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Nuclease-Sensitive Binding of an *Actinobacillus actinomycetemcomitans* Leukotoxin to the Bacterial Cell Surface

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A leukotoxin of *Actinobacillus actinomycetemcomitans* 301-b was solubilized from cell-associated membrane vesicles by treatment with externally added DNase and RNase and was further purified by a procedure which included ammonium sulfate fractionation, gel filtration chromatography, and ion-exchange chromatography. The purified toxin had a molecular mass of 113,000 Da by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and a high isoelectric point (approximately 8.8). From these characteristics, it was to be expected that the membrane vesicle toxin was almost identical to the leukotoxin extracted with polymyxin B in an earlier study (C.-C. Tsai, B. J. Shenker, J. M. DiRienzo, D. Malamud, and N. S. Taichman, Infect. Immun. 43:700–705, 1984). The treatment with DNase and RNase was also highly effective for solubilizing the leukotoxin directly from whole cells, suggesting that the toxin is secreted extracellularly but retained in nucleic acids on the outermost surface of bacterial cells.

Actinobacillus actinomycetemcomitans is a gram-negative, facultatively anaerobic, small coccobacillus which has been implicated as a possible etiological agent in some severe types of human periodontitis (24, 30). The important virulence factor of this organism is a polypeptide toxin (leukotoxin) which specifically destroys human polymorphonuclear leukocytes and monocytes (28). The genes encoding the leukotoxin structural protein have been cloned (8, 13), and their nucleotide sequence have been determined (9, 12). The DNA sequence analyses indicate that the *A. actinomycetemcomitans* leukotoxin shares extensive homology with the well-characterized RTX cytotoxins, including *Escherichia coli* alpha-hemolysin and the *Pasteurella haemolytica* leukotoxin. The RTX cytotoxin family is characterized by not only the presence of repeated domains in the structural toxin but also the presence of a specialized set of genes involved in the secretion of the toxin (26). In the case of the *A. actinomycetemcomitans* leukotoxin determinant, the secretion genes have been identified (9) and the secretion function of the gene products have also been indicated by nucleotide sequence analyses (4, 5). Nevertheless, in earlier works, it was reported that neither the leukotoxic activity nor the toxin itself could be detected in spent culture media (2, 19). Instead, it was suggested that the toxin was localized primarily in the periplasmic space of bacterial cell (2) or associated with membrane vesicles (6, 17).

Recently, we have studied the localization of leukotoxic activity in growing cultures of *A. actinomycetemcomitans* under controlled conditions in a chemostat (17). When the cultures were fractionated to cell-free soluble proteins, cell-free membrane vesicles, cell-associated membrane vesicles, and vesicle-free cells, the cell-associated membrane vesicles accounted for 97% of the leukotoxic activity. In this article, we report the effective isolation of a leukotoxin from such cell-associated membrane vesicles and more directly from the surface of whole cells by treatments with nucleases. We also provide evidence for the binding activity of the isolated leukotoxin to nucleic acids.

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MATERIALS AND METHODS

Bacterial strain and culture condition. *A. actinomycetemcomitans* 301-b, used throughout this study, was isolated from a Japanese adult patient with rapidly progressive periodontitis (18, 19). This strain was classified as belonging to serotype a (6a). The organism was grown in anaerobic chemostat cultures as described previously (16). The growth medium (16) was composed of 50 mM Tris-hydrochloride, various mineral salts, and 0.2% (wt/vol) yeast extract and supplemented with 11 mM fructose as the growth-limiting nutrient. Two fermentors (working liquid volume, 440 and 1,470 ml) were used. Cultures were maintained at 37°C and at pH 7.0 with the automatic additions of 2 N NaOH or 1 N HCl (440-ml fermentor). In the case of the 1,470-ml fermentor, no apparatus for pH control was provided but the culture pH was kept at 7.3 to 7.4 at a dilution rate of 0.15 h^{-1} by increasing the concentration of Tris to 100 mM in the medium.

Determination of leukotoxic activity. Human promyelocytic HL-60 leukemic cells were used as the leukotoxin-sensitive target cells. The cells were grown and maintained in RPMI 1640 medium (Nissui Pharmaceutical Co., Ltd., Tokyo, Japan) supplemented with penicillin G (100 U/ml), streptomycin (100 μg/ml), and 5% heat-inactivated fetal bovine serum (GIBCO Laboratories, Grand Island, N.Y.). Cytotoxicity was determined by the method of Rotman and Papermaster (21), with some modifications (17). Briefly, HL-60 cells were washed twice with 20 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES)-buffered Hanks balanced salt solution (HBSS; pH 7.6) and suspended in HBSS supplemented with 0.1% (wt/vol) gelatin (HBSS-gelatin). Cells (10^5) were distributed into polypropylene microtubes (1.5-ml volume); HBSS-gelatin and various test

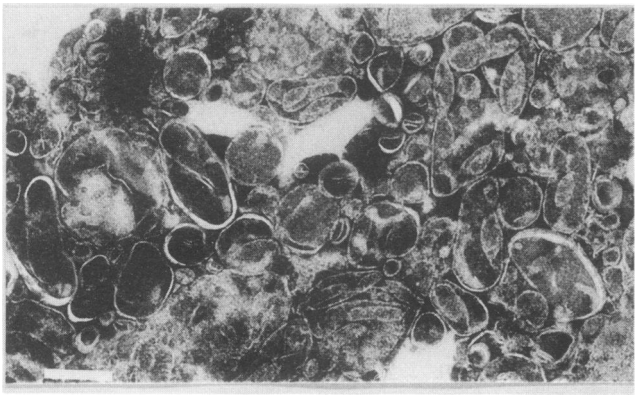

FIG. 1. Electron photomicrograph of the cell-associated membrane vesicle preparation. Bar = 0.2 μm.

samples were added in a total volume of 0.1 ml. Immediately after being mixed with a sample, the aliquot of cell suspension (0.025 ml, 2.5×10^4 cells) was removed for determination of zero time activity, rapidly transferred into a 1-cm cuvette containing 0.875 ml of prewarmed HBSS (at 37°C), and put into a Hitachi F-4000 fluorescence spectrophotometer fitted with a cuvette stirring accessory. The reaction was started by the addition of 0.1 ml of fluorescein diacetate solution (10 μg/ml in HBSS), and the reaction solution was stirred continuously (250 rpm). The initial rate of increase in fluorescence light intensity was measured for 1 min at 510 nm as the emission wavelength with excitation at 490 nm. HL-60 cells were incubated with test samples at 37°C for 25 min, and then the fluorescein-accumulating activity was also measured (25-min activity) as described above. The relative loss of the ability to accumulate fluorescein due to the incubation with samples was expressed as the ratio of 25-min activity to zero time activity (designated as RA_{sample}). Controls consisted of HL-60 cells in HBSS-gelatin alone ($RA_{control}$) and in HBSS-gelatin containing 0.5 mg of saponin (ICN Pharmaceutical Inc., Cleveland, Ohio) per ml ($RA_{saponin}$). The latter was defined as the total loss of the fluorescein-accumulating activity (21). Thus, from these ratios, the percent loss of the activity due to the incubation with a sample was standardized by the following equation: loss of activity (%) = $[(RA_{control} - RA_{sample})/(RA_{control} - RA_{saponin})] \times 100$. The leukotoxic activity was expressed as the 50% effective dose, the dose of protein required to cause 50% loss of the activity.

Isolation of membrane vesicles. The effluent cultures from the chemostat were collected in a sterile bottle kept on ice. Bacterial cells were harvested by centrifugation (10,000 × *g*, 20 min, 4°C) and suspended in 10 mM Tris-hydrochloride-buffered saline (Tris-saline; 0.9% NaCl, pH 7.5) (approximately 1/50 the original volume of culture). The cell suspension was then subjected to 30 s of light sonication to remove membrane vesicles attached to cell surfaces. The wash fluid containing membrane vesicles was separated by centrifugation at 10,000 × *g* for 15 min and kept on ice. The cell pellets were resuspended in Tris-saline, and the above wash procedure was repeated. After five washes, the wash fluids were collected and subjected to ultracentrifugation at 100,000 × *g* and 4°C for 60 min. The pellet was composed of a mass of membrane vesicles (30 to 200 nm in diameter) as shown in Fig. 1. For this wash, the percent lysis of bacterial cells was calculated to be as low as 0.5% when the activity of glucose-6-phosphate isomerase, which was used as a cytoplasmic marker enzyme, was measured.

Extraction and purification of leukotoxin from membrane vesicles. The membrane vesicle suspension in Tris-saline was mixed with an equal volume of 200 mM acetate buffer (pH 5.0) containing 300 mM NaCl and 10 mM $MgSO_4 \cdot 7H_2O$. The suspension was then treated with DNase I (90 U/ml; Sigma Chemical Co., St. Louis, Mo.) at 25°C for 60 min to remove DNA from vesicles. The membrane vesicles were collected by ultracentrifugation at 100,000 × *g* and 4°C for 60 min. The pellet was suspended in 50 mM acetate buffer (pH 5.0) containing 200 mM NaCl and incubated with RNase A (0.4 mg/ml; Sigma) at 25°C for 30 min. Subsequently, the suspension was centrifuged at 100,000 × *g* and 4°C for 60 min and the supernatant (crude toxin) was collected. The following procedures were performed at 4°C or on ice unless otherwise stated. A saturated ammonium sulfate (SAS) solution was added slowly to the crude toxin, with gentle stirring, to 50% saturation. After being stirred for 60 min, the precipitate was removed by centrifugation (20,000 × *g*, 15 min). Secondly, an additional amount of SAS solution was added to 80% saturation. After 12 h, the precipitate was collected by centrifugation and dissolved in a minimal amount of 100 mM sodium phosphate buffer (pH 6.5) containing 200 mM NaCl and 1 mM EDTA (buffer A). This protein solution (80% SAS–toxin) was further purified by gel filtration chromatography and subsequent ion-exchange chromatography. The gel filtration chromatography was carried out with a Toyopearl HW-50 column (2.5 by 87 cm; Tosoh Corporation, Tokyo, Japan) equilibrated with buffer A. The 80% SAS-toxin was fractionated at a flow rate of 31.2 ml/h. Fractions with high leukotoxic activity were combined, concentrated by precipitation at 80% ammonium sulfate, and dialyzed extensively against 100 mM sodium phosphate buffer (pH 6.5) containing 1 mM EDTA (buffer B). The dialyzed toxin solution was loaded at a flow rate of 32.4 ml/h onto a CM-Toyopearl 650 column (1 by 5.5 cm; Tosoh) equilibrated with buffer B. After nonbinding protein was removed by washing, elution was carried out with a linear gradient of 0 to 500 mM NaCl in buffer B.

Isolation of leukotoxin from whole cells. Bacterial cells were collected from the chemostat culture run at a dilution rate of 0.15 h^{-1} in the 440-ml fermentor; to obtain increased amounts of bacterial cells, the medium was supplemented with 20 mM fumarate. Cells were suspended in 100 mM acetate buffer (pH 5.0) containing 150 mM NaCl and 5 mM $MgSO_4 \cdot 7\ H_2O$ and incubated with DNase I (100 U/ml; Sigma) and RNase A (0.2 mg/ml; Sigma) at 25°C for 40 min. The cell suspensions were centrifuged at 12,000 × *g* for 20 min, and then the supernatant was further ultracentrifuged at 100,000 × *g* for 60 min. The resulting supernatant was further purified by the above procedures, with a minor modification: the gel filtration chromatography was performed with a Toyopearl HW-55 column (1.5 by 99 cm; Tosoh) in place of the Toyopearl HW-50 column.

Electrophoresis. Proteins were analyzed by discontinuous sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) with a 7.5 or 10% acrylamide gel as described by Laemmli (11). Protein was stained with Coomassie brilliant blue R-250 or silver (20). The molecular weight of the leukotoxin was determined by comparison with known standard. Isoelectric focusing was done with a Pharmacia Ampholine gel (pH range, 3.5 to 9.5) on an LKB Multiphor II apparatus under the conditions recommended by the manufacturer. Membrane vesicle-associated nucleic acids or extracted DNA was analyzed by electrophoresis on

0.75% agarose gels in Tris acetate buffer (40 mM Tris and 1 mM EDTA, pH 8.0). Gels were stained with ethidium bromide (0.5 μg/ml) for 30 min and photographed under UV illumination.

Preparation of antiserum. Antiserum against purified leukotoxin from vesicle extracts was raised in a Japanese White rabbit (male, 2.5 kg) after subcutaneous injections of 42 μg of purified vesicle toxin suspended in Freund's complete adjuvant. Four weeks later, subcutaneous booster injections of 0.6 mg of protein from 80% SAS-toxin suspended in Freund's incomplete adjuvant were made. After 1 week, the rabbit was bled and the serum was stored at −20°C.

Western blotting (immunoblotting). Separated proteins on the SDS-polyacrylamide gel were electrophoretically transferred to a polyvinylidene difluoride membrane (Bio-Rad Laboratories, Richmond, Calif.) by the methanol Tris-glycine method described by Towbin et al. (27) by using the LKB Multiphor II NovaBlot apparatus. Reaction with an antiserum was detected with swine anti-rabbit immunoglobulin G conjugated to peroxidase and then visualized by adding 4-methoxy-1-naphthol and hydrogen peroxide.

Isolation of DNA. Total DNA of *A. actinomycetemcomitans* 301-b was prepared as described by Saito and Miura (22).

Gel mobility shift assay for binding of leukotoxin to DNA. Different amounts of leukotoxin (190 to 1,710 ng) were incubated for 30 min at room temperature with a target DNA (145 to 150 ng) in buffer A (total volume, 10 μl). The reaction mixture was then separated by agarose gel electrophoresis as described above. The following DNAs were examined as the target DNA: total DNAs of *A. actinomycetemcomitans* 301-b and *Porphyromonas gingivalis* SUNY 1021 (donated by O. Isoshima, Department of Periodontology and Endodontology, Okayama University Dental School) and bacteriophage lambda DNA-*Hin*dIII digest (Takara Shuzo Co., Ltd., Kyoto, Japan).

Other methods. The amount of protein was determined by the method of Lowry by using bovine serum albumin (BSA) as standard. The content of nucleic acids in the vesicle preparation was measured with the fluorescence spectrophotometer by using a nucleic acid-binding fluorescent dye, propidium iodide, at an emission wavelength of 606 nm and an excitation wavelength of 488 nm (10). The purified DNA of *A. actinomycetemcomitans* 301-b was used as the standard. Transmission electron microscopy was performed with a Hitachi H-800 at 100 kV for observing the collected vesicles, which were negatively stained with 1% sodium phosphotungstate (pH 7.2).

RESULTS

Solubilization of leukotoxin from cell-associated membrane vesicles. Some attempts to solubilize leukotoxin from the membrane vesicles as in the ordinary procedures with membrane proteins were made by using several detergents. Zwitterionic CHAPS (3-[(3-cholamidopropyl)-dimethyl-ammonio]-1-propanesulfonate) was found to be an effective detergent for toxin solubilization. However, the further fractionation of CHAPS extract by gel filtration chromatography or ion-exchange chromatography was unsuccessful because of leukotoxin-nucleic acid interactions. The presence of membrane vesicle-associated nucleic acids was indicated by agarose gel electrophoresis of nuclease-treated membrane vesicles. As shown in Fig. 2, there was a diffuse band of nucleic acids in the undigested control vesicles (lane 1). The digestion with DNase resulted in the complete loss of

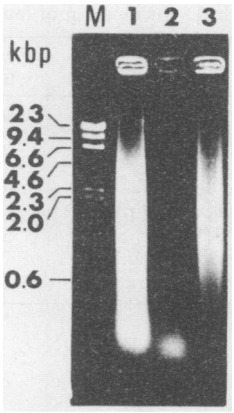

FIG. 2. Agarose gel electrophoresis of the membrane vesicle-associated nucleic acids of *A. actinomycetemcomitans* 301-b. The cell-associated membrane vesicles were incubated for 30 min with either DNase I or RNase A and then analyzed by 0.75% agarose gel electrophoresis. Lanes: M, molecular size standards (*Hin*dIII fragments of bacteriophage lambda); 1, undigested control; 2, DNase digest; 3, RNase digest.

the diffuse band between approximately 0.5 and 23 kbp (Fig. 2, lane 2). The presence of RNA was also suggested by hydrolysis with DNase-free RNase: a small diffuse band at the bottom of the gel (lane 2), probably RNA, disappeared in the RNase digest (lane 3). Hence, the digestion of membrane vesicle-associated nucleic acids with nucleases was applied to the initial step of toxin purification. The organism was grown in the fructose-limited chemostat culture at a dilution rate of 0.15 h^{-1} in the 1,470-ml fermentor. A total of 225 mg of protein from cell-associated membrane vesicles was isolated from 14.2 liters of the effluent cultures. This vesicle preparation contained 42 mg of nucleic acids. The membrane vesicles were treated with DNase and subsequently with RNase. The treatment with DNase resulted in not only the digestion of vesicle-associated DNA but also the 37% release of leukotoxin from vesicles (Table 1). The subsequent digestion with RNase yielded 236% leukotoxic activity compared with that of the original membrane vesicles (Table 1). These results suggest that the leukotoxin was masked with nucleic acids.

Purification of leukotoxin. The supernatant fluid of the RNase digest (crude toxin) revealed three major protein bands of 25, 29, and 113 kDa as well as many minor bands by SDS-PAGE (Fig. 3, lane 3). When the crude toxin was fractionated with ammonium sulfate, the majority of leukotoxic activity was found in the 50 to 80% fraction (Table 1). This fraction was passed over a Toyopearl HW-50 column, which removed nonproteinaceous low-molecular-weight contaminants (probably nucleotides). The subsequent CM-Toyopearl 650 chromatography was the most effective step. Any remaining proteins other than the 113-kDa protein did not bind to the column, and leukotoxic activity was detected in one peak eluted at 0.2 M NaCl (data not shown). This chromatography resulted in a 7.7-fold purification (Table 1) and yielded a homogeneous protein, as judged by a single band with a molecular mass of 113 kDa on SDS-PAGE (Fig. 3, lane 8). The isoelectric point (pI) was estimated to be approximately 8.8 in the Ampholine gel.

Extraction of leukotoxin directly from whole cells. From the above purification procedures, it is to be expected that the leukotoxin can be solubilized directly from whole cells by

TABLE 1. Purification of leukotoxin from cell-associated membrane vesicles of *A. actinomycetemcomitans*[a]

Step	Vol (ml)	Amt of protein (mg)	Total ED_{50} (%)	ED_{50} (μg of protein)	Purification (fold)	Yield (%)
Membrane vesicles	48	225	113,000 (100)	2.0		
DNase-solubilized material	45	45	40,900 (36)	1.1		
RNase-solubilized material after DNase treatment (crude toxin)	46	26.7	267,000 (236)	0.1	1.0	100.0
Ammonium sulfate (50-80%)	2.5	7.5	221,000 (196)	0.034	2.9	82.8
Toyopearl HW-50 chromatography	3.6	0.29	18,100 (16)	0.016	6.3	6.8
CM-Toyopearl 650 chromatography	2.0	0.064	4,900 (4.3)	0.013	7.7	1.8

[a] ED_{50}, 50% effective dose.

treatment with externally added nucleases. To confirm this, the bacterial cell suspensions (26 mg of cells [dry weight] per ml) were prepared from the chemostat cultures grown at 0.15 h^{-1} and incubated with DNase and/or RNase. Then the supernatants were subjected to SDS-PAGE. The supernatant of DNase-digested cell suspension contained a significant amount of protein whose molecular mass was in good agreement with that of leukotoxin (113 kDa) (Fig. 4A, lane 3). Moreover, in the DNase-plus-RNase digest, large amounts of the 113-kDa protein could be detected (Fig. 4A, lane 7). Further identification of the 113-kDa protein was carried out by Western blotting with an antiserum raised against the purified toxin from vesicles. As shown in Fig. 4B, the 113-kDa protein in the nuclease digests was recognized by the antileukotoxin serum. In addition, it should be noted that the immunopositive band was detected in the cell pellet of the undigested control (Fig. 4B, lane 2) and that of the RNase digest (lane 6) but was no longer detected in the cell pellet after digestion with DNase plus RNase (lane 8). This result indicates that the majority of toxin was extractable from whole cells by direct nuclease treatment.

Leukotoxin preparation from whole cells. The crude leukotoxin solubilized from whole cells with DNase plus RNase was further purified according to the above procedures with membrane vesicles. The CM-Toyopearl 650 chromatography at the final step showed the same elution profile as that with membrane vesicles, but the leukotoxin fractions contained trace amounts of the 140-kDa protein contaminant (Fig. 5A). When this preparation was analyzed by Western blotting, the 140-kDa protein band as well as the 113-kDa band were recognized by the antileukotoxin serum (Fig. 5B). No further attempts to characterize this immunoreactive 140-kDa protein were made.

Binding of leukotoxin to DNA. Gel mobility shift assay was performed to examine the binding of the isolated leukotoxin to DNA. A constant amount of strain 301-b DNA (150 ng) was incubated with different amounts of the highly purified leukotoxin prepared from whole cells. The free genomic DNA represented a major band at above 23 kbp in agarose gels (Fig. 6, lane 1). With an increase in the ratio of the amount of leukotoxin to that of DNA, the free DNA band

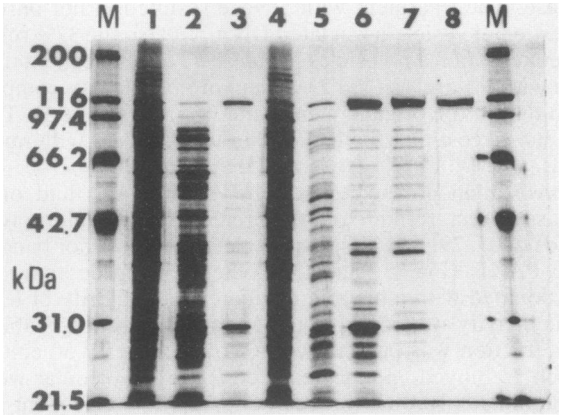

FIG. 3. SDS-PAGE of preparations obtained during purification of the *A. actinomycetemcomitans* leukotoxin. SDS-PAGE was performed with a 10% gel, and the gel was stained with silver. Lanes: M, molecular mass standards; 1, cell-associated membrane vesicles; 2, supernatant fluid of the DNase digest; 3, supernatant fluid of the RNase digest (crude toxin); 4, precipitate after digestion with DNase and RNase; 5, precipitate of the RNase digest after fractionation with 50% ammonium sulfate; 6, precipitate of the RNase digest after fractionation with between 50 and 80% ammonium sulfate (80% SAS-toxin); 7, combined active fraction after Toyopearl HW-50 chromatography; 8, purified toxin after CM-Toyopearl 650 chromatography. Two micrograms (lanes 1 to 6), 0.88 μg (lane 7), and 0.35 μg (lane 8) of protein were loaded.

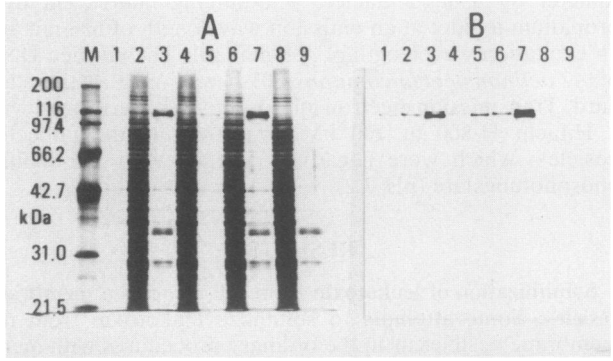

FIG. 4. Effects of DNase and RNase on the solubilization of leukotoxin from whole cells of *A. actinomycetemcomitans* 301-b. The bacterial cell suspensions (26 mg [dry weight]/ml) were incubated for 30 min with DNase and/or RNase. The supernatant fluids (lanes 1, 3, 5, and 7) and the cell pellets after the digestions (lanes, 2, 4, 6, and 8) were subjected to SDS-PAGE with a 10% gel. (A) Silver-stained gel; (B) Western blot with antileukotoxin serum. Lanes: M, molecular mass standards; 1 and 2, undigested control; 3 and 4, DNase digest; 5 and 6, RNase digest; 7 and 8, DNase-plus-RNase digest; 9, enzyme control containing DNase and RNase alone. In the lanes with cell pellets, samples containing 7.2 μg (dry weight) of cell (4.0 μg of cell protein) were applied to SDS-PAGE gels.

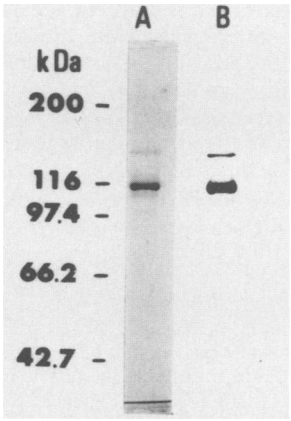

FIG. 5. SDS-PAGE of the final leukotoxin preparation from *A. actinomycetemcomitans* 301-b whole cells. SDS-PAGE was performed with a 7.5% gel. (A) Coomassie blue-stained gel; (B) Western blot with antileukotoxin serum.

became faint while the amount of DNA in the original well (toxin-associated DNA) increased (lanes 2 to 5). The complete loss of free DNA was observed at a leukotoxin/DNA ratio of 7.6 (lane 6). BSA had no known DNA interaction. In fact, BSA did not influence the migration of the DNA even at a BSA/DNA ratio of 12 (lane 8). The specificity of DNA binding was also examined by using DNAs from different sources. When the leukotoxin was mixed with *Hin*dIII fragments of bacteriophage lambda DNA and *P. gingivalis* DNA, the inhibition of DNA migration due to the binding of leukotoxin to DNA could be seen (lanes 10, 11, and 13). These results indicated that the leukotoxin binds to nucleic acids nonspecifically, as observed with very basic proteins such as cytochrome *c*.

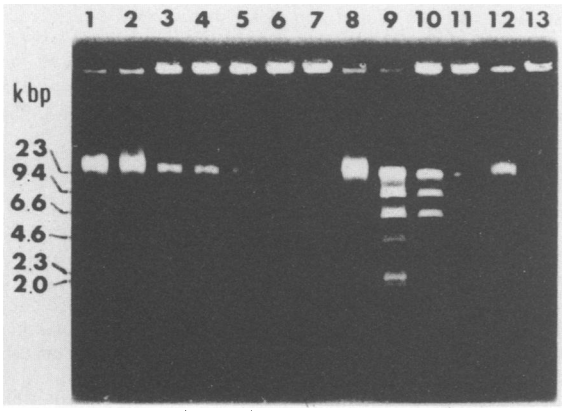

FIG. 6. Gel mobility shift assay for the binding of *A. actinomycetemcomitans* leukotoxin to different DNAs. The leukotoxin was incubated with different DNAs, and then the mixtures were separated by 0.75% agarose gel electrophoresis. Different amounts of leukotoxin were incubated with a constant amount of strain 301-b DNA (150 ng) (lanes 1 to 7). The ratios of leukotoxin to DNA were as follows (per lane): 1, 0; 2, 1.3; 3, 2.5; 4, 3.8; 5, 5.1; 6, 7.6; 7, 11.4. The ratio of BSA to strain 301-b DNA (150 ng) (lane 8) = 12. The ratios of leukotoxin to *Hin*dIII fragments of bacteriophage lambda DNA (150 ng) (lanes 9 to 11) were as follows (per lane): 9, 0; 10, 5.7; 11, 11.4. The ratio of leukotoxin to *P. gingivalis* DNA (145 ng) (lanes 12 and 13) were as follows (per lane): 12, 0; 13, 12.4.

DISCUSSION

In the present study, chemostat cultures of *A. actinomycetemcomitans* were maintained for vesicle preparation and direct extraction of toxin from whole cells for the following two reasons. First, the growth conditions suited for the production of toxin can be characterized in a well-defined manner and reproducibly established in chemostats. Some of these conditions were described previously (17). By contrast, many attempts to find and to keep the optimum conditions for toxin production in batch cultures of *A. actinomycetemcomitans* 301-b were unsuccessful. On the basis of the results with batch cultures, strain 301-b may be classified as a leukotoxin-variable strain as Spitznagel et al. (25) described with strain ATCC 29523. Secondly, beyond a doubt, bacteria in the oral environment will usually grow at submaximal rate because of a nutrient deficiency or an inhibitory environment. In fact, according to the calculation of Loesche (14), the doubling time of the oral flora is 5 h ($\mu = 0.14\ h^{-1}$). This is considerably slower than 1.4- to 1.5-h doubling times in the pure batch cultures of *A. actinomycetemcomitans* (16, 25). Hence, to understand the production of toxin in vivo, work with a chemostat is required.

Tsai et al. (29) first reported that leukotoxin was purified from the whole cells of *A. actinomycetemcomitans* JP2 by using polymyxin B for the solubilization. The toxin had a molecular mass of 115 kDa by SDS-PAGE and 180 kDa by gel filtration. Their subsequent work revealed that the toxin was a very basic protein (pI, 8.2 to 8.5) (2). In the present study, leukotoxin was purified from the membrane vesicles of *A. actinomycetemcomitans* 301-b. The molecular mass of this toxin was determined to be 113 kDa by SDS-PAGE, and this value seems to be comparable with that of the polymyxin B-extracted leukotoxin from strain JP2. In our preliminary experiment, the crude toxin was prepared by incubating the membrane vesicles with DNase alone and then extracting with CHAPS. When this CHAPS-extracted material was fractionated by gel filtration chromatography on a Toyopearl HW-60 column (2.5 by 82 cm; Tosoh), maximal leukotoxic activity was detected in a large defined peak eluted at approximately 180 kDa (data not shown). This elution profile on the gel filtration also seems to be analogous to that of the polymyxin B-extracted toxin (29). In addition, it should be noted that the purified toxin was also highly basic (pI, approximately 8.8). Thus, from these considerations, it may be concluded that the leukotoxin from the membrane vesicles of strain 301-b is almost identical to the toxin from strain JP2.

The complete solubilization of leukotoxin from the vesicles could be achieved by treatment with DNase plus RNase (Table 1). This result indicates that the nucleases can get access to nucleic acids complexed with the leukotoxin; i.e., the leukotoxin-nucleic acid complex is present on the outside of vesicles. The outside location of nucleic acids seems to be opposed to the well-characterized DNA-membrane complex of *Haemophilus influenzae*, in which DNA is internalized into membrane vesicles (transformasomes) and is resistant to digestion with external DNase (1, 7). However, it was also reported that *Neisseria gonorrhoeae* produced not only the transformasome-type complex but also the membrane bleb-DNA complexes which were hydrolyzable with external DNase (3). This bleb-DNA complex appears to exhibit similarities to the vesicles of *A. actinomycetemcomitans*. The transformasome-type complex may aid intercellular transfer of DNA (3, 7), whereas in the case of the

nuclease-sensitive DNA-vesicle complex, the role and mechanisms of complex formation are currently unknown.

The RTX cytotoxin family is characterized by the presence of four contiguous genes (*lktC*, *lktA*, *lktB*, and *lktD*, in order of their genetic organization) including a specialized set of secretion genes (*lktB* and *lktD* genes) and the presence of a series of repeated domains within the structural toxin itself (*lktA* gene product) (26). In the case of the *A. actinomycetemcomitans* leukotoxin determinant, the function of *lktB* and *lktD* gene products (designated LktB and LktD) was not clear since the leukotoxin could not detect in the culture supernatants (2, 19). However, in a very recent study, it has been suggested that the LktB and LktD proteins of *A. actinomycetemcomitans* have the same secretion function as in the *lktB* and *lktD* gene products of *E. coli* hemolysin and *P. haemolytica* leukotoxin (4, 5). Moreover, Northern (RNA) blot analyses have indicated that the *lktB* and *lktD* genes are expressed in *A. actinomycetemcomitans* (25). These seemingly incompatible facts may be explained by the findings of the present study; i.e., the result that the leukotoxin was completely solubilized from whole cells by treatment with nucleases suggests that the toxin is secreted extracellularly but retained on the cell surface by associating with nucleic acids. Very little is known about the cell surface locality of nucleic acids. To our knowledge, one example of extracellular DNA exists in which DNA is directly involved in the cell association of a *Pseudomonas* sp. (23). In the case of *A. actinomycetemcomitans*, one may assume that at submaximal growth rates, the viability of the culture is low and a significant proportion of the cells is lysed, which allow the formation of leukotoxin-nucleic acid complex from the lysed cells. However, this is not the case for the culture grown at 0.15 h^{-1} in the present study because the percent lysis of cells in the culture was estimated to be fully low (approximately 1.4% from the assay for glucose-6-phosphate isomerase in the culture supernatant).

When the crude toxin prepared from whole cells by the digestion with DNase plus RNase was subjected to Western blotting, the antiserum against the purified toxin reacted with two polypeptides of 113 and 140 kDa (Fig. 5). Because a preimmune serum control did not react with both polypeptides, the possibility that the rabbit serum contains a natural antibody against the 140-kDa protein will be excluded. An alternative possibility is that the original antigen preparation contained a trace amount of the 140-kDa protein, but it was virtually impossible to detect the protein in not only the silver-stained SDS-PAGE gel (Fig. 3) but also in the Western blot (data not shown). Further interpretation requires the isolation and characterization of the protein.

The cell surface locality of toxin may imply that the toxin is not accessible to target host cells until the contact of bacterial cells with target cells or the release of membrane vesicles occurs. However, this consideration does not seem to provide an exact picture of the in vivo pathogenesis of leukotoxin since the periodontal pocket harbors complex microbial communities. In the case of a species producing nuclease extracellularly in the microbiota, such nucleases would degrade the leukotoxin-nucleic acid complex of *A. actinomycetemcomitans*, resulting in the release of leukotoxin from the bacterial cell. Thus, in complex microbial communities the leukotoxin may be directly accessible to target host cells without the bacterium-target cell contact. Previously, we reported the bacterial flora of the periodontal pocket from which *A. actinomycetemcomitans* 301-b had been isolated (18). In this bacterial flora, the major species were *Fusobacterium* species (34%), *A. actinomycetemcomitans* (19%), *Streptococcus* species (18%), and *Wolinella recta* (16%). An attempt to find the activity for DNA digestion among the isolates of such predominant species was made, and some isolates of *W. recta* have been found to produce DNase extracellularly. This finding may support the above speculation on the in vivo pathogenesis of leukotoxin. Further studies are now in progress to ascertain the large release of leukotoxin from *A. actinomycetemcomitans* cells by coculturing with DNase-producing *W. recta* under controlled conditions in a chemostat.

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