Suppression of Murine Lymphocyte Mitogen Responses by Exopolysaccharide from *Capnocytophaga ochracea*

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An extracellular polysaccharide was purified from culture supernatants of *Capnocytophaga ochracea* 25, a gram-negative bacillus associated with human periodontal disease. The extracellular polysaccharide suppressed in vitro mitogenic responses of murine splenic lymphocytes to concanavalin A and lipopolysaccharide. This suppression was dose dependent, persisted up to 120 h, and was not caused by direct toxicity of the extracellular polysaccharide.

Modulation of immune responses by bacterial components has received considerable attention in recent years (1, 2, 4, 5). Since a number of these substances are produced by members of the normal bacterial flora, they may have considerable impact on host-parasite interactions. Indeed, there are several diseases of microbial etiology, both clinical and experimental, which have been associated with immune suppression or enhancement (reviewed in reference 12).

A consequence of the immunomodulatory action of bacterial products may be human periodontal disease. Bacteria are essential agents in the etiology of this disease; however, the progress of the lesion has been attributed to altered host responses to bacterial products which may penetrate gingival tissue (10). Recently, much attention has been given to identifying the bacteria associated with periodontal disease and characterizing host responses to these organisms (reviewed in reference 10); however, little attention has been given to the immunomodulatory potential of these bacteria. Recently, sonicated cells of *Actinobacillus actinomycetemcomitans*, a gram-negative bacterium implicated in certain forms of periodontal disease, were shown to suppress human lymphocyte activation in vitro (14). The authors suggested that such immunosuppressive agents may act in vivo by enhancing the pathogenicity of their parent microorganism or that of some other opportunistic pathogen.

In this investigation an extracellular polysaccharide (EP) was purified from culture supernatants of *Capnocytophaga ochracea* 25, a gram-negative bacterial species found in the human oral cavity and associated with certain forms of periodontal disease (3, 7, 15). The modulatory effect of EP on murine splenic lymphocyte responses to the mitogens concanavalin A (ConA) and lipopolysaccharide (LPS) was evaluated in vitro.

C. *ochracea* 25, a gift from S. S. Socransky, Forsythe Dental Center, Boston, was cultivated at 37°C in Trypticase soy broth supplemented with 1% yeast extract and 0.1% NaHCO₃. After 48 h the cultures were centrifuged at 10,000 × g for 15 min at 4°C. EP was isolated from liquid culture supernatants by cold 95% ethanol precipitation. The precipitate was dissolved in water and treated with cold 10% trichloroacetic acid for 2 h, dialyzed against water, and reprecipitated with ethanol. The ethanol precipitate was dissolved in 0.05 M Tris buffer (pH 7.2) and further purified by Sephadex G-100 gel filtration. Fractions were analyzed for carbohydrate by the anthrone method (16) and for protein by the BioRad protein assay. Purified EP was hydrolyzed in 1 N HCl for 4 h at 100°C. The hydrolysates were dried in vacuo over P₂O₅ and NaOH pellets. Monosaccharides in the reconstituted hydrolysates were determined by thin-layer chromatography with silica gel 60 (EM Laboratories, Elmsford, N.Y.) and n-butanol–95% ethanol–water (52:32:16, three ascends). Purified EP was examined for the presence of endotoxin by the *Limulus* amoebocyte lysate test. Pyrogen-free water was used for the preparation of all reagents used in this investigation. The antigenic purity of the Sephadex G-100 column fraction was evaluated by agar-gel immunodiffusion against rabbit anti-C. *ochracea* 25 serum prepared by subcutaneous injection of whole cells mixed with incomplete Freund adjuvant.

Spleen cells from C57BL/6 mice (aged 5 months) were cultured at a concentration of 2 × 10⁶ cells per ml of RPMI 1640 medium supplemented with gentamycin sulfate (100 µg/ml), L-glutamine (2 mM), and 10% fetal calf serum, as described previously (2). Optimal concentrations of ConA (0.25 µg per culture) (Pharmacia Fine Chemicals, Piscataway, N.J.) or LPS (7 µg per culture) (*Escherichia coli* 055:B5; Difco Lab-
oratories, Detroit, Mich.) were added in 0.1-ml volumes to the appropriate tubes, and the cultures were incubated for 72 h or as indicated. During the last 4 h of incubation, each culture received a 1-μCi pulse of either methyl-[3H]thymidine (TdR) (specific activity, 2 Ci/mmol), [3H]uridine (specific activity, 5 Ci/mmol), or [3H]leucine (specific activity, 10 Ci/mmol) (all from New England Nuclear Corp., Boston, Mass.) for evaluation of DNA, RNA, and protein syntheses, respectively. The radioactivity of trichloroacetic acid-precipitable material was evaluated by liquid scintillation, and counting efficiency was determined by channels ratio.

Modulation experiments were performed by diluting EP in culture medium (see the figure legends) and adding 0.1 ml of each dilution to triplicate spleen cell cultures at various times before or after the addition of LPS or ConA; however, the elapsed time from addition of mitogen to culture termination remained constant except in the kinetics study, when cultures were terminated at 24-h intervals. A control preparation extracted from the bacterial culture medium was evaluated similarly. Results were compared with controls which contained only LPS or ConA. The effect of EP alone on murine spleen cell activity was evaluated by incubating various concentrations of EP with the cells in the absence of LPS or ConA for 48 h, with a pulse of radiolabeled precursor during the final 4 h. Representative cultures were then evaluated for uptake of labeled precursor, and others were examined for viability by trypan blue dye exclusion.

C. ochracea produced 5.2 g of EP per 100 g of cells (dry weight). EP eluted from Sephadex G-100 as a single peak immediately after the void volume. This fraction produced a single band against rabbit anti-strain 25 serum in agar-gel immunodiffusion. Preliminary analyses revealed this material to be a polysaccharide, with mannose as a major constituent (80%). Protein was present at a concentration of 27 μg/mg of EP. Our preparation was free of endotoxin at the concentrations used in this investigation, since 1 mg/ml produced a negative Limulus test. A complete chemical analysis of EP will be forthcoming. The extraction procedure for EP was used on uninoculated culture medium and produced a precipitate, which was used as a control in the lymphocyte stimulation experiments.

EP produced a dose-dependent suppression of murine spleen cell responses to the T lymphocyte mitogen ConA (Fig. 1). DNA, RNA, and protein syntheses were suppressed to similar degrees, with 80% suppression occurring at 40 μg of EP per culture. EP also produced significant but less marked suppression of radiolabeled precursor uptake in LPS-stimulated cultures (Fig. 2). The time at which EP was added to the cell cultures in relation to that of mitogen determined the degree of suppression (Fig. 3). The most significant suppression of both the LPS and ConA responses was observed when the cells were preincubated with EP for 30 min. EP added to the cultures after LPS had little effect on stimulation; however, suppression of ConA responses remained significant, although diminished, when EP was added afterward. The control preparation extracted from bacterial culture medium produced no significant effects on lymphocyte responses when used at the same concentrations as EP.

EP did not mediate the observed suppression of cell activation by delaying the response. Cultures preincubated with 10 μg of EP before LPS or ConA was added and harvested at 24-h intervals revealed a continual increase in suppression up to 120 (Fig. 4). Suppression was not caused by toxic effects produced by EP, as cell viability did not significantly differ from controls during the incubation times and at the EP concentrations employed in this investigation. EP in the absence of LPS or ConA produced no significant
FIG. 2. Effect of EP on DNA, RNA, and protein syntheses in murine spleen cells preincubated with EP for 30 min before the addition of an optimal mitogenic dose of LPS. Results are plotted as a percentage of radiolabeled precursor incorporation compared to cultures that received LPS only. Each point represents the mean value ± the standard error for triplicate assays from two experiments. Incorporation in cells receiving LPS alone averaged 99,921, 5,026, and 2,284 dpm for [3H]TdR, [3H]uridine, and [3H]leucine, respectively.

FIG. 3. Effect of pre- and post-mitogen exposure of cells to EP (20 µg per culture). Cultures were incubated for 72 h after the addition of LPS or Con A regardless of the time at which EP was added. Results are plotted as a percentage of [3H]TdR incorporation in cultures receiving LPS or Con A alone and represent the mean ± the standard error for six assays. Incorporation of [3H]TdR averaged 59,812 dpm in cultures receiving Con A alone and 59,474 dpm in those receiving LPS alone.

nate explanations include EP binding to cell surface glycoproteins, with subsequent inhibition of B cell activation by interferene with essential membrane conformational changes, or

FIG. 4. Kinetics of [3H]TdR incorporation in murine spleen cell cultures preincubated with 10 µg of EP for 30 min before the addition of either LPS or Con A. A pulse of [3H]TdR was added during the final 4 h. Results are plotted as a percentage of radiolabeled precursor incorporation compared to cultures that received LPS or Con A alone and represent the mean ± the standard error for triplicate assays.

changes in viability or in DNA, RNA, and protein syntheses in 24-h cultures (Table 1).

The suppression of lymphocyte responses to Con A may have resulted from the interference of EP with the binding of Con A to lymphocyte surfaces. Supportive of such a hypothesis are our observations that the greatest suppression occurred when the cells were preincubated with EP before the addition of Con A, that the suppression persisted, and that EP alone produced no significant effects on cell viability or on DNA, RNA, or protein synthesis. Binding inhibition by EP is further supported by the significant levels of mannose found in our preparation, since α-D-mannose is known to inhibit Con A binding and mitogenicity (13).

The EP-mediated suppression of mitogenic responses to LPS was probably not the result of direct inhibition of LPS binding to cell surfaces. LPS interactions with lymphocytes involve a nonspecific lipid A-membrane lipid binding, which can be inhibited with phospholipid (6), as opposed to Con A binding to cell surface glycoproteins, which is inhibitable by α-D-mannose (13). Thus, it would be unlikely that EP could interfere with LPS-lymphocyte binding. Alter-
TABLE 1. Effect of EP on the uptake of radiolabeled precursors by murine spleen cells in vitro

<table>
<thead>
<tr>
<th>EP conc (µg)</th>
<th>Incorporation (dpm) (mean ± SE)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>[³H]Tdr</td>
</tr>
<tr>
<td>0</td>
<td>5,819 ± 756</td>
</tr>
<tr>
<td>5</td>
<td>6,000 ± 685</td>
</tr>
<tr>
<td>10</td>
<td>6,247 ± 117</td>
</tr>
<tr>
<td>20</td>
<td>6,196 ± 473</td>
</tr>
<tr>
<td>40</td>
<td>5,416 ± 387</td>
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</tbody>
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* Per 2 × 10⁵ cells. Cell viability remained greater than 85% at all concentrations used after 48 h of incubation.

EP inhibition of responses to LPS by interference with the generation of regulatory T cells (8, 9).

An alternate site of action for the immunomodulatory effects of EP may be the macrophage. EP may interfere with production of immunoregulatory substances (reviewed in reference 18) and, in the ConA experiments, with presentation of the mitogen to T lymphocytes (11). The results presented here are inconclusive with regard to cellular adherence and the site of action of EP. Cell separation and EP adherence experiments are in progress and should clarify the mechanism of action.

It may be hypothesized that EP-mediated immunosuppression is an experimental artifact resulting from alteration of the natural biological properties of EP by the trichloroacetic acid treatment. Our preliminary studies would refute such an argument, since we initially used crude ethanol precipitates of spent culture fluid and found them to be immunosuppressive (unreported data). In addition, we have found that further purification of EP enhances its immunosuppressive properties as well as its reactivity in agar-gel immunodiffusion. The validity of our observations is further supported by the failure of extracts from uninoculated culture fluid to produce immunosuppression.

Components of the *Capnocytophaga* species found in the oral cavity may elicit a complex series of immunological events, some of which may contribute to periodontal disease. The presence of LPS (17), an immunostimulatory agent (5), and immunosuppressive EP in the same species poses a considerable challenge in dissecting the influence this organism may have on the immune system. Our preliminary studies indicate that EP can also suppress human lymphocyte responses, thus lending credence to the hypothesis that *Capnocytophaga* species may contribute to periodontal pathology by causing altered immune reactions to substances entering the gingival tissue.

LITERATURE CITED