in vivo. This response could, in theory, contribute to the gastrointestinal effects characteristic of many CPE-associated illnesses. While animal studies strongly suggest that intestinal tissue damage appears necessary for the initiation of CPE-induced fluid loss (30), leukocyte infiltration is often noted in rabbit ileal loops after several hours of CPE exposure (8). Since C. perfringens food poisoning typically persists for several hours, and non-foodborne CPE-associated illnesses often continue for days or even weeks, inflammation and cytokine production could possibly contribute to the diarrheal and cramping symptoms frequently described in patients.

In summary, our study shows that CPE does not exhibit “typical” superantigen behavior by widely accepted criteria; i.e., CPE does not cause lymphocyte proliferation or elicit cytokines commonly associated with superantigenic activity. An explanation for the discrepancy between our conclusions and those of a previous study (1), suggesting that CPE is a superantigen, is not obvious. However, it is clear that these differences cannot be explained by the possibility that CPE variants were used with different superantigenic properties, since the CPE used in both studies apparently was purified from the same strain of C. perfringens, NCTC 8239.

At least two plausible explanations exist for the different conclusions about CPE superantigenicity. First, as conceded by the authors of the previous study (1), their CPE preparation may have become contaminated with another molecule that has superantigenic properties. Their reported purity of CPE by electrophoretic analysis does not rule out a possible contaminant, given that superantigens are active at very low concentrations. The converse possibility, i.e., that we inactivated the putative superantigenic properties of our CPE preparation during purification, seems less likely, considering that (i) neither purified CPE preparation from C. perfringens or recombinant E. coli exhibited superantigenic activities, although both toxin preparations possessed high levels of accepted biological activities attributed to CPE, including cytotoxicity, enterotoxicity, and lethality (data not shown); and (ii) we used the same purification method as the previous study to purify CPE from C. perfringens.

Alternatively, it is possible that the contradictory conclusions from our study and that previously published (1) resulted from the different experimental approaches used to assess superantigenicity. Only one assay for superantigenicity, i.e., examining the proliferation of human PBMC incubated with different concentrations of CPE, was shared by both studies. Upon further examination, it is noteworthy that the previous study (1) concludes that CPE induces lymphocyte proliferation based upon results obtained with PBMC from a single, possibly anomalous, donor. In contrast, our conclusion that CPE does not induce PBMC proliferation is based upon results from two independent laboratories with PBMC cultures prepared from eight different donors. Finally, it is clear that the different conclusions about CPE-induced proliferation of human PBMC cannot be explained simply as a dose effect, since overlapping CPE concentrations were used in these experiments.

To confirm our PBMC proliferation results indicating that CPE lacks superantigenicity, we used another standard assay for determining superantigenicity (i.e., measuring whether CPE treatment affects cytokine profiles from PBMC cultures, as expected for a superantigen). In contrast, the previous study (1) relied upon less standard techniques, including a cytolyis assay and an anchored PCR assay to confirm the results from their PBMC proliferation experiments. As with the proliferation experiment, positive results from the cytolyis assay in the previous study (1) were based on data from only one donor. We contend that the PBMC of different donors should have been analyzed to confirm the hypothesis that CPE is, or is not, a superantigen.

Conceivably, the anchored PCR assay, while potentially very sensitive, also may not have generated definitive results. For example, the increased mRNA levels for CPE-treated Vβ6.9 and Vβ22 lymphocytes detected in the previous study may not be directly correlated with increased levels of these proteins on the cell surface. The use of anchored PCR to study the T-cell receptor repertoire of superantigen-stimulated lymphocytes is not commonly used for determining superantigenicity, and so the reliability of this assay for this purpose remains unclear. Although CPE was considered a superantigen in the study by Bowness et al. (1), it is extremely difficult to reconcile how CPE could be a superantigen without reproducibly inducing PBMC proliferation and cytokine profiles that are indicative of other well-characterized bacterial superantigens (3–5, 7, 12, 14, 16, 24, 34).

Finally, while our current data strongly suggest that CPE is not a superantigen by conventional criteria, further studies are necessary to determine what role, if any, the CPE-induced increases in levels of the proinflammatory cytokine IL-6 observed in our study may play in sequelae associated with CPE-induced diarrhea and disease.

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