Murine Macrophage Interleukin-1 Release by Capsularlike Serotype-Specific Polysaccharide Antigens of Actinobacillus actinomycetemcomitans

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Serotype-specific polysaccharide antigens (SPAs) were extracted from whole cells of Actinobacillus actinomycetemcomitans ATCC 29523 (serotype a), Y4 (serotype b), and NCTC 9710 (serotype c) by autoclaving and purified by chromatography on DEAE-Sephadex A-25 and Sephacryl S-300 columns. Y4 SPA induced interleukin-1 (IL-1) release by P388D1 murine macrophages. Polymyxin B had virtually no effect on the release of IL-1. Rabbit anti-murine IL-1 serum strongly suppressed the proliferation of C3H/HeJ mouse thymocytes induced with the culture supernatants of Y4 SPA-stimulated P388D1 cells and a submitogenic dose of concanavalin A. Gel filtration of the culture supernatants of Y4 SPA-stimulated macrophages on Sephacryl S-200 showed that an IL-1 peak at a point corresponding to approximately 16.5 kDa was eluted. The ability of SPAs from strains ATCC 29523 and NCTC 9710 to induce the release of IL-1 was lower than that of Y4 SPA. The IL-1-releasing ability of serotype a and c antigens was enhanced by deacetylation of both polysaccharides, suggesting that acetyl groups of these antigens might hinder the interaction between the antigens and macrophages.

Actinobacillus actinomycetemcomitans is a small gram-negative coccobacillus. The organism has been implicated as an etiologic agent in localized juvenile periodontitis (19, 23) and severe nonoral human infections (8).

Oral A. actinomycetemcomitans strains are serologically classified into three serotypes, a, b, and c (25). Serotype a and b are the main isolates from the human oral cavity, whereas serotype c is isolated with less frequency. Serotype b is isolated most frequently from patients with localized juvenile periodontitis (25). It has been shown that the immunodominant antigen of serotype b in periodontitis patients is a capsularlike serotype-specific antigen (5). However, the periodontopathic mechanism of this potent antigen of serotype b is unknown.

Amano et al. (1) extracted a serotype-specific polysaccharide antigen (SPA) from whole cells of A. actinomycetemcomitans Y4 (serotype b) by autoclaving and purified it by ion-exchange chromatography and gel filtration. They showed that the serotype b antigen is a polymer consisting of a disaccharide repeating unit, →3)-α-D-fucopyranosyl-(1→2)-β-L-rhamnopyranosyl-(1→7). We have recently found that the serotype a and c antigens are 6-deoxy-t-tal and 6-deoxy-t-talan, respectively (18). Both polysaccharides carry approximately one acetyl group per two sugar residues.

Clinically, localized juvenile periodontitis is characterized by alveolar bone loss mainly affecting the permanent first molars and incisors (24). Several monocyte products, such as interleukin-1 (IL-1), tumor necrosis factor, and pros-taglandins, are known to stimulate osteoclastic bone resorption (11). In this study, we compared the serotype b antigen with the serotype a and c antigens and lipopolysaccharide (LPS) from serotype b for their ability to induce IL-1 release by murine macrophages. These studies will give us information on the basis for the high periodontopathic potential of serotype b A. actinomycetemcomitans.

MATERIALS AND METHODS

Microorganisms. A. actinomycetemcomitans Y4 (serotype b) was mainly used in this study. In some experiments, A. actinomycetemcomitans ATCC 29523 (serotype a) and NCTC 9710 (serotype c) were used. These strains were grown in Todd-Hewitt broth (Difco Laboratories, Detroit, Mich.) supplemented with 1% (wt/vol) yeast extract at 37°C for 3 days in a 5% CO2 atmosphere (15). Organisms were harvested by centrifugation, washed three times with pyrogen-free water, and lyophilized.

Preparation of SPAs. SPAs were extracted from lyophilized cells of A. actinomycetemcomitans by autoclaving (1). The extracts were purified by chromatography on DEAE-Sephadex A-25 (Pharmacia Fine Chemicals, Piscataway, N.J.) and Sephacryl S-300 (Pharmacia) columns and lyophilized. All SPAs purified in this study contained more than 90% (wt/wt) carbohydrate and a trace amount of fatty acid. No protein, d-glycero-d-mannoheptose, l-glycero-d-mannoheptose, 2-keto-3-deoxyoctulosonic acid, ribose, deoxyribose, hexosamine, or phosphorus was detected. These materials from A. actinomycetemcomitans ATCC 29523, Y4, and NCTC 9710 were designated 29523 SPA, Y4 SPA, and 9710 SPA, respectively.

Deacetylation of SPAs. SPAs (2 mg each) were deacetylated with 1 ml of 0.1 M sodium hydroxide overnight at room temperature under a nitrogen stream. The solutions were deionized with Amberlite CG-120 (H+ form; Rohm & Haas Co., Philadelphia, Pa.) and lyophilized.

Preparation of Y4 LPS. LPS was extracted from lyophi-
lized cells of *A. actinomycetemcomitans* Y4 by the hot phenol-water procedure (20). The extract was treated with nuclease and washed extensively with pyrogen-free water by ultracentrifugation (12). The crude LPS was purified by chromatography on Sephadex G-200 (Pharmacia) equilibrated with 10 mM Tris hydrochloride (pH 8.0) containing 0.2 M NaCl, 0.25% (wt/vol) deoxycholate, 1 mM EDTA, and 0.02% (wt/vol) sodium azide (9). The purified material was dialyzed against pyrogen-free water, lyophilized, and used as Y4 LPS. No protein, ribose, deoxyribose, rhamnose, or fucose was detected in Y4 LPS.

**IL-1 production.** P388D1 murine macrophages were kindly provided by K. S. Akagawa and T. Tokunaga, National Institute of Health, Tokyo. The cells were cultured in RPMI 1640 medium (GIBCO Laboratories, Grand Island, N.Y.) supplemented with 10% (vol/vol) heat-inactivated fetal calf serum, penicillin (100 U/ml), and streptomycin (100 μg/ml) at 37°C in a humidified atmosphere of 5% CO₂ in air. The adherent cells were removed with a rubber policeman, washed with Hanks balanced salt solution (GIBCO), and suspended to a density of 10⁶ cells/ml in RPMI 1640 medium containing 10% (vol/vol) fetal calf serum and antibiotics. One milliliter of P388D1 cell suspension (10⁶/ml) was incubated in triplicate in a 24-well culture plate at 37°C in an atmosphere of 5% CO₂ in air for 3 h. Nonadherent cells were removed by repeated washings with Hanks balanced salt solution. Adherent cells were incubated in 1 ml of RPMI 1640 medium with or without stimulant. After incubation for 16 to 96 h, the cell-free supernatants were collected and centrifuged at 1,000 rpm for 10 min. After sterilization by passage through a filter (0.45-μm pore size; Millipore Corp., Bedford, Mass.), the supernatant samples were assayed for IL-1 activity. The effect of polymyxin B (Sigma Chemical Co., St. Louis, Mo.) on IL-1 release was examined as follows. P388D1 cells (10⁶) were cultured at 37°C for 48 h in 1 ml of RPMI 1640 medium containing various amounts of polymyxin B and Y4 LPS (6.25 μg) or Y4 SPA (50 μg), and then the IL-1 activity of the cell-free supernatants was determined as described below.

**IL-1 assay.** IL-1 activity was assayed by measuring the proliferative response of mouse thymocytes (18). In brief, thymocytes from C3H/HeJ mice were suspended to a density of 1.5 × 10⁶ cells/ml in RPMI 1640 medium containing antibiotics, 10% (vol/vol) fetal calf serum, and 15 mM HEPES (N-2-hydroxyethylpipperazine-N′-2-ethanesulfonic acid) buffer, pH 7.2. The cell suspension (0.1 ml) was seeded in triplicate into a 96-well microculture plate with an equal volume of serial dilutions of IL-1 samples and a submicrogram concentration of concanavalin A (ConA; 1 μg/ml; Sigma). Cultures were incubated in an atmosphere of 5% CO₂ in air for 72 h. They were pulsed for the final 18 h with 0.5 μCi of [³H]thymidine ([³H]TdR; 24 Ci/mmol; Amersham Corp., Buckinghamshire, England). After the labeling period, cells were harvested onto glass fiber filters by using a multiple-cell harvester. The radioactivity incorporated was assayed in a liquid scintillation counter. Data are expressed as the mean counts per minute ± standard deviation (SD) of [³H]TdR uptake in triplicate cultures.

For inhibition studies, normal rabbit serum, rabbit antimurine soluble IL-1 serum (13), or monospecific rabbit anti-murine recombinant IL-1α (rIL-1α) serum (Genzyme Corp., Boston, Mass.) was added to test cultures to give a final concentration of 5% (vol/vol). To examine whether SPAs and Y4 LPS exhibit IL-1-like activity, C3H/HeJ thymocytes (1.5 × 10⁶) were cultured for 72 h with various amounts of these stimuli in the presence or absence of ConA (1 μg/ml). [³H]TdR uptake by the thymocytes was determined as described above.

**Gel filtration of Y4 SPA-stimulated IL-1.** P388D1 cells (2 × 10⁶) were incubated at 37°C for 48 h in 200 ml of RPMI 1640 medium with Y4 SPA (50 μg/ml). Culture supernatants were combined with solid ammonium sulfate, resulting in 45% saturation. The mixture was centrifuged at 10,000 × g for 30 min, and the supernatant was brought to 65% saturation with ammonium sulfate. The precipitate was collected by centrifugation at 10,000 × g for 30 min, dissolved in 5 ml of 50 mM Tris hydrochloride, pH 7.5, containing 100 mM NaCl, and dialyzed against this buffer. The suspension (5 ml) was applied to a column (1.5 by 100 cm) of Sephacryl S-200 (Pharmacia) equilibrated with 50 mM Tris hydrochloride, pH 7.5, containing 100 mM NaCl. The column was eluted with this buffer. Fractions of 5 ml were collected at a flow rate of 15 ml/h. The fractions were dialyzed against phosphate-buffered saline (8.0 g of NaCl, 2.9 g of Na₂HPO₄·12H₂O, 0.2 g of KH₂PO₄, and 0.2 g of KCl per liter), sterilized by passage through a filter (0.22-μm pore size), and assayed for IL-1 activity. Molecular size standards (Pharmacia) included blue dextran 2000 (2,000 kDa), aldolase (158 kDa), bovine serum albumin (67 kDa), chymotrypsinogen A (25 kDa), and RNase A (13.7 kDa).

**Limulus amebocyte lysate clotting activity.** The colorimetric blood endotoxin determination reagent (Pyrodisc; Seikagaku Kogyo Co., Tokyo, Japan) was used for the measurement of *Limulus* amebocyte lysate clotting activity of Y4 SPA, Y4 LPS, and LPS from *Escherichia coli* O111:B4 (Sigma). The reagent (0.1 ml) was incubated for 30 min at 37°C with 0.1 ml (0 to 1 ng/ml) of sample. After the reaction was terminated by adding 1 ml of 0.6 N acetic acid, the A₅₀₅ was determined.

**RESULTS**

SPA and LPS were extracted from whole cells of *A. actinomycetemcomitans* Y4 (serotype b) by autoclaving and the hot phenol-water procedure, respectively, and purified by chromatography. The *Limulus* amebocyte lysate clotting activity of these purified materials was compared with that of LPS from *E. coli* O111:B4 by a colorimetric endotoxin determination. Both LPSs strongly activated the clotting enzyme (Fig. 1). On the other hand, Y4 SPA did not activate
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FIG. 2. IL-1 release by P388D1 cells stimulated with various amounts of Y4 SPA (A) or Y4 LPS (B). P388D1 cells (10⁶) were cultured in 96-well plates with various concentrations of Y4 SPA or Y4 LPS for 48 h. Culture supernatants at a 1:2 dilution were assayed for IL-1 activity. Data are shown as the mean ± SD for triplicate cultures. The experiments were performed five times, and similar results were obtained in each experiment.

FIG. 3. IL-1 activity in various dilutions of culture supernatants of P388D1 cells stimulated with Y4 SPA or Y4 LPS. P388D1 cells (10⁶) were cultured without addition (□) or with Y4 SPA (50 μg/ml) (○) or Y4 LPS (6.25 μg/ml) (●) for 48 h. Various dilutions of the culture supernatants were assayed for IL-1 activity. Data are shown as the mean ± SD for triplicate cultures. The experiments were performed three times, and similar results were obtained in each experiment.

FIG. 4. Time course of IL-1 release by P388D1 cells stimulated with Y4 SPA or Y4 LPS. P388D1 cells (10⁶) were cultured without addition (□) or with Y4 SPA (50 μg/ml) (○) or Y4 LPS (6.25 μg/ml) (●) for 16 to 96 h. Culture supernatants at a 1:2 dilution were assayed for IL-1 activity. Data are shown as the mean ± SD for triplicate cultures. The experiments were performed five times, and similar results were obtained in each experiment.

were 50 and 6.25 μg/ml, respectively (Fig. 2). Maximal IL-1 activity in culture supernatants of P388D1 cells stimulated with Y4 SPA and Y4 LPS was detected after 48 to 72 h and 72 h, respectively, of culture (Fig. 4).

The effect of polymyxin B on the release of IL-1 by P388D1 cells stimulated with Y4 SPA and Y4 LPS was examined. The addition of polymyxin B to cultures markedly inhibited IL-1 release by Y4 LPS-stimulated P388D1 cells, but it had virtually no effect on the release of IL-1 by Y4 SPA-stimulated cells (Fig. 5).

To elucidate whether IL-1 induces the proliferation of C3H/HeJ thymocytes in culture supernatants of Y4 SPA-stimulated P388D1 cells in the presence of a suboptimal dose of ConA, anti-IL-1 sera were added to the IL-1 assay. Rabbit anti-murine soluble IL-1 and anti-murine rIL-1α sera

FIG. 5. Effect of polymyxin B on IL-1 release by P388D1 cells stimulated with Y4 SPA or Y4 LPS. P388D1 cells (10⁶) were cultured with Y4 SPA (50 μg/ml) (○) or Y4 LPS (6.25 μg/ml) (●) in the presence of polymyxin B (0 to 30 μg/ml) for 48 h. Culture supernatants at a 1:2 dilution were assayed for IL-1 activity. The proliferation (counts per minute per well) of C3H/HeJ thymocytes incubated with ConA (1 μg/ml) plus Y4 SPA-stimulated IL-1 and ConA (1 μg/ml) plus Y4 LPS-stimulated IL-1 was 5,947 ± 310 and 6,015 ± 983 cpm, respectively. The percent inhibition of IL-1 release was calculated by the following formula: % inhibition = 100 × [(cpm of stimulant) – (cpm of stimulant plus polymyxin B)/(cpm of stimulant)]. Data are shown as the mean ± SD for triplicate cultures. The experiments were performed four times, and similar results were obtained in each experiment.
TABLE 1. Inhibitory effects of anti-murine IL-1 sera on IL-1 activity

<table>
<thead>
<tr>
<th>Antiserum</th>
<th>Mean [3H]Tdr uptake (cpm) ± SD</th>
<th>% Inhibition*</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>7,907 ± 531</td>
<td>0</td>
</tr>
<tr>
<td>Normal rabbit serum</td>
<td>7,327 ± 624</td>
<td>7</td>
</tr>
<tr>
<td>Anti-soluble IL-1 serum</td>
<td>1,077 ± 289</td>
<td>86</td>
</tr>
<tr>
<td>Anti-IL-1α serum</td>
<td>756 ± 81</td>
<td>90</td>
</tr>
</tbody>
</table>

* P388D1 cells (10⁶) were cultured with Y4 SPA (50 μg/ml) for 48 h, and then the cell-free supernatants were collected. C3H/HeJ thymocytes (1.5 × 10⁶) were cultured in 0.2 ml of RPMI 1640 medium containing supernatant (0.1 ml) and ConA (1 μg/ml) in the presence or absence of antiserum (5%). Data are shown as the mean ± SD for triplicate cultures. The experiments were performed three times, and similar results were obtained in each experiment.

strongly suppressed the proliferation of C3H/HeJ thymocytes, but normal rabbit serum did not (Table 1). Crude IL-1 was prepared from culture supernatants of Y4 SPA (50 μg/ml)-stimulated P388D1 cells by 45 to 65% ammonium sulfate precipitation. The preparation was applied to a column of Sephacryl S-200. An IL-1 peak at a point corresponding to approximately 16.5 kDa was eluted (Fig. 6).

The ability of SPAs from whole cells of A. actinomycetemcomitans ATCC 29523 (serotype a) and NCTC 9710 (serotype c) to induce the release of IL-1 was compared with that of Y4 SPA. IL-1 activity in culture supernatants of P388D1 cells stimulated with 29523 SPA and 9710 SPA was lower than that in culture supernatants of Y4 SPA-stimulated cells (Fig. 7A). To examine the role of the acetyl groups of 29523 SPA and 9710 SPA in the release of IL-1, both SPAs were deacetylated with 0.1 M sodium hydroxide. Y4 SPA was also treated with sodium hydroxide. The ability of 29523 SPA and 9710 SPA to induce the release of IL-1 was enhanced by deacetylation (Fig. 7B). Both deacetylated antigens showed the same IL-1-releasing ability as Y4 SPA. Treatment of Y4 SPA with sodium hydroxide had no effect on IL-1-releasing ability.

To examine whether SPAs from A. actinomycetemcomitans exhibit IL-1-like activity, C3H/HeJ thymocytes were cultured with various amounts of SPAs or Y4 LPS without ConA and with ConA at 1 or 20 μg/ml. A mitogenic dose (20 μg/ml) of ConA strongly induced the proliferation of C3H/HeJ thymocytes (Table 2). On the other hand, neither Y4 LPS nor SPAs from three serotypes of A. actinomycetemcomitans induced the proliferation of C3H/HeJ thymocytes in the presence of absence of ConA (1 μg/ml).

DISCUSSION

IL-1 is a hormonelike cytokine secreted mainly by monocytes and macrophages (17). IL-1 plays a central role in the regulation of immunological and inflammatory reactions (17). In particular, IL-1 is known to induce osteoclastic bone resorption (11). In this study, we showed that capsularlike SPAs from A. actinomycetemcomitans strongly induced the release of IL-1 by P388D1 macrophages. Our preliminary experiments showed that the polysaccharide antigens also induced the release of IL-1 by BALB/c murine peritoneal macrophages (unpublished data). The ability of the serotype b-specific antigen to induce the release of IL-1 by P388D1 cells was higher than that of the serotype a- and serotype c-specific antigens. These findings suggest that the high periodontopathic potential of serotype b might be due to the
TABLE 2. Effects of SPAs and LPS from A. actinomycetemcomitans on proliferation of C3H/HeJ thyocytes in the presence and absence of a submitogenic dose of ConA

<table>
<thead>
<tr>
<th>Stimulant</th>
<th>Dose (µg/ml)</th>
<th>Mean [3H]TdR uptake (cpm) ± SD</th>
<th>Mean [3H]TdR uptake (cpm) ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ConA</td>
<td>20</td>
<td>16,444 ± 2,075</td>
<td>17,361 ± 1,427</td>
</tr>
<tr>
<td>Y4 LPS</td>
<td>50</td>
<td>650 ± 104</td>
<td>684 ± 115</td>
</tr>
<tr>
<td></td>
<td>0.5</td>
<td>807 ± 83</td>
<td>658 ± 71</td>
</tr>
<tr>
<td>Y4 SPA</td>
<td>50</td>
<td>590 ± 72</td>
<td>489 ± 62</td>
</tr>
<tr>
<td></td>
<td>0.5</td>
<td>380 ± 48</td>
<td>602 ± 98</td>
</tr>
<tr>
<td>29523 SPA</td>
<td>50</td>
<td>361 ± 106</td>
<td>647 ± 94</td>
</tr>
<tr>
<td></td>
<td>0.5</td>
<td>381 ± 103</td>
<td>369 ± 43</td>
</tr>
<tr>
<td>9710 SPA</td>
<td>50</td>
<td>596 ± 88</td>
<td>617 ± 92</td>
</tr>
<tr>
<td></td>
<td>0.5</td>
<td>376 ± 87</td>
<td>480 ± 27</td>
</tr>
</tbody>
</table>

a C3H/HeJ thyocytes (1.5 x 10⁶) were cultured in 0.2 ml of RPMI 1640 medium containing various amounts of stimulant in the presence or absence of ConA (1 µg/ml) for 72 h. They were pulsed for the final 18 h with 0.5 µCi of [3H]TdR. Data are shown as the mean ± SD for triplicate cultures. The experiments were performed twice, and similar results were obtained in each experiment.

ability of the serotype b-specific antigen to induce greater release of IL-1.

Our ¹³C and ¹H nuclear magnetic resonance analyses suggested that both the serotype a and serotype c antigens carry approximately one acetyl group per two sugar residues (18a). The present study showed that the ability of both polysaccharides to induce the release of IL-1 was markedly enhanced by their acetylation. The acetyl groups of these serotype antigens might hinder the interaction between the polysaccharides and macrophages.

It has been shown that LPS from A. actinomycetemcomitans can stimulate macrophages to secrete various inflammatory mediators, such as prostaglandins (6), IL-1 (16), and acid phosphatase (2). It is possible that the IL-1-releasing ability of the SPAs used in this study is the result of contamination with LPS. However, Y4 SPA did not exhibit Limulus amebocyte lysate clotting activity. The Y4 LPS-stimulated release of IL-1 was suppressed by polymyxin B, which is an inhibitor of lipid A, but the Y4 SPA-stimulated release of IL-1 was not. Moreover, d-glycerol-d-mannoheptose, L-glycerol-d-mannoheptose, and 2-keto-3-deoxyoctulosonic acid, which are characteristic components of LPSs from A. actinomycetemcomitans (4), were undetectable in all the SPAs used in this study. These findings indicate that contamination with LPS does not explain the ability of SPAs to stimulate the release of IL-1.

Among a variety of cytokines produced by murine macrophages, IL-6 as well as IL-1 is known to be active in the mouse thyocyte proliferation assay (3). In this study, anti-murine soluble IL-1 serum and monospecific anti-murine rIL-1α serum strongly inhibited the proliferation of C3H/HeJ thyocytes that were induced with culture supernatants of Y4 SPA-stimulated P388D1 cells in the presence of a submitogenic dose of ConA. The IL-1 and IL-6 produced by P388D1 cells were reported to be approximately 16 (10) and 23 (16a) kDa, respectively. Gel filtration of the culture supernatants of Y4 SPA-stimulated P388D1 cells showed that an active peak in the thyocyte proliferation assay was eluted at a point corresponding to approximately 16.5 kDa.

These results suggest that IL-1 in the culture supernatants induced the proliferation of C3H/HeJ thyocytes. It is, however, difficult to rule out the possibility that a small amount of IL-6 might be released by Y4 SPA-stimulated macrophages.

Wilson et al. (21) extracted a capsular material from whole cells of A. actinomycetemcomitans NCTC 9710 (serotype c) with cold saline. They demonstrated that the capsular material consists of both protein and carbohydrate in the ratio 2.2:1. The capsular material induces bone resorption in an in vitro mouse calvarial system and suppresses the synthesis of collagen and DNA in the system (21, 22). In addition, the material enhances the proliferation of mitogen-induced BALB/c mouse thyocytes, indicating that it exhibits IL-1-like activity (7). On the other hand, SPAs extracted from whole cells of A. actinomycetemcomitans by autoclaving did not contain protein and did not show any IL-1-like activity in the thyocyte proliferation assay. The IL-1-like activity of the capsular material from strain NCTC 9710 might be due to proteinaceous substances in the material. In this regard, Nishihara et al. (14) reported that A. actinomycetemcomitans Y4 produces extracellular proteinaceous mitogenic substances as well as LPS.

The serotype antigens extracted from the culture supernatants of A. actinomycetemcomitans have been characterized as mannos-containing polysaccharide antigens suggestive of mannans (26, 27). On the other hand, we showed that a serotype b-specific antigen extracted from whole cells of A. actinomycetemcomitans Y4 by autoclaving is composed of L-rhamnose and D-fucose (1). In addition, serotype a- and serotype c-specific antigens from whole cells of A. actinomycetemcomitans were demonstrated to be composed of 6-deoxy-d-talose and 6-deoxy-l-talose, respectively (18a). Further studies should clarify these differences in the chemical composition of serotype-specific antigens from A. actinomycetemcomitans.

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