Biological Response Modifier Activity of an Exopolysaccharide from *Paenibacillus jamilae* CP-7

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An extracellular polysaccharide was purified from culture supernatants of *Paenibacillus jamilae* CP-7, a gram-positive bacillus that was isolated from compost prepared with olive mill wastewaters. The extracellular polysaccharide was produced under aerobic conditions in a medium containing olive mill wastewaters (80% [vol/vol]). This exopolymer had a low level of acute toxicity when it is administered to BALB/c mice by the intraperitoneal route. Interesting immunomodulatory effects were detected when mice were given 10 mg of exopolysaccharide per kg of body weight; the proliferative responses of splenocytes to B-cell and T-cell mitogens were suppressed, the in vitro levels of production of gamma interferon and granulocyte-macrophage colony-stimulating factor by unstimulated and lipopolysaccharide-stimulated splenocytes were enhanced, and the levels of resistance to the intracellular pathogen *Listeria monocytogenes* was increased in mice. Also, the exopolysaccharide was able to induce lymphocyte proliferation in vitro. We conclude that *P. jamilae* produces an exopolysaccharide with interesting immunomodulatory properties.

Microbial exopolysaccharides (EPSs) often show clearly identified properties that form the basis for a wide range of applications in food, pharmaceutical, petroleum, and other industries (32). Thus, several EPSs have been shown to possess immunological activities with potential pharmacological applications as biological response modifiers (BRMs). BRMs are agents that alter the normal immune response and whose mechanisms of action include induction of cytokines (29). Research on pharmacological applications of BRMs has led to development of both immunosuppressive and immunostimulating drugs that are effective in preventing the rejection of transplanted organs, for the treatment of some autoimmune diseases, as cancer immunotherapy, or as adjuvants for vaccine construction (14). Lentinan and other fungal glucans, yeast mannoproteins, and a number of bacterial EPSs have been identified as BRMs and have been found to have the ability to stimulate tumor rejection (for a review, see reference 40). In recent years, research has focused on the mechanisms of action of these compounds (12, 41), as well as on the discovery of new ones (7, 33).

Although polysaccharides are considered to be T-cell-independent antigens, a number of microbial EPSs are immunomodulators, with activities for T cells and macrophages (for a review, see reference 35). Polysaccharide A, a component of the capsular complex of *Bacteroides fragilis*, possesses mitogenic activity for T lymphocytes (6), and the production of interleukin-2 (IL-2) by CD4+ T cells appears to play an essential role in the in vivo immunomodulation by this EPS (37). A number of fungi and yeasts produce β-(1,3)-glucans with immunomodulatory properties (4, 35). Studies on the mechanisms of immunomodulation by a soluble derivative of β-(1,3)-glucan have shown that it has the ability to prime granulocytes and macrophages for enhanced cytokine release (30), reactive nitrogen intermediate production (11), and bactericidal capacity (39) in response to a secondary stimulus. In addition, this polymer modulates cytokine production by lymphocytes (31). Other levels of the immune response may be also affected by polysaccharides: mannanuronic an EPS from *Pseudomonas aeruginosa*, enhances natural cytotoxicity by increasing Fas ligand expression in NK cells (17). As a consequence of their BRM properties, a number of EPSs are able to induce resistance to bacterial infections in experimental models (20, 36), and some of them have been evaluated in clinical trials (3).

In the investigation described here, an EPS was purified from culture supernatants of a *Paenibacillus jamilae* strain growing on olive mill wastewaters under aerobic conditions, and the BRM properties of this EPS were evaluated.

**MATERIALS AND METHODS**

Production and isolation of EPSs. Strain CP-7 was isolated from compost prepared with olive mill wastewaters and was identified as *P. jamilae* on the basis of phenotypic and phylogenetic analyses and DNA-DNA relatedness studies (1).

The growth medium for seed cultures contained olive mill wastewaters (80% [vol/vol]), NH4Cl (0.1% [wt/vol]), and yeast extract (0.1% [wt/vol]); and the pH was adjusted to 7.0 before sterilization by autoclaving at 112°C. Bacteria were grown in this medium at 30°C for 48 h, and 10 ml of seed culture was transferred to a 300-ml preculture flask containing 90 ml of the same medium. The flasks were incubated on a shaker (120 rpm) at 30°C for 48 h, and each culture (100 ml) was used as an inoculum for 900 ml of a culture medium containing olive mill wastewaters (80% [vol/vol]) and NH4Cl (0.1% [wt/vol]). EPS was produced by bacteria during incubation at 30°C for 72 h in a 2.0-liter jar-fermentor (Biostat M; Braun-Biotec, Melsungen AG, Germany), with aeration provided by bubbling (850 ml/min) and agitation at 150 rpm. Bacterial cells were separated from the fermented broth by centrifugation, and the EPS present in the supernatant was precipitated by the addition of 2 volumes of cold ethanol. The precipitated material was collected by centrifugation, dissolved in distilled water, and dialyzed against distilled water. The EPS was purified by chromatography on a column of Sepharose CL-2B and elution with 50 mM phosphate (pH 7.0) containing 0.5 M NaCl. Carbohydrates and proteins were determined in the eluted fractions by the methods of Dubois et al. (13) and Bradford (5), respectively. The elution profile showed two EPS fractions with molecular masses of 500 kDa and >2,000 kDa,
The bacteria were counted by using a Petroff-Hausser chamber, and the phosphate-buffered saline (PBS) solution, washed twice, and resuspended in was kindly provided by M. De La Rosa (Hospital Virgen de las Nieves, Granada, Spain).

Cytokine assays. Spleen cells were cultured with LPS or ConA as described above, and supernatants were removed after 24 h for determination of IL-2 levels and after 72 h for determination of gamma interferon (IFN-γ) and granulocyte-macrophage colony-stimulating factor (GM-CSF) levels. Supernatants were stored at -20°C until they were assayed. Cytokines were quantified by enzyme immunoassays (Endogen, Cambridge, Mass.); the concentrations of IL-2, IFN-γ, and GM-CSF were interpolated from the standard curve for the appropriate recombinant cytokine.

Challenge with Listeria monocytogenes. A virulent isolate of L. monocytogenes was kindly provided by M. De La Rosa (Hospital Virgen de las Nieves, Granada, Spain). Bacteria were grown on blood agar at 37°C for 24 h, harvested in sterile phosphate-buffered saline (PBS) solution, washed twice, and resuspended in PBS. The bacteria were counted by using a Petroff-Hausser chamber, and the bacterial suspension was adjusted to infect 10^3 organisms into a mouse tail vein. The mice were observed daily, and deaths were recorded.

Statistical analysis. The differences between the treated and the control groups were analyzed by using Student’s t test. A P value of less than 0.05 was considered significant.

RESULTS

EPS production. The culture conditions used in the present study were determined in previous work as appropriate to obtain the maximal level of EPS production (26). After 72 h of incubation, the EPS yield was 5.5 g/liter. EPS was obtained as a water-soluble, white powder.

EPS toxicity. In the acute toxicity tests, no mortality was observed after intraperitoneal administration of 100, 10, and 1 mg of EPS per kg of body weight (data not shown). Mice that received 100 mg/kg showed a significant decrease in body weight at the first day after injection (P < 0.0001), but this effect dissipated on the second day (Fig. 1). When these animals were killed at 10 days after injection, significant splenomegaly was observed. For EPS dosages of 0, 1, 10, and 100 mg/kg of body weight, splenic indices (measured on day 10 after treatment and expressed as spleen weight [in grams] per 20 g of body weight) were 0.1016 ± 0.01024, 0.1080 ± 0.00707, 0.1072 ± 0.00563, and 0.1254 ± 0.01024 (P < 0.01), respectively (the results represent the means ± standard deviations for five mice). Each mouse received a single injection by the intraperitoneal route. Mice in the control group (0 mg/kg) were given sterile water. The mean values of the splenic index for treated mice represented 123% of the splenic indices for untreated controls (P < 0.01). No variations in body weight or splenomegaly were observed in mice treated with EPS doses of 10 or 1 mg/kg. For in vivo immunomodulation studies, the dosage of 10 mg/kg was selected on the basis of the lack of adverse effects in toxicity tests.

Effect of treatment with EPS on mitogen-induced responses of splenocytes. The capacity of splenocytes to proliferate in response to mitogens was assayed at days 1, 4, and 7 after single-dose EPS treatment. Results are shown in Fig. 2. EPS given 1 day before the assay suppressed 49% (P < 0.002) of the proliferation in response to LPS and 37% (P < 0.01) of the proliferation in response to ConA. Treatment of mice with EPS on day 4 before the assay was also suppressive: the response to LPS was decreased by 17% (P < 0.02), although the
response to ConA was not significantly suppressed. The administration of EPS on day 7 before the test did not modify spleen cell proliferation in response to either mitogen.

To determine whether the suppression of lymphoproliferative responses observed in cultures of spleen cells from EPS-treated mice was accompanied by changes in the production of cytokines, mice were killed on the first day after EPS administration, and the production of IL-2, IFN-\(\gamma\), and GM-CSF by unstimulated and LPS- or ConA-stimulated splenocytes was measured in vitro. The results are shown in Fig. 3. In the absence of mitogenic stimuli, the basal levels of IFN-\(\gamma\) and GM-CSF in cultures of splenocytes from untreated controls were higher than those in cultures of splenocytes from untreated controls (mean increases, 1.8- and 2.2-fold, respectively, with \(P\) values of <0.05 for both cytokines). LPS did not modify EPS-induced increases from basal levels of IFN-\(\gamma\) and GM-CSF production (mean increases, 2.1- and 2.0-fold, respectively, with \(P\) values of <0.05 for both cytokines). In contrast, ConA induced higher levels of GM-CSF secretion in cultures from untreated controls than in EPS-treated mice (\(P < 0.002\)). The production of IL-2 by unstimulated or mitogen-stimulated splenocytes was not affected by the treatment with EPS.

**Protective effect of EPS against experimental infection with *L. monocytogenes***. To determine whether EPS-induced modifications in splenocyte responsiveness could affect cell-mediated immunity and resistance against intracellular pathogens, mice were challenged with a lethal inoculum of *L. monocytogenes* on day 1 after EPS treatment. This bacterium causes a systemic infection in mice, and both activated T cells and macrophages are required to overcome infection (38). As shown in Fig. 4, 80% of the EPS-treated animals survived a challenge by the intravenous route with a dose of *L. monocytogenes* that was lethal for 100% of the control mice.

**Lymphocyte-proliferating activity of EPS**. To investigate the in vitro effect of EPS on lymphocytes, spleen cells from untreated mice were incubated with a range of EPS concentrations: 100, 10, and 1 \(\mu\)g/ml. The lymphoproliferation assay was performed as described above. Results from experiments that compared the mitogenic abilities of LPS, ConA, and EPS are presented in Fig. 5. An EPS concentration of 100 \(\mu\)g/ml elicited a proliferative response representing 60 and 37% of those elicited by LPS (2.5 \(\mu\)g/ml) and ConA (1 \(\mu\)g/ml), respectively. Little proliferation was observed at low EPS concentrations.

**DISCUSSION**

EPS can be produced by *P. jamilae* strain CP-7 in a medium containing olive oil wastewater (80%). Olive oil waste effluents are major pollutants in the Mediterranean area and represent a severe environmental problem because of its content of large
amounts of phenolic compounds, which have antimicrobial and phytotoxic activities (9, 10, 15, 22). Ramos-Cornenzana et al. (27) suggested that olive mill wastewater could be used as an alternative, low-cost substrate for EPS production because of its high carbon/nitrogen ratio, which is similar to that of standard media used for polysaccharide production. In the study described here, an EPS with interesting BRM properties was purified from culture supernatants of strain CP-7 grown in an olive oil wastewater-based medium.

EPS had a low level of acute toxicity: no adverse effects were observed in mice given a single dose of 10 mg/kg by the intraperitoneal route. However, this treatment caused a marked immunomodulation: there was an inhibitory effect on the proliferation of splenocytes to B-cell and T-cell mitogens (19). Furthermore, the level of production of GM-CSF by activated macrophages was decreased by EPS treatment, it is likely that activated macrophages contributed to increased levels of this cytokine in unstimulated or LPS-stimulated cultures of splenocytes from EPS-treated mice. The central role of GM-CSF in the differentiation of precursor cells into antigen-presenting cells (25) could also be important in the immunomodulation by EPS.

In conclusion, our results demonstrate the production of EPS with BRM properties by P. jamilae. Additional studies on the chemical characterization of this EPS are in progress.

FIG. 5. Lymphoproliferative activity of EPS. Splenocytes from untreated mice were incubated with control mitogens (LPS and ConA) or EPS (100, 10, and 1 µg/ml). The results are the means for three mice (with eight replicate wells per mitogen and per mouse). Error bars represent standard deviations.
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