Cytotoxic effect of *Aggregatibacter actinomycetemcomitans* Lipopolysaccharide on Human Leukemia Cell Lines and Human Gingival Fibroblasts

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Abstract

Aggregatibacter actinomycetemcomitans is implicated in the etiology of aggressive periodontitis and chronic periodontitis. Lipopolysaccharide (LPS) is a major constituent of the outer membrane of Gram-negative bacteria. LPS from *Aggregatibacter actinomycetemcomitans* (Aa-LPS) and *Escherichia coli* (Ec-LPS) was isolated by the hot phenol-water method and partially purified on a column. The purpose of this study was to compare the properties of Aa-LPS with Ec-LPS. Aa- and Ec-LPS showed different patterns in the results of SDS-PAGE. Furthermore, the total sugar and limulus activity of Aa-LPS were different from those of Ec-LPS. The cytotoxic effect of Aa-LPS was in a time- and dose-dependent manner. Aa-LPS had a strong cytotoxic effect on human leukemia cell lines and human gingival fibroblasts compared with Ec-LPS. These results suggested that the difference in structure, total sugar amount and limulus activity of Aa-LPS from those of Ec-LPS or the cell difference may participate in the cytotoxic effect.

Introduction

Aggregatibacter actinomycetemcomitans, a Gram-negative bacillus and a member of the oral microflora, is thought to play a major role in the development of aggressive periodontal disease, involving rapid gingival and alveolar bone destruction (1, 2). This bacterium produces several virulence factors, including the production of lipopolysaccharide (LPS), cytolethal distending toxin, a chaperonin and a leukotoxin (3, 4, 5, 6, 7, 8, 9). It is likely that leukotoxin plays an important role in *A. actinomycetemcomitans* pathogenesis by helping the bacterium evade host immune responses (5, 6). The lipopolysaccharide molecules of Gram-negative bacterial cell walls are the antigen (O antigen) as well as the source of endotoxic activity of these bacteria (10). LPS from *A. actinomycetemcomitans* (Aa-LPS) is a potent inducer of pro-inflammatory mediator, related with adverse pregnancy outcomes (11, 12), osteoclast activation (13), complement activation (14) and direct B cell activation (15). In this study, we investigated the cytotoxic effect of

Aa-LPS on human promyelocytic leukemia cells (HL60 cells), the human acute monocytic leukemia cell line (THP-1 cells) and human gingival fibroblasts (HGFs) compared with LPS from *Escherichia coli* (Ec-LPS).

Materials and Methods

Cell culture

HL60 cells, THP-1 cells (Cell Bank, Riken Bioresource Center, Ibaraki, Japan) and HGFs (obtained from the Department of Renascent Dentistry, Nihon University School of Dentistry at Matsudo, Chiba, Japan) were maintained in RPMI 1640 medium (Wako, Osaka, Japan) supplemented with 10% fetal bovine serum (FBS; Invitrogen, Carlsbad, CA) for 48 h at 37°C under a 5% CO₂ atmosphere. Before use, the medium was discarded and the adherent HGFs were harvested with Trypsin-EDTA (0.25 w/v% trypsin, 1 mmol L⁻¹ EDTA-4Na, Wako, Osaka, Japan) for 5 min at 37°C. HL60 cells, THP-1 cells and float HGFs were washed twice in phosphate-buffered saline (PBS; Wako, Japan) to remove the medium and then resuspended in PBS.

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Bacterial cultures and LPS preparation

A. actinomycetemcomitans strain Y4 and *E. coli* strain K12 were grown in brain-heart infusion broth supplemented with 1% yeast extract (Becton, Dickinson and Co., Sparks, MD) at 37°C in a 5% CO₂ atmosphere. LPS was extracted using the hot phenol-water method and subsequently treated with DNase 1, RNase A and proteinase K as described by Moran *et al.* (16). Furthermore, crude LPS was partially purified by Sephacryl S-200 HR (Amersham Pharmacia Biotech AB, Uppsala, Sweden) as described by Takada *et al.* (17). The protein concentration in LPS was measured in a standard protein assay (DC protein assay; Bio-Rad, Richmond, CA). The total sugar in LPS was determined by the phenol-sulfuric acid method (18). LPS was quantitated by the Toxin Sensor Chromogenic Limulus amoebocyte Lysate (LAL) endotoxin assay kit (GenScript, NJ, USA), according to the manufacturer's instructions. In brief, standards, samples and LAL reagent were dispensed into endotoxin-free vials; 1 mg ml⁻¹ Aa- and Ec-LPS were diluted prior to analysis. Activity (endotoxin units ml⁻¹; EU ml⁻¹) were determined by comparison to an *Escherichia coli* standard solution. Partially purified LPS (10 µg dry weight) was analyzed by 12.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). As a molecular weight marker, a dual-color, prestained Precision Plus protein standard (Bio-Rad Laboratories, Richmond, CA) was used. After electrophoresis, the gel was stained using a Silver Stain Kit (Wako, Osaka, Japan).

Measurement of cytotoxic effect of Aa-LPS at various doses

Measurement of the dose-dependent cytotoxic effect of Aa-LPS for each cell type was performed by broth dilution and calculation of the percent cell lysis. One milliliter of the reaction mixture, including 2×10^6 cells of each cell type and 0 to 500 µg dry weight of Aa-LPS in PBS, was incubated at 37°C for 30 min in an atmosphere of 5% CO₂ in air. After incubation, the reaction mixtures were placed on ice, 100 µl trypan blue (0.4%) was added, and surviving cells were counted in a hemocytometer. At least four fields were counted and cell numbers were averaged for each assay. Percent lysis was calculated by dividing the number of surviving cells by the number of cells in the negative control. The cytotoxic effect of Ec-LPS was examined as a comparison of Aa-LPS.

Measurement of cytotoxic effect of Aa-LPS at various times

The time-dependent cytotoxic effect of Aa-LPS for each cell was measured by calculating the percent of cell lysis. One milliliter of the reaction mixture, including 2×10^6 cells of each cell type and 500 µg dry weight of Aa-LPS, was incubated at 37°C for 0 to 45 min in an atmosphere of 5% CO₂ in air. Calculations were performed as described above.

Statistical analysis

The data are presented as the mean ± the standard deviation and compared using the two-tailed Student's t test. A P value of < 0.05 was considered significant.

Results

Characterization of Aa- and Ec-LPS

The amount of Aa-LPS obtained from 1L culture was almost one-third of *E. coli* (Table 1). The concentration of sugar in Aa- and Ec-LPS was 70.2 and 91.7 µg mg⁻¹, respectively; this difference was statistically significant ($P < 0.05$). The concentration of protein in Aa- and Ec-LPS was 43.9 and 39.8 µg mg⁻¹, respectively; The difference was not statistically significant ($P > 0.05$). Activities of Aa- and Ec-LPS were 9.7×10^6 and 7.3×10^6 EU mg⁻¹, respectively; this difference was statistically significant ($P < 0.05$). SDS-PAGE pattern of Aa-LPS showed strong silver staining only in the lower-molecular-weight region of the gel (Fig. 1A). On the other hand, the SDS-PAGE pattern of Ec-LPS by silver staining revealed progressive ladder-like bands up the gel (Fig. 1B).

Dose-dependent cytotoxicity of Aa-LPS against HL60 cells, THP-1 cells and HGFs

The dose-dependent cytotoxicity of Aa-LPS on each cell was examined. Aa- and Ec-LPS-untreated HL60 cells as a control showed 4.9% of cell lysis after 30 min incubation. HL60 cells treated with 500, 250, and 125 µg ml⁻¹ Aa-LPS accounted for 69.0%, 28.7% and 18.3% of cell lysis, respectively (Fig. 2A). HL60 cells treated with 500, 250, and 125 µg ml⁻¹ Ec-LPS accounted for 32.3%, 21.3% and 13.3% of cell lysis, respectively.

LPS-untreated THP-1 cells as a control showed 6.2% of cell lysis after 30 min incubation. THP-1 cells treated with 500, 250, and 125 µg ml⁻¹ Aa-LPS accounted for 92.2%, 70.2% and 55.3% of cell lysis, respectively (Fig. 2B). THP-1 cells treated with 500, 250, and 125 µg ml⁻¹ Ec-LPS accounted for 37.1%, 21.0% and 15.2% of cell lysis, respectively.

Table 1 Purification of Aa- and Ec-LPS from 1L culture

	dry weight (mg)	sugar (mg)	protein (mg)	limulus activity (EU)
Aa-LPS	11.4	0.8	0.5	1.1×10^8
Ec-LPS	32.7	3.0	1.3	2.4×10^8

Fig. 1 Silver-stained SDS-PAGE pattern of Aa-(A) and Ec-LPS (B). A 10 μ g amount of partially purified LPS was applied and stained by the silver staining method. M: molecular weight marker.

LPS-untreated HGFs as a control showed 7.8% of cell lysis after 30 min incubation. HGFs treated with 500, 250, and 125 μ g ml⁻¹ Aa-LPS accounted for 93.4%, 58.8% and 29.8% of cell lysis, respectively (Fig. 2C). HGFs treated with 500, 250, and 125 μ g ml⁻¹ Ec-LPS accounted for 27.3%, 24.3% and 14.7% of cell lysis, respectively. The cytotoxic effect of Aa-LPS on HL60 cells, THP-1 cells and HGFs was in a dose-dependent manner and stronger than Ec-LPS.

Time-dependent cytotoxicity of Aa-LPS against HL60 cells, THP-1 cells and HGFs

Fig. 3 shows the time-dependent cytotoxic effect of Aa-

LPS on HL60 cells, THP-1 cells and HGFs. Each surviving cell was decreased immediately and rapidly with Aa-LPS. Moreover, the cytotoxic effect of Aa-LPS on HL60 cells after 45 min incubation was almost 20% lower than on other cells. Each surviving cell treated with Ec-LPS decreased gradually and slowly with increasing incubation time. Moreover, the cytotoxic effects of Ec-LPS on HGFs at 45 min incubation were almost 15% lower than on other cells.

Discussion

It was reported that leukotoxin may play a role in host cell killing and immune evasion in vivo (5, 6). *A. actinomycetemcomitans* strain JP2 is well known to have high leukotoxicity (19); however, all *A. actinomycetemcomitans* which we isolated from patients with chronic and aggressive periodontitis had low leukotoxicity (data not shown); therefore, we thought that cell components of *A. actinomycetemcomitans* other than leukotoxin participated in periodontitis. We particularly focused on LPS of *A. actinomycetemcomitans*.

The cytotoxic effect of Aa-LPS and Ec-LPS occurred in a dose- and time-dependent manner. A high concentration of Aa-LPS showed strong and rapid cytotoxicity on each cell. The cytotoxic effect of Aa-LPS was stronger than Ec-LPS. Similar results were obtained in each cell type; however, when comparing the effect of Aa-LPS in each cell type, cell lysis of HL60 cells by Aa-LPS was lower than THP-1 cells and HGFs. It was thought that the cytotoxic effect of Aa-LPS depended on cell differences. In addition, it was thought that the cytotoxic difference between HL60 cells and HGFs depended on the difference between the cell line and primary cells. Furthermore, the cytotoxic effect of Ec-LPS was slightly different between the human leukemia cell line and HGFs ($P > 0.05$).

It is difficult to consider that the high concentration of Aa-LPS used in these experiments could accumulate in the oral cavity; however, it was suggested that Aa-LPS participates in periodontal disease by having shown a cytotoxic effect that was stronger than Ec-LPS.

Aa-LPS mainly consisted of lower-molecular-weight components. Moreover, the total sugar and limulus activity of Aa- and Ec-LPS were different. It was thought that structural differences between Aa-LPS and Ec-LPS or cell differences participated in cytotoxicity.

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Fig. 2 Dose-dependent effect of LPS on HL60 cells (A), THP-1 (B) cells and HGFs (C). Thirty min incubation with various concentrations of Aa- (white bar) and Ec-LPS (black bar). The results are expressed as the mean $\pm$ standard deviation of four independent experiments. Single asterisk indicates $P < 0.05$; double asterisk indicates $P < 0.01$.

Fig. 3 Time-dependent effect of LPS on HL60 cells (A), THP-1 (B) cells and HGFs (C). 500 μg ml⁻¹ LPS incubation for various times; ○, Aa-LPS; ●, Ec-LPS; △, PBS. The results are expressed as the mean $\pm$ standard deviation of four independent experiments. Single asterisk indicates significant difference from PBS ($P < 0.05$); double asterisk indicates significant difference from PBS ($P < 0.01$).

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