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Monoclonal Antibodies to Leukotoxin of *Actinobacillus actinomycetemcomitans*

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Hybridoma cell lines which produce monoclonal antibodies to a leukotoxin from *Actinobacillus actinomycetemcomitans* were prepared. The monoclonal antibodies were selected for their ability to neutralize the cytotoxic activity of the leukotoxin and recognize the toxin on nitrocellulose blots. The antibodies belonged to either the immunoglobulin G1 (IgG1) or IgG2 subclass and differed in their ability to bind to the leukotoxin on nitrocellulose blots. However, only slight differences in neutralization titers were observed. Use of the monoclonal antibodies revealed that polymyxin B-extracted or osmotic shock-released leukotoxin could be separated into several high-molecular-weight polypeptides by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Immunoblot analysis with the monoclonal antibodies also demonstrated that the leukotoxin was present in eight oral strains of *A. actinomycetemcomitans* that had been previously classified by a biological assay as leukotoxic. The availability of these monoclonal antibodies should facilitate and expand studies concerning the role of the leukotoxin in the pathogenicity of *A. actinomycetemcomitans*.

The gram-negative, capnophilic bacterium *Actinobacillus actinomycetemcomitans* has been implicated as a periodontopathic agent in young adults. This microorganism has been isolated, with relatively high frequencies, from diseased sites in juvenile periodontitis patients (17, 22–24); individuals with this form of periodontal disease characteristically have high serum antibody titers to the bacterium (12, 15, 18, 26). Ebersole et al. (5) and Taubman et al. (26) reported that approximately 70% of juvenile periodontitis patients have detectable immunoglobulin G (IgG) antibodies to specific bacterial components of *A. actinomycetemcomitans* Y4, as measured by the enzyme-linked immunosorbent assay. Furthermore, Taichman et al. (25) have found that greater than 90% of juvenile periodontitis sera neutralize the cytotoxic activity of the leukotoxin.

Human isolates of *A. actinomycetemcomitans* have been divided into three major serogroups, designated a, b, and c, on the basis of immunodiffusion and indirect immunofluorescence methods (30). The strains most commonly isolated from juvenile periodontitis patients are representatives of serotypes a and b (30), and only serotype a and b strains produce a leukotoxin which specifically kills human and monkey polymorphonuclear leukocytes (PMNs) (25). This molecule has recently been purified and partially characterized (29).

In view of the important role that the leukotoxin may play in the pathogenicity of *A. actinomycetemcomitans* and as a possible indicator of juvenile periodontitis, this study was undertaken to prepare monoclonal antibodies for use as immunological probes in the identification, localization, and further characterization of the leukotoxin.

MATERIALS AND METHODS

Bacterial strains and culturing conditions. *A. actinomycetemcomitans* strains Y4, JP2, 650, 651, 652, 627, 511, ATCC 29522, ATCC 29523, and ATCC 29524 were used in this study. Other bacteria included *Haemophilus aphrophilus* strains 80 and 81 and *Capnocytophaga ochracea* 25. The original sources of strains have been reported elsewhere (3, 29). All of the bacterial strains were grown in a peptone-yeast extract-glucose medium (10) supplemented with 0.5% sodium bicarbonate for 24 h at 37°C in a carbon dioxide atmosphere.

Cell fractionation procedures. Bacterial cell fractions which contained leukotoxin were obtained by either extracting whole cells with polymyxin B or subjecting cells to osmotic shock. The polymyxin extraction procedure has been described previously (29) and was used to obtain the antigen preparation for hybridoma production. Briefly, washed cells were suspended in 10 mM Tris hydrochloride–0.9% sodium chloride (pH 7.4) containing 4.0 mg of polymyxin B sulfate per ml (Sigma Chemical Co., St. Louis, Mo.). The suspension was incubated for 30 min at 37°C and then centrifuged at 27,000 × *g* for 15 min to remove remaining whole cells and debris. The extract was dialyzed against 10 mM Tris hydrochloride–1 mM EDTA (pH 7.4) and applied to a DEAE-Sephacel column (1.5 by 28 cm) previously equilibrated with the same buffer. The material which did not bind to the column was collected and concentrated by ultrafiltration. This preparation contained 89% of the leukotoxic activity present in the initial polymyxin extract.

In other experiments, the cells were subjected to the osmotic shock procedure of Neu and Heppel (16). Cells were harvested from 24-h-old cultures (100 ml) and washed three times with 10 mM Tris hydrochloride (pH 8.0). The cells were suspended in 0.5 ml of 20% sucrose–30 mM Tris hydrochloride (pH 8.0) at room temperature. The cell suspension was treated with 1 mM EDTA, final concentration, and mixed for 10 min. The cells were then removed by centrifugation and rapidly suspended in 0.5 ml of ice-cold water. This suspension was incubated in an ice bath for 10 min, after which the cells were removed by centrifugation. The resulting supernatant fluid contained periplasmic space proteins.

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Immunization. BALB/cJ female mice (Jackson Laboratories, Bar Harbor, Maine), 12 to 16 weeks old, were immunized with 10 μg of polymyxin-extracted protein from *A. actinomycetemcomitans* JP2. The protein preparation was emulsified in complete Freund adjuvant. The mice were boosted on days 10, 20, and 30 with protein in incomplete Freund adjuvant and bled via the retroorbital plexus on day 37. Sera were tested for the presence of anti-leukotoxin antibody by the biological assay described below. Mice whose sera showed inhibition of leukotoxin in the biological assay were segregated and allowed to rest for 60 days.

Fusion protocol. Three days before fusion, the animals received 10 μg of polymyxin-extracted protein intravenously. On the day of the fusion, spleens were removed aseptically, and a single cell suspension was made with a loose-fitting tissue homogenizer. The cells were washed once in Dulbecco minimal essential medium (10% fetal calf serum), and the erythrocytes were lysed with 0.17 M ammonium chloride–Tris buffer. Spleen cells recovered in this manner had a viability of >95% as assessed by trypan blue exclusion.

The fusion procedure was a modification of the method of Gefter et al. (7). Sp2/0-Ag14 myeloma cells (20) were mixed with spleen cells (1:10) and centrifuged. A 1-ml portion of 50% polyethylene glycol solution (PEG 1000; J. T. Baker Chemical Co., Phillipsburg, N.J.) was slowly added to the cell pellet. The pellet was gently stirred, allowed to set for 1 min, and then dispersed by the addition of 50 ml of Dulbecco minimal essential medium. After centrifugation, the cells were suspended in 30 ml of medium, and 0.1-ml portions were placed in 96-well tissue culture plates.

The next day, an additional 0.1 ml of medium containing aminopterin (0.04 μM) was added to each well. Cells were fed every 3 to 4 days by drawing off 0.1 ml of spent medium. Clones were visible 7 to 9 days after the fusion, and when they covered approximately one-half of the bottom of the well, they were screened for the presence of anti-leukotoxin antibody by inhibition in the biological assay and for recognition on the electrophoretic blots.

Cloning. Cultures which inhibited leukotoxin activity and recognized the leukotoxin on the nitrocellulose blots were cloned according to a modification of the method of Sato et al. (19).

Isotype determination. Immunoglobulin subclass and light chain determinations were achieved using monospecific antisera that had been prescreened for cross-reactivity according to the method of Slack et al. (21).

Isolation of human PMNs and the leukotoxin inhibition assay. Human peripheral blood PMNs were isolated from healthy donors by dextran sedimentation and centrifugation on Ficoll-Hypaque and suspended in Hanks balanced salt solution supplemented with 0.1% gelatin as described previously (28). Inhibition of cytotoxicity was measured by a modification of the ^{51}Cr-release assay (29). To test the inhibitory activity of the monoclonal antibodies, 50 μl of polymyxin-extracted leukotoxin preparation (3.6 μg of protein) and various dilutions of ascites fluid or the spent medium from cloned cells were added to the ^{51}Cr-labeled PMNs in the 96-well microculture plates. Background ^{51}CR release from labeled PMNs in Hanks balanced salt solution with 0.1% gelatin alone ranged from 5 to 10% of that released from the cells by disruption in 0.05% Triton X-100.

Polyacrylamide gel electrophoresis and electrophoretic blotting. Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis was performed on 17.5% slab gels as described previously (3). Two-dimensional gel electrophoresis was performed essentially as described by Ames and Nikaido (1). The isoelectric focusing gels contained urea and a nonionic detergent, Nonidet P-40. Gels were cast in acid-washed glass tubes (3 [inside diameter] by 130 mm [length]) and contained 2% Ampholine (LKB Instruments Inc., Rockville, Md.) (pH ranges 5 to 7, 8 to 10, and 3.5 to 10; 2:2:1 [vol/vol/vol]). The anode and cathode buffers were 10 mM H_3PO_4 and 20 mM NaOH, respectively, and electrophoresis was performed at 300 V for 18 h, followed by 500 V for 2 h. Isoelectric focusing gels without sample were sliced after electrophoresis for determination of the pH gradient. Additional focusing gels containing trypsinogen (pI 9.3), lentil lectin (pI 8.15, 8.45, and 8.65), and hemoglobin (pI 7.2) were also used as standards. A 17.5% polyacrylamide slab gel was used in the second dimension. Gels were stained and destained as described previously (1). Electrophoretic transfer of polypeptides to nitrocellulose and the immunodetection of these polypeptides were performed with either one- or two-dimensional gels by the procedure of Towbin and co-workers (27).

Analytical procedures. Protein concentration was determined by the method of Lowry et al. (14) with bovine serum albumin as a standard.

RESULTS

Identification and characterization of monoclonal antibodies which recognize the leukotoxin. A crude leukotoxin fraction was prepared from *A. actinomycetemcomitans* JP2 by polymyxin B extraction and DEAE-Sephacel chromatography. This material demonstrated potent toxic activity as measured by the ^{51}Cr-release assay and served as the antigen preparation for hybridoma production. The polypeptide composition of this antigen mixture is shown in Fig. 1, lane A. The purified leukotoxin (115,000-molecular-weight polypeptide), characterized in a previous study (29), is shown in Fig. 1, lane B for comparison. The corresponding leukotoxin band in the polymyxin B-extracted material was actually a cluster of polypeptides ranging in apparent molecular weight from 135,000 to 115,000 (compare the samples in lanes A and B of Fig. 1).

Sera collected from mice which had been immunized with the polymyxin B-extracted antigen were screened for the presence of neutralizing antibodies. To demonstrate that these neutralizing antibodies also recognized the leukotoxin polypeptide, the crude antigen was subjected to SDS-polyacrylamide gel electrophoresis, and the separated polypeptides were transferred to nitrocellulose. Duplicate nitrocellulose strips were incubated with either the neutralizing serum from an immune mouse or with human juvenile periodontitis serum. The results of one such experiment are shown in Fig. 2. The mouse serum recognized several polypeptides, one of which was the leukotoxin (Fig. 2, lane B). The leukotoxin polypeptide was also recognized by the human juvenile periodontitis serum as shown in Fig. 2, lane C. All neutralizing juvenile periodontitis sera tested to date recognize this leukotoxin polypeptide (data not shown). Normal mouse and normal human sera served as controls in these experiments. Both of these sera failed to show any reaction with the polymyxin B-extracted antigen preparation (data not shown).

Hybridomas were subsequently selected by a double screening procedure. Those hybridomas that produced neutralizing antibody, as tested in the ^{51}Cr-release assay, were examined for leukotoxin recognition on nitrocellulose filters. Ultimately, seven anti-leukotoxin-neutralizing hybridomas were obtained. The monoclonal antibodies produced by these hybridomas all recognized the leukotoxin polypeptide

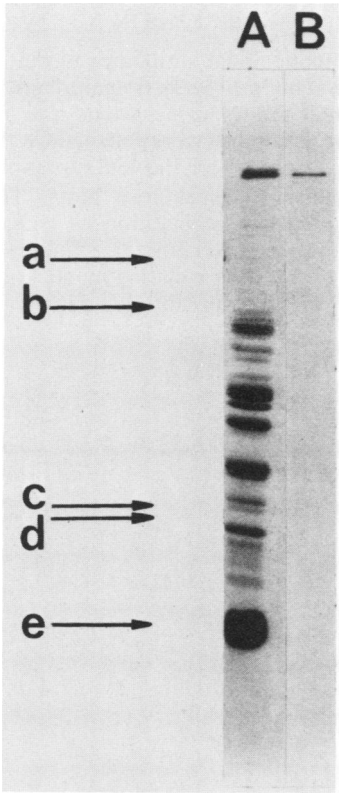

FIG. 1. SDS-polyacrylamide gel electrophoresis of leukotoxin isolated from *A. actinomycetemcomitans* JP2. Cells were treated with polymyxin B, and the extracted material was chromatographed on DEAE-Sephacel, Sepharose 6B, and CM-Sephadex as described previously (29). Lane A, polymyxin B-extracted antigen preparation; lane B, purified leukotoxin from the CM-Sephadex column. Molecular weight standards are represented by: a, bovine serum albumin (68,000); b, ovalbumin (43,000); c, lysozyme (14,300); d, cytochrome *c* (11,700); e, insulin (6,000).

and demonstrated only slight differences in neutralization titers when crude bacterial sonically treated material or purified leukotoxin was used as the antigen. However, three monoclonal antibodies showed some variability in binding to the leukotoxin on nitrocellulose blots (Fig. 3). The isotypes of these three monoclonal antibodies were IgG2,κ ($46A_3C_2$), IgG1,κ ($83A_4A_3$), and IgG1,κ ($107A_3A_3$). The monoclonal antibody produced by one representative hybridoma, $107A_3A_3$, was chosen for use as a probe to examine the heterogeneity and strain distribution of the leukotoxin.

Analysis of the heterogeneity of the leukotoxin by two-dimensional polyacrylamide gel electrophoresis. The polymyxin B-extracted leukotoxin fraction from *A. actinomycetemcomitans* JP2 was subjected first to two-dimensional gel electrophoresis and then to electrophoretic blotting (Fig. 4). The leukotoxin, as detected with monoclonal antibody, had an isoelectric point in the range of 8.2 to 8.5, as determined in urea gels. In addition to the major polypeptide (Fig. 4B small arrow), several minor spots were detected with monoclonal antibody. These additional polypeptides had either reduced apparent molecular weights or a more acidic character and would appear to represent subunits or degradation products of the major leukotoxin polypeptide. The leukotoxin polypeptide could be distinctly separated from other contaminating polypeptides by two-dimensional gel electrophoresis.

Use of monoclonal antibodies to examine the distribution of leukotoxin in oral strains of *A. actinomycetemcomitans*. Previous work showed that leukotoxin could be extracted from *A. actinomycetemcomitans* with polymyxin B (29). These results indicated that the leukotoxin may be localized in the periplasmic space or outer membrane of this microorganism. Consequently, various strains of oral gram-negative bacteria were subjected to osmotic shock, and the released polypeptides were separated by SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose by electrophoretic blotting (Fig. 5). Incubation of the blot with monoclonal antibody $107A_3A_3$ revealed the presence of one or two (depending upon the strain) peroxidase-stained polypeptides (see arrow in Fig. 5B). These polypeptides had apparent molecular weights of 135,000 and 130,000, respectively, as determined by comparison to protein standards. Eight strains of *A. actinomycetemcomitans*, previously shown to be leukotoxic in a bioassay (25), contained one or both of the monoclonal-specific polypeptides (Fig. 5B, lanes A through H). *A. actinomycetemcomitans* strains 652 and 627, *C.*

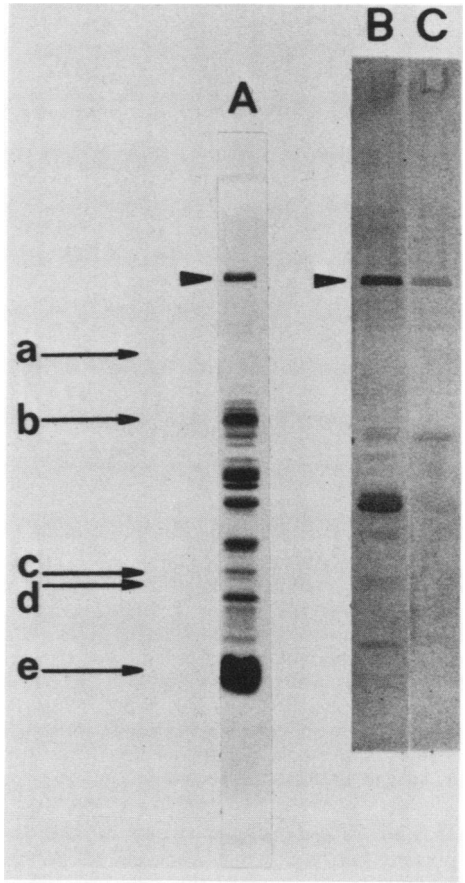

FIG. 2. Immunodetection of leukotoxin using heterologous sera. Crude polymyxin B-extracted polypeptides from strain JP2 were separated by SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose by electroblotting. Replicate nitrocellulose blots were then incubated with various sera, followed by peroxidase-conjugated second antibody. Lane A, SDS-polyacrylamide gel of polymyxin B extract (42 μg of protein); lane B, nitrocellulose blot incubated with serum (1:50 dilution) from a mouse immunized with polymyxin B-extracted protein and peroxidase-conjugated sheep anti-mouse immunoglobulin (1:2,000 dilution); lane C, blot incubated with juvenile periodontitis serum (1:10 dilution) and peroxidase-conjugated goat anti-human immunoglobulin (1:2,000 dilution). The arrows indicate the position of the leukotoxin polypeptides. Molecular weight standards (a through e) are the same as described in the legend to Fig. 1.

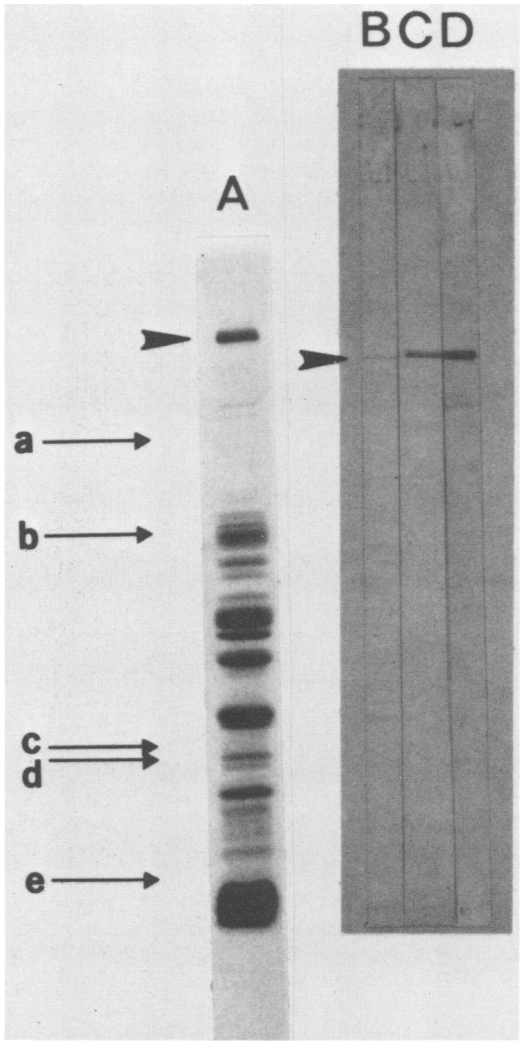

FIG. 3. Identification of monoclonal antibodies which recognize the leukotoxin. Spent culture medium from three hybridomas, which neutralized leukotoxic activity in the biological assay, reacted with electroblotted polymyxin B-extracted protein (42 μg). All culture supernatants were obtained after 4 days of growth (2×10^5 cells plated) and were used at a 1:30 dilution with each nitrocellulose strip. Lane A, SDS-polyacrylamide gel of polymyxin B extract; lanes B through D, nitrocellulose blots of crude antigen, shown in lane A, incubated with spent culture medium from hybridomas $46A_3C_2$, $83A_4A_3$, and $107A_3A_3$, respectively.

ochracea 25, and *H. aphrophilus* strains 80 and 81 did not contain a polypeptide which was recognized by the monoclonal antibody (Fig. 5B, lanes I through M). These strains are known to be "minimally" leukotoxic or nonleukotoxic in the bioassay (25). The cytoplasmic and membrane protein fractions were also prepared from each of the bacterial strains used in this study and were examined on electrophoretic blots with the monoclonal antibody against the leukotoxin. In some of the leukotoxic strains of *A. actinomycetemcomitans*, trace amounts of leukotoxin could be detected in the cytoplasmic protein fraction (data not shown). These results suggested that the leukotoxin was localized primarily in the periplasmic space of *A. actinomycetemcomitans*. After 24 h of growth of strain Y4, spent culture medium was also analyzed by SDS-polyacrylamide gel electrophoresis and electrophoretic blotting; no leukotoxin could be detected with the monoclonal antibody.

DISCUSSION

In this study, monoclonal antibodies to the leukotoxin of *A. actinomycetemcomitans* were prepared and used to characterize and detect the toxin in various oral strains of this bacterium. These antibodies were selected for their ability to neutralize the cytotoxicity of the leukotoxin and to recognize this protein on nitrocellulose blots. The use of a partially purified protein preparation as antigen and electrophoretic blotting as a screening technique significantly increased the probability of isolating monoclonal antibodies that recognized the leukotoxin. Although various mono-

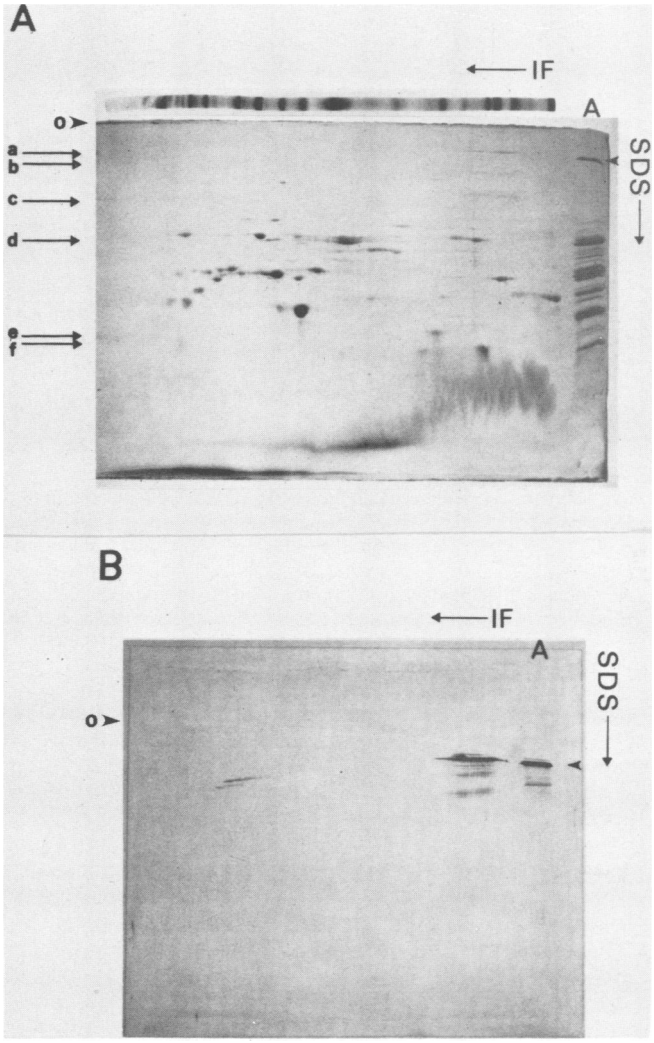

FIG. 4. Two-dimensional gel electrophoresis of polymyxin B-extracted leukotoxin fraction. Polymyxin B-extracted protein (100 μg) was applied to isoelectric focusing gels. One gel was stained with Coomassie brilliant blue and two gels were each overlaid on a 17.5% SDS-polyacrylamide slab gel. Polymyxin B-extracted protein (42 μg) was applied to a well at one end of the slab gels (lane A) and molecular weight standards were applied at the other end. One two-dimensional gel was stained for protein (panel A) and the second gel was subjected to electrophoretic blotting (panel B). Leukotoxin was detected by incubating the two-dimensional blot with monoclonal antibody $107A_3A_3$ (in ascites fluid, 1:200 dilution). The origin of the running gel is designated by the letter O. The small arrows in panels A and B indicate the position of the leukotoxin. Molecular weight standards are represented by: a, β galactosidase (130,000); b, phosphorylase *a* (94,000); c, bovine serum albumin; d, ovalbumin; e, lysozyme; f, cytochrome *c*.

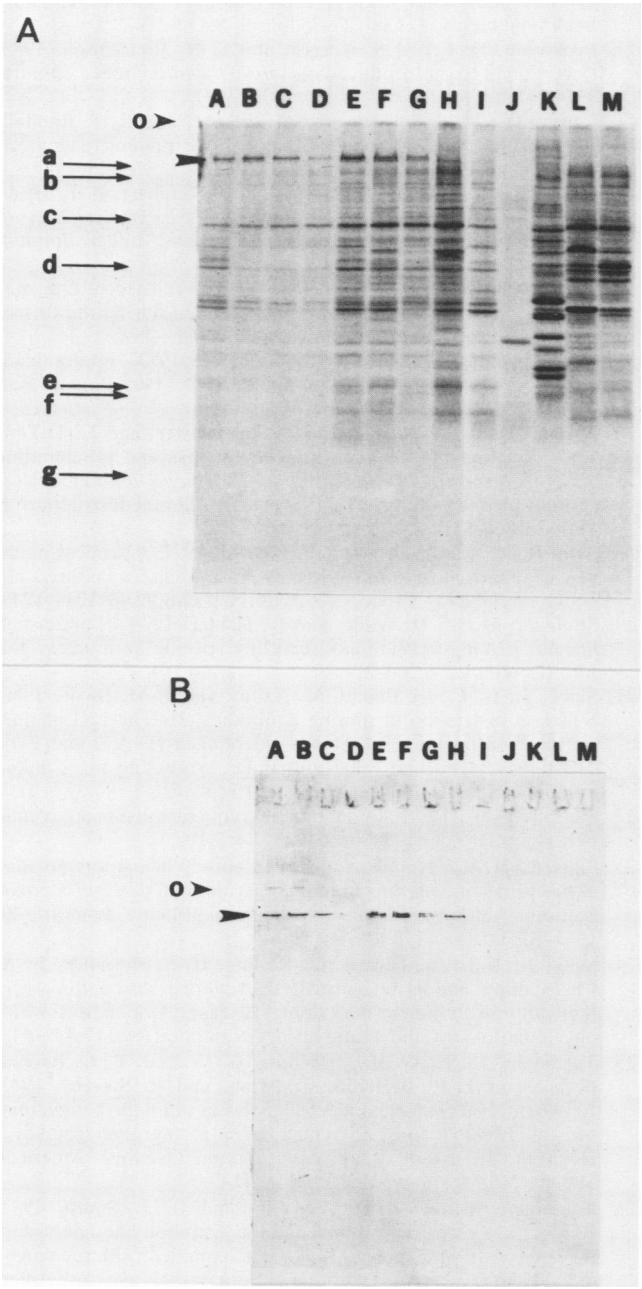

FIG. 5. Immunodetection of leukotoxin in strains of *A. actinomycetemcomitans*. The polypeptides released by osmotic shock treatment of various bacterial strains were separated by SDS-polyacrylamide gel electrophoresis. The same volume of osmotic shock fluid was applied to each lane and routinely contained 15 to 30 μg of protein. One gel was stained for protein (panel A) and a duplicate gel was subjected to electrophoretic blotting (panel B). The nitrocellulose blot was incubated with monoclonal antibody $107A_3A_3$ (in ascites fluid, 1:100 dilution) and peroxidase-conjugated sheep anti-mouse immunoglobulin (1:2,000 dilution). The following *A. acinomycetemcomitans* strains and other organisms are shown in the lanes: A, Y4; B, JP2; C, 650; D, 651; E, ATCC 29522; F, ATCC 29523; G, ATCC 29524; H, 511; I, 652; J, 627; K, *C. ochracea* 25; L, *H. aphrophilus* 80; M, *H. aphrophilus* 81. The origin of the running gel is designated by the letter O. The arrows in panels A and B indicate the position of the leukotoxin. Molecular weight standards are the same as in Fig. 4, except that g represents insulin.

clonal antibodies against *A. actinomycetemcomitans* had been isolated previously, none of these were reported to be specific for the leukotoxin (J. Klass, P. Berthold, and W. McArthur, J. Dent. Res. **61**:333, 1982; V. Cohn, M. E. Neiders, P. Hammond, and R. J. Genco, J. Dent. Res., **62**:208, 1983). This was probably due to the use of crude bacterial sonically treated material as antigens, which were bound to plastic microtiter plates in an enzyme-linked immunosorbent assay. Two factors which limit the sensitivity of this type of enzyme-linked immunosorbent assay system are the competition among antigens for adsorption sites on the plastic plates and the differences in affinities of various types of molecules for the plastic. Cantarero et al. (2) showed that the amount of radiolabeled protein which adsorbed to the plastic was dependent upon the concentration of the protein until saturation of the plastic surface was achieved. After saturation, a constant amount of protein was bound regardless of the amount of protein in solution. Thus, the important factor was the ratio of competitor to antigen. Kenny and Dunsmoor (11) reported that the specificity and sensitivity of the enzyme-linked immunosorbent assay system were reduced when crude antigen mixtures were used in place of purified antigens. At high ratios of competitor to specific antigen, the antigen could not be detected. These problems are circumvented when antibodies are screened by using electrophoretic blots because of the high affinity of all proteins for nitrocellulose.

The following criteria were used to establish that the monoclonal antibodies identified by this dual-screening protocol were directed against the leukotoxin. (i) The monoclonal antibodies recognized the same polypeptide on nitrocellulose blots as that isolated by Tsai and co-workers in a previous study (29). (ii) The antibodies neutralized the cytotoxicity of the leukotoxin as measured in the ^{51}Cr-release assay. (iii) All tested juvenile periodontitis sera which neutralized the biological activity of the leukotoxin also recognized the same polypeptides as those recognized by the monoclonal antibodies. (iv) The leukotoxin polypeptides were detected with the monoclonal antibodies only in strains of *A. actinomycetemcomitans* previously shown to be leukotoxic in the ^{51}Cr-release assay. These polypeptides were not detected in *C. ochracea* 25, *H. aphrophilus* strains 80 and 81, and minimally leukotoxic strains 652 and 627 (29).

In terms of recognition of the leukotoxin by the monoclonal antibodies, several interesting observations were made. The monoclonal antibodies bound to the leukotoxin even though the protein was heated (50°C, 20 min) in the presence of SDS and presumably denatured for gel electrophoresis and electrophoretic blotting. The antigen preparation which was used for immunization was obtained by nondenaturing methods. Also, the monoclonal antibodies recognized at least three polypeptides ranging in molecular weight from 135,000 to 115,000. The structural and biological relationships among these polypeptides are currently under investigation. This size heterogeneity may be related to the subunit structure or physical degradation of the toxin; however, more extensive experimentation is necessary to confirm these possibilities.

The most surprising finding in this study was that the leukotoxin may be concentrated in the periplasmic space of *A. actinomycetemcomitans*. Earlier results showed that the leukotoxin could be released from whole cells by extraction with the membrane-directed antibiotic, polymyxin B (29). These results suggested that the leukotoxin was either associated with the outer membrane or localized in the periplasmic space (4, 6, 8, 13). When *A. actinomycetemcomitans* was subjected to osmotic shock, a biologically active

leukotoxin was released. The leukotoxin could not be found in spent culture medium or in the membrane fraction from these cells. Recently, Hirst et al. (9) established that both the A and B subunits of the heat-labile enterotoxin of *Escherichia coli* are located in the periplasmic space. Therefore, a mechanism must exist for the release of the enterotoxin for disease to occur. The periplasmic localization of the leukotoxin presents a similar situation since this protein must be translocated across the outer membrane to be accessible to PMNs.

The monoclonal antibodies described in this study have important applications in light of the potential contribution of the leukotoxin to the pathogenicity of *A. actinomycetemcomitans*. These antibodies can be used to examine toxin structure and mode of binding to PMNs as well as the biosynthesis and release of the leukotoxin from the bacterial cell. In clinical applications these antibodies can be used as probes to identify and localize the toxin in vivo and examine the role of the leukotoxin in the disease process.

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