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Lethal Effects of *Actinobacillus actinomycetemcomitans* Leukotoxin on Human T Lymphocytes

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The majority of strains of *Actinobacillus actinomycetemcomitans* isolated from patients with periodontal diseases secrete a leukotoxin that destroys human myeloid cells within minutes but has no effect on viability of peripheral blood lymphocytes in culture for 1.5 h. However, since this organism persists in the gingival crevice and thus may continuously release toxin over extended periods of time, we assessed the viability of T cells cultured with leukotoxin (0 to 250 ng/ml) for up to 2 days. Although the total numbers of cells recovered from cultures with or without leukotoxin were equivalent, leukotoxin killed up to 70% of the T cells in a time- and concentration-dependent manner. Cell death was associated with uptake of propidium iodide, release of ^{51}Cr from the cytoplasm, and morphological evidence of damage to the plasma membrane and apoptosis. Leukotoxin also induced increased cleavage of chromosomal DNA into nucleosome-sized fragments, suggesting activation of an endogenous nuclease in the T cells. These data suggest that leukotoxin kills T cells by pathways resembling necrosis and programmed cell death. Leukotoxin-induced lymphotoxicity may represent a critical mechanism by which *A. actinomycetemcomitans* suppresses the host local immune response and contributes to the pathogenesis of diseases involving this microorganism.

Actinobacillus actinomycetemcomitans is a gram-negative bacterium implicated in the pathogenesis of juvenile and adult periodontitis (2; reviewed in reference 18). A majority of fresh clinical isolates of *A. actinomycetemcomitans* synthesize a heat-labile exotoxin which rapidly kills human polymorphonuclear neutrophils and monocytes (19, 20). Whereas human platelets, fibroblasts, endothelial cells, and epithelial cells are relatively resistant to the cytotoxic effects of this leukotoxin (20), there exists some confusion about its action on human lymphocytes. Freshly isolated lymphocytes treated with crude leukotoxin for 1 h remained viable as determined by trypan blue exclusion and morphology (20), whereas human lymphocyte cell lines treated with purified leukotoxin were killed within 45 min (17). Furthermore, Rabie et al. (15) reported that, although freshly isolated lymphocytes exposed to purified leukotoxin for ≤1.5 h remained viable, there was a striking decrease in mitogen- and antigen-induced proliferation and immunoglobulin production.

Since leukotoxin could be continuously released into the gingival tissues during chronic infection with *A. actinomycetemcomitans* (2, 7), we have examined its long-term (up to 2 days) effects on viability of T lymphocytes (T cells), cells that play a critical role in the regulation of host immune responses. Our results clearly indicate that purified leukotoxin kills T cells in a time- and concentration-dependent manner. Death was associated with damage to the plasma membranes and increased cleavage of chromosomal DNA into oligonucleosome-sized fragments. These toxic effects may not have been observed in previous studies because of the resistance of peripheral blood T cells to killing and complete lysis by brief exposure to leukotoxin. The capacity of leukotoxin to kill T cells may represent an important immunosuppressive mechanism involved in the pathogenesis of periodontal diseases involving *A. actinomycetemcomitans*.

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MATERIALS AND METHODS

Leukotoxin. Leukotoxin was isolated from 18-h cultures of *A. actinomycetemcomitans* JP2 and purified by polymyxin B extraction and ion-exchange and molecular sieve chromatography as described by Tsai et al. (21). Lyophilized leukotoxin was reconstituted in RPMI 1640 (GIBCO, Gaithersburg, Md.) and maintained on ice for <30 min before being added to lymphocyte cultures. Heat-inactivated toxin was prepared by incubating leukotoxin (1 μg/ml in RPMI 1640) at 70°C for 30 min.

Lymphocyte isolation and culture. Lymphocytes were isolated from peripheral venous blood of healthy donors undergoing leukapheresis in the Department of Transfusion Medicine at the National Institutes of Health. This leukocyte-enriched blood was centrifuged on Ficoll-sodium diatrizoate (Organon Teknika, Durham, N.C.), and mononuclear cells were then separated by centrifugal elutriation as described previously (23). Lymphocyte preparations were highly enriched for T lymphocytes (>95% T cells) with <3% B cells, <10% natural killer cells, and <1% monocytes as determined by labeling with cell-specific fluorochrome-conjugated antibodies and analysis by flow microfluorometry (FACScan; Becton Dickinson, Mt. View, Calif.). In additional experiments, lymphocytes were labeled with anti-CD4 or anti-CD8 fluorochrome-conjugated monoclonal antibodies (Becton Dickinson) for 30 min at 5°C, washed, and sorted into fluorescence-positive and -negative populations on a FACStar Plus flow cytometer (Becton Dickinson). Lymphocytes were washed twice and resuspended in RPMI 1640 supplemented with a final concentration of 2% fetal bovine serum (GIBCO; medium).

Lymphocytes were cultured at 5×10^6/0.5 ml of medium in polypropylene tubes (17 by 100 mm) (Falcon, Becton Dickinson, Lincoln Park, N.J.). Leukotoxin was diluted in medium and added in 50-μl aliquots to the cultures, which were then incubated at 37°C in 5% CO_2 and high humidity. After the specified period, the cells were centrifuged at 150 × *g* for 5 min, washed, and resuspended in cold phosphate-buffered saline (PBS) (0.05 M PO_4, 0.15 M NaCl).

Viability and cell size. Lymphocyte viability was assessed by propidium iodide (PI) uptake and ^{51}Cr release. In PI assays, cells (10^6/ml) that had been incubated with or without leukotoxin were suspended in PBS containing PI (5 μg/ml) and incubated at 23°C for 5 min. PI uptake, indicative of membrane permeability that occurs in dying cells, was then determined by flow microfluorometry. PI binds to DNA and causes the cells to fluoresce red when activated with UV light (11). Cell size was simultaneously determined by the amount of forward light scatter which is the amount of light deflected as the cell passes through the laser beam and is proportional to the size of the cell.

^{51}Cr release was determined as described previously (12). Lymphocytes (5×10^7/ml) were labeled with 0.5 μCi of $Na_2{}^{51}CrO_4$ (Dupont, NEN Research Products, Boston, Mass.), washed, and divided into aliquots (5×10^5 per well) in microculture plates. Various concentrations of leukotoxin were added to triplicate wells before incubation at 37°C in 5% CO_2–95% air for 3 h. After sedimentation of cells, the amount of radiolabel released into 100 μl of the supernatant was measured by gamma spectroscopy. Total incorporated label was determined by lysis of cells in 0.1% Triton X-100 (12).

DNA fragmentation. DNA fragmentation was assessed as previously described (13, 16). In brief, lymphocytes were lysed and centrifuged at 13,000 × *g* for 10 min. DNA in both the supernatant (low molecular weight, fragmented) and pellet (high molecular weight, intact) were quantified by the diphenylamine reaction (4). The percentage of DNA fragmentation equals the amount of DNA in the supernatant divided by the total amount of DNA recovered in the sample (pellet plus supernatant) times 100.

DNA fragmentation was characterized by electrophoresis in agarose (1, 13, 16). DNA from 10^7 cells was purified from the supernatants of cell lysates centrifuged at 13,000 × *g* for 10 min. The samples were treated with proteinase K (0.1 μg/ml) at 50°C for 20 h, extracted with phenol-chloroform-isoamyl alcohol (25:24:1), and precipitated with cold isopropanol. Contaminating RNA was digested with DNase-free RNase (Sigma; 1 μg/ml) at 37°C for 3 h, and DNA was reprecipitated with ethanol. Total recovered DNA was electrophoresed for 90 min in 1% agarose in 40 mM Tris–acetate–2 mM EDTA buffer (pH 8) (Advanced Biotechnologies, Columbia, Md.). The molecular weights of DNA fragments were estimated from a 1-kb standard ladder (Bethesda Research Laboratories, Gaithersburg, Md.).

Transmission electron microscopy. Control and toxin-treated cells were centrifuged at 50 × *g* (5 min, 5°C), washed in 0.1 M cacodylate buffer, and fixed in PBS containing 2% paraformaldehyde and 2% glutaraldehyde for 2 h. Fixed cells were washed in cacodylate buffer and postfixed in 1% osmium tetroxide in cacodylate buffer for 1 h. After washing, cells were stained with 0.5% aqueous uranyl acetate for 2 h, dehydrated in ethanol, embedded in Spurr epoxy resin, sectioned, and examined with a Zeiss model 10A electron microscope.

Data analysis. Results from PI uptake and DNA fragmentation assays are expressed as the mean (± standard deviation) of replicate cultures of cells from individual donors and are representative of data from three or more donors. Statistical differences between tests and controls were calculated by Student's *t* test with significance at $P < 0.05$.

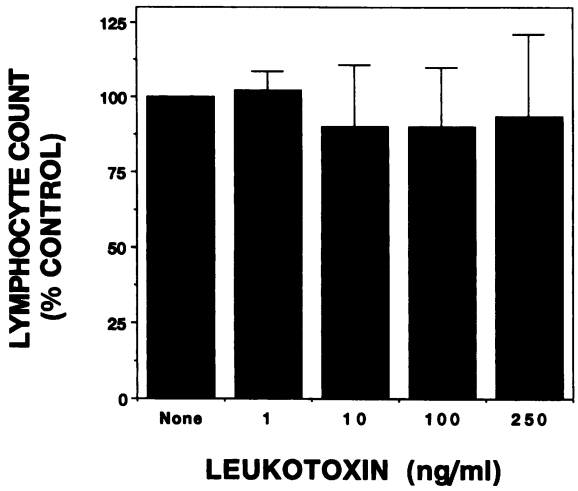

FIG. 1. Effects of *A. actinomycetemcomitans* leukotoxin on total number of human T cells. Lymphocytes (5×10^6/0.5 ml) were incubated with the indicated concentrations of leukotoxin. After 24 h, the cells were washed and then counted on a Coulter Counter. Results are averaged data from three donors (± standard deviation) and are expressed as percentage of cells recovered from control cultures incubated with no leukotoxin (3×10^6).

RESULTS

Cell recovery and propidium iodide uptake. Human peripheral blood lymphocytes, highly enriched for T cells, were incubated at 37°C with leukotoxin at final concentrations of 0 to 250 ng/ml. In contrast to the marked destruction of myeloid cells which occurs during even shorter intervals (12, 19, 20), leukotoxin had no significant effect on the total number of lymphocytes recovered from 24-h cultures (Fig. 1). However, leukotoxin caused a concentration-dependent increase in PI uptake, a more sensitive assay of cell death (11) (Fig. 2). Nearly 70% of the cells were PI positive (PI^+) when treated with leukotoxin at 250 ng/ml, indicating that most T cells were susceptible to the lethal effects of the toxin under these conditions. T cells labeled with fluorochrome-conjugated anti-CD4 (helper cell) or anti-CD8 (suppressor cell) monoclonal antibodies and separated into $CD4^+$, $CD4^-$, $CD8^+$, and $CD8^-$ subsets by flow cytometry demonstrated equivalent numbers of PI^+ cells when treated with leukotoxin (100 ng/ml) (not shown), suggesting that susceptibility to toxin was unrelated to these phenotypically and functionally defined populations.

On the basis of the ability of leukotoxin to induce PI uptake in T cells after 24 h, we carried out kinetic studies to establish the rate of progression of cytotoxicity. The percentage of PI^+ cells in cultures incubated with leukotoxin increased rapidly during the first 10 h of culture and then more slowly over the remaining 32 h (Fig. 3). Control cultures with no leukotoxin demonstrated a slower, progressive increase in PI^+ cells over the entire period. Statistical analyses of these data indicated that leukotoxin is lethal for T cells, provided that the cells are exposed to the toxin for >1.5 h.

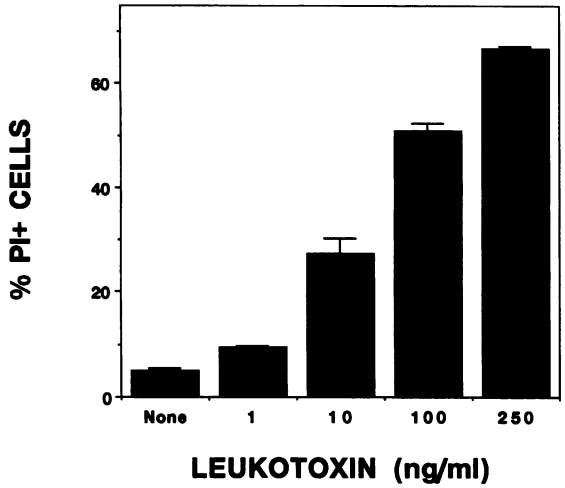

FIG. 2. Effects of leukotoxin on viability of human T cells. Lymphocytes were incubated with the indicated concentrations of leukotoxin as described in the legend to Fig. 1 and evaluated for percentage of PI^+ cells. Results are means (± standard deviations) of triplicate samples from one of three representative donors.

^{51}Cr release. To evaluate the effect of leukotoxin on plasma membrane integrity, lymphocytes were labeled with ^{51}Cr and exposed to toxin for 3 h. ^{51}Cr incorporates into the cytoplasmic compartment and is released when the plasma membrane is damaged during toxic insult or cell death (12). As shown in Fig. 4, leukotoxin caused the release of ^{51}Cr, clearly demonstrating an increase in membrane permeability. Inactivation of leukotoxin by heating (70°C for 30 min) eliminated enhanced PI uptake (not shown) and ^{51}Cr release (Fig. 4) even at toxin concentrations as high as 100 ng/ml. Furthermore, lymphocytes did not become PI^+ or release ^{51}Cr when cultured with leukotoxin at 5°C (not shown), indicating that lymphotoxicity was an active process that occurs at physiological temperatures.

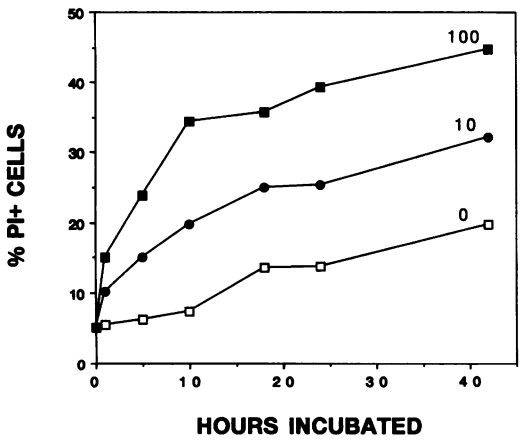

FIG. 3. Kinetics of leukotoxin-induced killing of T cells. Lymphocytes were incubated with leukotoxin at concentrations of 0 (□), 10 (●), or 100 (■) ng/ml as described in the legend to Fig. 1. At the indicated times, samples were analyzed for percentage of PI^+ cells. Data are means of duplicate or triplicate samples and are representative of data from three donors. Standard deviations were <10% of the mean.

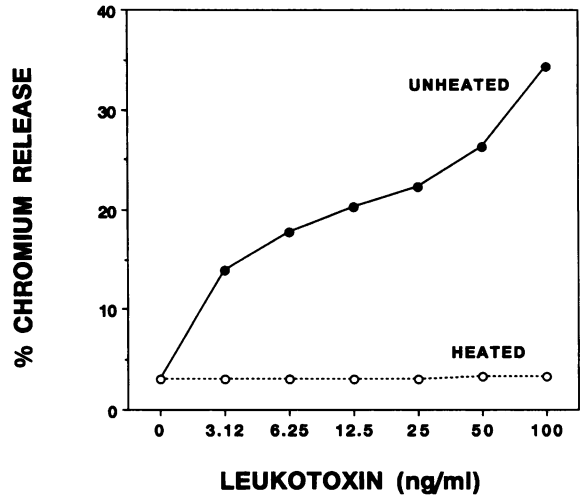

FIG. 4. ^{51}Cr release from T cells treated with leukotoxin. Lymphocytes (5×10^7) were labeled with ^{51}Cr, washed, and incubated with the indicated concentrations of unheated or heated (70°C, 30 min) leukotoxin for 3 h. Results are means from triplicate cultures and are expressed as percentage of total incorporated ^{51}Cr (12,138 cpm) that was released into the culture supernatant. Standard deviations were ≤10% of the mean. Data are from one of five representative experiments.

Ultrastructural morphology. Since the morphology of a dying cell can provide valuable information about the mechanism of cell death (25), lymphocytes were cultured with or without leukotoxin for 15 h and examined at the ultrastructural level by transmission electron microscopy. Compared with control cells, represented in Fig. 5A, T cells incubated with leukotoxin (100 ng/ml) exhibited three distinct morphological profiles (Fig. 5B). In the majority of treated cells (~60%), there was apparent damage to the plasma membrane, dissolution of cytoplasmic organelles, and poor staining of the nucleus, which are characteristic features of necrosis (25). Approximately 30% of the cells appeared similar if not identical to untreated control cells, suggesting that some cells were resistant to damage by toxin under these conditions. A third subset of cells (~10%) demonstrated cell shrinkage, an intact plasma membrane, and a condensed nucleus and cytoplasm, which are consistent with apoptosis associated with programmed cell death (PCD) (9, 25). Thus, these morphological data suggest that leukotoxin either had no effect, induced necrosis, or initiated PCD in lymphocytes.

DNA fragmentation. PCD is characterized by cleavage of chromosomal DNA into low-molecular-weight fragments that remain in the supernatants of cell lysates after centrifugation at 13,000 × *g* for 10 min (8, 9, 13, 16, 24). Quantification of low-molecular-weight DNA in leukotoxin-treated lymphocytes indicated that leukotoxin in concentrations of ≥10 ng/ml induced a significant increase in the proportion of fragmented DNA, with maximal fragmentation occurring in cells treated with leukotoxin at 100 ng/ml (Fig. 6). Cultures treated with leukotoxin at >100 ng/ml typically exhibited less-measurable DNA fragmentation.

To further evaluate the characteristics of DNA fragmentation, low-molecular-weight DNA was isolated from T cells treated with leukotoxin and electrophoresed in 1% agarose (Fig. 7). Consistent with the data in Fig. 6, cells cultured for 24 h with leukotoxin at concentrations of 10 to 100 ng/ml

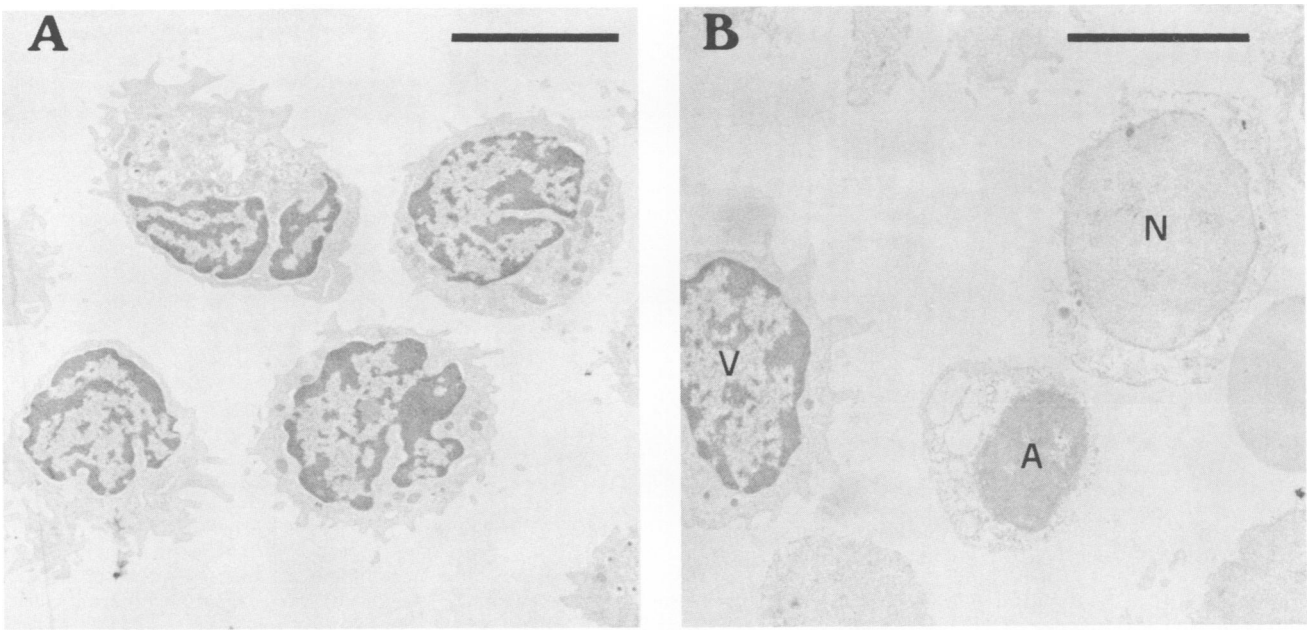

FIG. 5. Ultrastructural morphology of leukotoxin-treated T cells. Lymphocytes were incubated without (A) or with (B) leukotoxin (100 ng/ml) for 15 h and prepared for examination by transmission electron microscopy. Cells in panel B had morphological characteristics consistent with viable (V), apoptotic (A), or necrotic (N) cells. Bar = 5 μm.

contained the largest amounts of fragmented DNA. Moreover, DNA migrated in bands which were multiple integers of approximately 200 bp, indicating cleavage between chromosomal nucleosomes, a cardinal feature of PCD (8, 9, 24).

To better characterize the progression of DNA cleavage in leukotoxin-treated lymphocytes, DNA fragmentation was assessed over a 2-day period after exposure to leukotoxin. Leukotoxin induced rapid DNA fragmentation that was concentration dependent and peaked at 20 h (Fig. 8). Approximately 30% of the total DNA in these cultures was fragmented. By comparison, control cells showed a slower increase in DNA fragmentation which peaked at 20 h. Differences in DNA fragmentation in control and leukotoxin-treated (10 or 100 ng/ml) cells were statistically significant at all time points after 1.5 h.

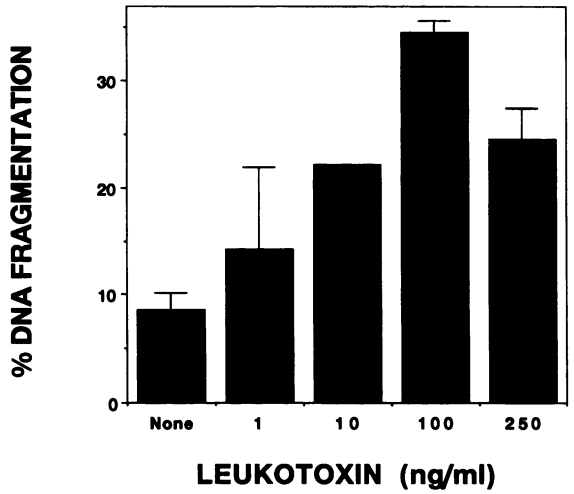

FIG. 6. Effects of leukotoxin on DNA fragmentation in human T cells. Lymphocytes were incubated with the indicated concentrations of leukotoxin as described in the legend to Fig. 1 and evaluated for percentage of DNA fragmentation. Results are means (± standard deviations) of triplicate samples from one of three representative donors. At 10 ng/ml, the standard deviation was too small to be shown in this figure.

DISCUSSION

During periodontal infections with toxin-producing *A. actinomycetemcomitans*, lymphocytes that are present in the chronic lesions may be exposed to leukotoxin for extended periods of time (2, 7, 18). Since previous studies reported that human lymphocytes are not killed after exposure to purified leukotoxin (100 ng/ml) for ≤1.5 h (15), in this study we investigated the effects of leukotoxin on lymphocytes during culture for up to 2 days. Our data indicate that T cells exposed to leukotoxin in concentrations of ≥10 ng/ml demonstrated significant PI uptake and ^{51}Cr release, which are sensitive markers of cell death (11, 12). After 24 h, approximately 50% of the lymphocytes were PI^+ when exposed to leukotoxin at a concentration of 100 ng/ml. Although this concentration is substantially higher than that needed to kill 50% of neutrophils (1 ng) or HL-60 cells (2 ng) within 1 h (12, 17), such concentrations may be achieved in tissues in immediate juxtaposition to toxin-secreting *A. actinomycetemcomitans*.

In general, cells in multicellular organisms die by one of two pathways, necrosis or PCD (9, 25). Necrosis occurs in cells injured by physical or toxic factors, such as complement activation, heating, or freeze-thawing, which damage the plasma membrane and cause osmotic swelling and rapid lysis. In contrast, PCD is an endogenous process induced in a cell-specific manner by exogenous factors including antibodies or ligands to surface receptors (22), glucocorticoid hormones (24), cytotoxic effector cells (8), ionizing radiation (16), and chemical toxins (14). In PCD, the plasma

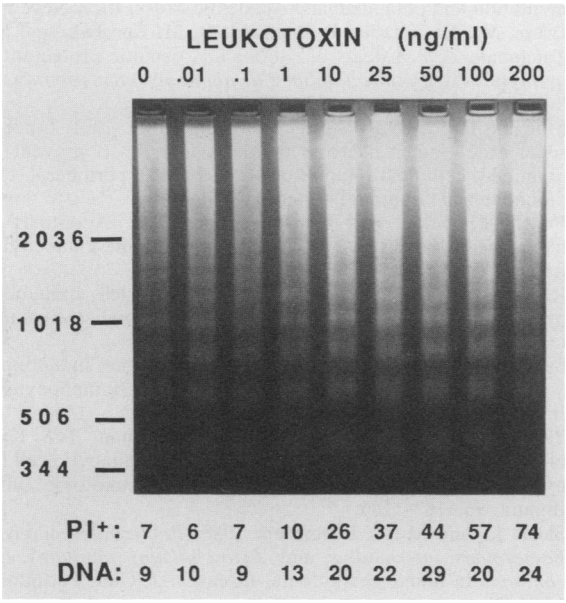

FIG. 7. Electrophoretic analysis of fragmented DNA from T cells. Lymphocytes (10^7) were incubated with the indicated concentrations of leukotoxin for 16 h, lysed, and centrifuged at 13,000 × *g* for 10 min. Low-molecular-weight DNA was isolated from the supernatants and electrophoresed in 1% agarose. Similar DNA electrophoretic patterns were obtained with toxin-treated cells from four other donors. Standard DNA sizes are indicated in base pairs. The percentages of PI^+ cells and DNA fragmentation in the cultures are indicated at the bottom.

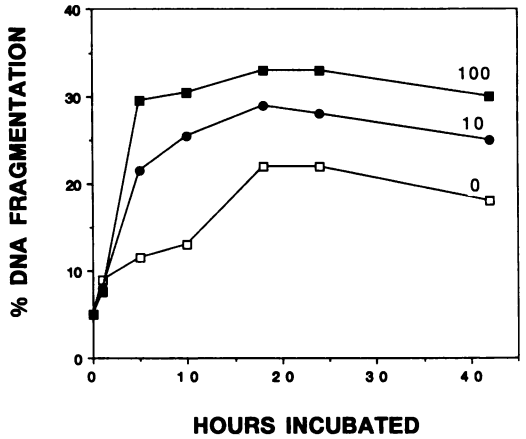

FIG. 8. Kinetics of leukotoxin-induced DNA fragmentation in T cells. Lymphocytes were incubated with leukotoxin at concentrations of 0 (□), 10 (●), or 100 (■) ng/ml. At the indicated times, samples were analyzed for percentage of DNA fragmentation. Data are means of duplicate or triplicate samples and are representative of data from three donors. Standard deviations were <10% of the mean.

membranes remain relatively intact, there is little or no cell lysis, and there is cell shrinkage and condensation of the cytoplasmic and nuclear material. In addition, PCD is characterized by cleavage of chromosomal DNA into oligonucleosome-sized (~200 bp) fragments (8, 24). The two pathways of cell death can be distinguished by ultrastructural morphology and demonstration of 200-bp DNA fragments (9, 25).

Our data suggest that leukotoxin kills T cells by pathways resembling both necrosis and PCD. Damage to the plasma membrane, as demonstrated by the ultrastructural morphology and release of ^{51}Cr from the cytoplasmic compartment of toxin-treated cells, suggested death by necrosis, whereas the appearance of apoptotic cells in the cultures and increased cleavage of DNA into multiple integers of 200-bp (i.e., oligonucleosome) fragments suggested death by PCD. The capacity to induce cell death by both pathways is not unusual and has been described for other pore-forming bacterial and mammalian cytolysins (3, 10). Whether the toxin induces necrosis or PCD appears to be related to how quickly the cell membrane is damaged and the extent of such damage (10). Rapid or extensive damage to the plasma membranes by toxin favors necrosis and little DNA fragmentation, whereas minimal or slow damage appears to favor PCD and extensive DNA fragmentation. In this regard, we found a maximum amount of DNA fragmentation in T cells treated with 10 to 100 ng of leukotoxin per ml, whereas at concentrations of >100 ng/ml, DNA fragmentation remained unchanged or decreased. Thus, PCD may be induced in T cells at low concentrations of leukotoxin, whereas necrosis appears to be the primary mode of cell death at high concentrations of the toxin. However, it remains unclear why the number of T cells did not decrease sharply in cultures treated with high concentrations of leukotoxin for periods as long as 24 h, since a substantial decrease in cell number due to lysis is characteristic of necrosis (9, 25).

Whether specific subsets of T cells demonstrate differential susceptibility to leukotoxin or undergo death by a particular pathway remains to be determined. T cells separated by flow cytometry into $CD4^+$, $CD4^-$, $CD8^+$, and $CD8^-$ subsets demonstrated equivalent PI uptake, suggesting that susceptibility to leukotoxin is not biased in favor of one or the other of these major T cell populations.

The mechanism by which leukotoxin induces DNA fragmentation in T cells is currently unknown. Although it is possible that leukotoxin caused DNA fragmentation by directly functioning as a nuclease, as was recently reported for diptheria toxin (5, 6), we found no cleavage of purified T cell DNA after 24 h of incubation with leukotoxin (100 ng/ml) (unpublished data), which argues against toxin-associated nuclease activity. Leukotoxin may directly or indirectly cause the activation of an endogenous nuclease that cleaves chromosomal DNA in the linker region between nucleosomes (8, 24). Activation of this enzyme is typical of PCD and may be associated with the nuclear condensation in apoptotic cells seen in leukotoxin-treated T cell cultures (9, 24, 25).

The results presented here provide new insight into a possible role of *A. actinomycetemcomitans* leukotoxin in the pathogenesis of chronic periodontal diseases. In addition to its dramatic cytotoxic effects on neutrophils and monocytes, leukotoxin clearly is lethal to T lymphocytes. The capacity of leukotoxin to initiate a process resembling necrosis as well as PCD in lymphocytes implicates important molecular mechanisms by which *A. actinomycetemcomitans* could compromise the local cellular and humoral immune responses in periodontal lesions.

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