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Evidence for Apoptosis of Murine Macrophages by *Actinobacillus actinomycetemcomitans* Infection

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The gram-negative bacterium *Actinobacillus actinomycetemcomitans* is considered an important etiological agent in periodontal diseases. In this study, we show that *A. actinomycetemcomitans* strains are cytotoxic for the murine macrophage cell line J774.1. On the other hand, *Porphyromonas gingivalis* strains, other gram-negative oral species implicated in adult periodontitis, showed weak cytotoxic effects. For this to occur, *A. actinomycetemcomitans* had to gain entry into the macrophages, since cytotoxicity was prevented by cytochalasin D. We demonstrate that cell death induced by *A. actinomycetemcomitans* Y4 occurs through apoptosis, as shown by changes in nuclear morphology, an increase in the proportion of fragmented DNA, and the typical ladder pattern of DNA fragmentation indicative of apoptosis. We further sought to determine whether the cytotoxicity induced by *A. actinomycetemcomitans* Y4 could be modulated by the protein kinase inhibitors H7 and HA1004. Apoptotic cell death induced by *A. actinomycetemcomitans* Y4 was suppressed by H7 but was relatively unaffected by HA1004. These findings suggest that the signals of protein kinases may regulate apoptosis induced by *A. actinomycetemcomitans* Y4. The ability of *A. actinomycetemcomitans* to promote the apoptosis of macrophages may be important for the initiation of infection and the development of periodontal diseases.

The important roles of specific bacteria, such as *Actinobacillus actinomycetemcomitans*, *Porphyromonas gingivalis*, *Bacteroides forsythus*, *Eikenella corrodens*, *Fusobacterium nucleatum*, and *Wolinella recta*, in the etiology and pathogenesis of periodontal diseases are well documented (18, 25). *A. actinomycetemcomitans*, a nonmotile, capnophilic, and gram-negative bacterium, has been recovered from within periodontally diseased gingival tissues (3) and implicated in the pathogenesis of juvenile and adult periodontitis (26, 27). *A. actinomycetemcomitans* elaborates a multiplicity of virulence factor and tissue-damaging products. A majority of isolates of *A. actinomycetemcomitans* synthesizes leukotoxin, which rapidly kills human polymorphonuclear neutrophils and monocytes (28, 31). It has been reported that leukotoxin may be continuously released into the gingival tissue during chronic infection with *A. actinomycetemcomitans* (2). In addition, Mangan et al. (15) reported that purified leukotoxin kills human T lymphocytes in a time- and dose-dependent manner.

The initial event in the pathogenesis of most bacterial diseases is microbial invasion of host cells and tissues (5). *Shigella* spp., *Salmonella* spp., *Yersinia* spp., and *Escherichia coli* can invade epithelial cells and cause several disease symptoms in humans (5). Recently, Meyer et al. (16) developed an in vitro cell culture invasion model for *A. actinomycetemcomitans* and provided evidence for the invasion of human epithelial cells by *A. actinomycetemcomitans*. It was demonstrated that *A. actinomycetemcomitans* invasion occurs through cytochalasin D and cycloheximide-sensitive processes (16).

Apoptosis has been shown to play important roles in the control of the immune, hematopoietic, and developmental systems (23). Its hallmark biochemical feature is endonuclease-mediated cleavage of internucleosomal DNA linker sections. Recently, it has been shown that *Shigella flexneri*, the etiological agent of dysentery, and *Bordetella pertussis*, the causative agent of whooping cough in humans, induce apoptosis in macrophages (11, 32). In this study, we report that the periodontopathic bacterium *A. actinomycetemcomitans* is cytotoxic for the murine macrophage cell line J774.1. Furthermore, we have investigated whether *A. actinomycetemcomitans* can induce apoptosis and whether protein kinase inhibitors have the potential to suppress apoptosis induced by *A. actinomycetemcomitans.*

MATERIALS AND METHODS

Bacterial strains and growth conditions. *A. actinomycetemcomitans* ATCC 29523 (serotype a), Y4 (serotype b), NCTC 9710 (serotype c), ST-1 (24), and TN-1 (clinically isolated, serotype a), which were maintained in our laboratory, were used in this study. These strains were grown in Todd-Hewitt broth (Difco Laboratories, Detroit, Mich.) supplemented with 1% (wt/vol) yeast extract at 37°C for 2 days in an atmosphere of 5% CO_2 in air (20). *P. gingivalis* 381 and W83 were grown in Trypticase soy broth (BBL Microbiology Systems, Cockeysville, Md.) supplemented with 1% (wt/vol) yeast extract, 0.0005% (wt/vol) hemin, and 0.0001% (wt/vol) menadione at 37°C for 3 days in an atmosphere of 80% N_2, 10% CO_2, and 10% H_2.

J774.1 cell culture. The murine macrophage cell line J774.1 was obtained from the Japanese Cancer Research Resources Bank and maintained in RPMI 1640 medium (GIBCO Laboratories, Grand Island, N.Y.) supplemented with 10% heat-inactivated fetal calf serum, penicillin G (100 U/ml), and streptomycin (100 μg/ml).

Cytotoxicity assay. J774.1 cells were plated in a 96-well plate at a concentration of 2×10^4 cells per well 1 day before the experiment. Bacterial strains were harvested by centrifugation and resuspended in RPMI 1640 medium without antibiotics to an optical density of 0.55 at 550 nm, corresponding to approximately 5×10^9 bacteria. Bacterial suspension was added to the wells, and the plates were centrifuged at $1,000 \times g$ for 10 min at 4°C prior to incubation at 37°C for 1 h in an atmosphere of 5% CO_2 in air. Macrophages infected at bacterium/cell ratios of 50:1, 500:1, and 5,000:1 were washed three times with RPMI 1640 medium containing penicillin G, streptomycin, and gentamicin (200 μg/ml) to remove extracellular bacteria. The infected cells were cultured with RPMI 1640 medium containing 5% fetal calf serum and antibiotics for 24 to 72 h (4). In some experiments, the infected cells were cultured with various concentrations of a DNA endonuclease inhibitor, aurintricarboxylic acid (ATA), an inhibitor of actin

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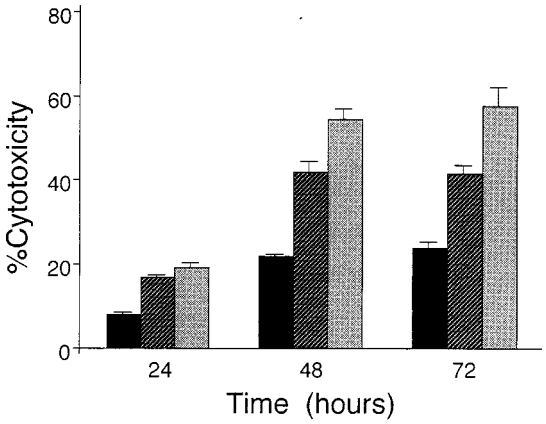

FIG. 1. Cytotoxic effect of *A. actinomycetemcomitans* Y4 on J774.1 cells. J774.1 cells (2×10^4) were infected with *A. actinomycetemcomitans* Y4 at bacterium/cell ratios of 50:1 (■), 500:1 (▨), and 5,000:1 (▤). The percent cytotoxicity was determined at 24, 48, and 72 h postinfection by the MTT viability assay as described in Materials and Methods. Data are expressed as the means ± standard deviations of six cultures. The experiment was performed three times, and similar results were obtained from each experiment.

polymerization, cytochalasin D, and a protein kinase inhibitor such as 1-(5-isoquinolinesulfonyl)-2-methylpiperazine dihydrochloride (H7; Seikagaku Kogyo Co., Tokyo, Japan) and *N*-(2-guanidinoethyl)-5-isoquinolinesulfonamide hydrochloride (HA1004; Seikagaku Kogyo Co.) in concentrations that are below their toxicity limits. Stock MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide, 2.5 mg/ml; Sigma Chemical Co., St. Louis, Mo.) solution (20 μl/well) was added to the wells, and the plates were incubated for the final 4 h. After acid-isopropanol (100 μl of 0.04 N HCl in isopropanol) was added and mixed thoroughly, the plates were read on a Multiskan Bichromatic microplate reader (Labsystems, Helsinki, Finland), using a test wavelength of 570 nm and a reference wavelength of 620 nm (the MTT viability assay) (19). The percent cytotoxicity was calculated by the following formula: percent cytotoxicity = 100 × (1 − optical density at 570 to 620 nm with stimulants/optical density at 570 to 620 nm without stimulants).

Cytotoxicity was also determined with the CytoTox 96 assay (Promega, Madison, Wis.), which measured lactate dehydrogenase (LDH) activity. J774.1 cells (2×10^4) were infected with various amounts of *A. actinomycetemcomitans* Y4 as described above. After being cultured for 48 h, the culture supernatants were collected. LDH activity was measured in cell lysates, and culture supernatants were measured by the CytoTox 96 assay. The percent cytotoxicity was calculated according to the manufacturer's instructions (the LDH assay).

DNA fragmentation. J774.1 cells infected with *A. actinomycetemcomitans* Y4 were prepared as described above. The infected cells (2×10^4/well) were cultured in 96-well culture plates in an atmosphere of 5% CO_2 in air. After 10 h of culturing, the cells were washed with phosphate-buffered saline (PBS, pH 7.2) and lysed in hypotonic lysing buffer. Cell death detection by enzyme-linked immunosorbent assay (ELISA; Boehringer GmbH, Mannheim, Germany), a photometric enzyme immunoassay, was used for the quantitative in vitro determination of cytoplasmic histone-associated DNA fragments as described in the manufacturer's instructions. In addition, DNA fragmentation was characterized by electrophoresis in a 2% agarose gel (17). J774.1 cells were infected as described above at bacterium/cell ratios of 50:1, 500:1, and 5,000:1. After 24 h of culturing, the cells were lysed with 10 mM Tris (pH 7.4)–5 mM EDTA–1% Triton X-100. The lysates were centrifuged to remove integral nuclei. The supernatants were digested with RNase (0.5 mg/ml) for 1 h at 37°C, incubated with proteinase K (10 mg/ml) for 1 h at 50°C, and extracted with phenol-chloroform (1:1, vol/vol) before precipitation with ethanol. The precipitates were dried and solubilized in 10 mM Tris (pH 8.0)–1 mM EDTA. Electrophoresis was performed with a 2% agarose gel, which was stained with ethidium bromide.

Cytochemical staining of apoptotic cells. Morphological changes in the nuclear chromatin of J774.1 cells were detected with Apop Tag (Oncor, Inc., Gaithersburg, Md.), an in situ apoptosis detection kit. In brief, J774.1 cells were infected as described above at bacterium/cell ratios of 500:1 and 5,000:1 in eight-well chamber slides (Nunc, Inc., Naperville, Ill.). The cells were washed with PBS, fixed with 4% formaldehyde in PBS, and stained as described in the manufacturer's instructions. The labeling target of the Apop Tag kit is the multitude of new 3′-OH DNA ends generated by DNA fragmentation (6).

RESULTS

Cytotoxic effects of *A. actinomycetemcomitans* strains. *A. actinomycetemcomitans* cytotoxicity was tested on the murine macrophage cell line J774.1 cells by the MTT viability assay. *A. actinomycetemcomitans* Y4 was able to induce cytotoxicity after 24 h of incubation. J774.1 cells infected with *A. actinomycetemcomitans* Y4 at a bacterium/cell ratio of 5,000:1 showed 20.3% ± 1.6%, 54.4% ± 2.3%, and 57.6% ± 4.3% cytotoxicity after being cultured for 24, 48, and 72 h, respectively (Fig. 1). We compared cytotoxic activities of *A. actinomycetemcomitans* ATCC 29523, Y4, NCTC 9710, ST-1, and TN-1, and *P. gingivalis* 381 and W83 after 48 h of incubation by the MTT viability assay. All *A. actinomycetemcomitans* strains used in this study strongly induced cytotoxicity at bacterium/cell ratios of 500:1 and 5,000:1. However, *P. gingivalis* 381 and W83 showed weak cytotoxic effects on J774.1 cells at the same bacterium/cell ratios. J774.1 infected with *P. gingivalis* strains showed below 22% cytotoxicity even after 72 h of incubation (Fig. 2). We also examined the cytotoxic effect of *A. actinomycetemcomitans* Y4 on J774.1 cells with the CytoTox 96 assay, which measured LDH activity released into the medium. *A. actinomycetemcomitans* Y4 killed J774.1 effectively, with maximal cytotoxicity (42.8% ± 1.6% cytotoxicity) at a bacterium/cell ratio of 5,000:1 (Fig. 3).

To examine whether *A. actinomycetemcomitans* needed to be within the cytoplasm of J774.1 cells to induce cell death, we examined cytotoxic effects of *A. actinomycetemcomitans* Y4 on J774.1 cells in the presence or absence of cytochalasin D (1 μg/ml), an inhibitor of actin polymerization. Under these conditions, cytochalasin D decreased the cytotoxicity of *A. actinomycetemcomitans* Y4 on J774.1 cells at bacterium/cell ratios of 50:1, 500:1, and 5,000:1 (Fig. 4). Various concentrations of ATA, a DNA endonuclease inhibitor, were added to infected J774.1 cells. It was found that ATA doses dependently decreased the percentage of cytotoxicity of J774.1 cells infected with *A. actinomycetemcomitans* Y4 at bacterium/cell ratios of 50:1, 500:1, and 5,000:1 (Table 1).

DNA fragmentation of J774.1 cells infected with *A. actinomycetemcomitans* Y4. To determine whether *A. actinomycetem-*

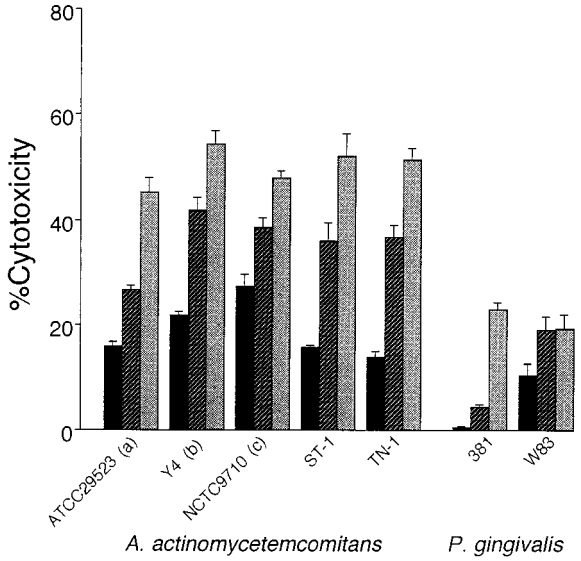

FIG. 2. Cell death of J774.1 cells induced by several strains of *A. actinomycetemcomitans* and *P. gingivalis*. J774.1 cells (2×10^4) were infected with *A. actinomycetemcomitans* ATCC 29523, Y4, NCTC 9710, ST-1, and TN-1, and *P. gingivalis* 381 and W83 at bacterium/cell ratios of 50:1 (■), 500:1 (▨), and 5,000:1 (▤). The percent cytotoxicity was determined at 48 h postinfection by the MTT viability assay as described in Materials and Methods. Data are expressed as the means ± standard deviations of six cultures. The experiment was performed three times, and similar results were obtained from each experiment.

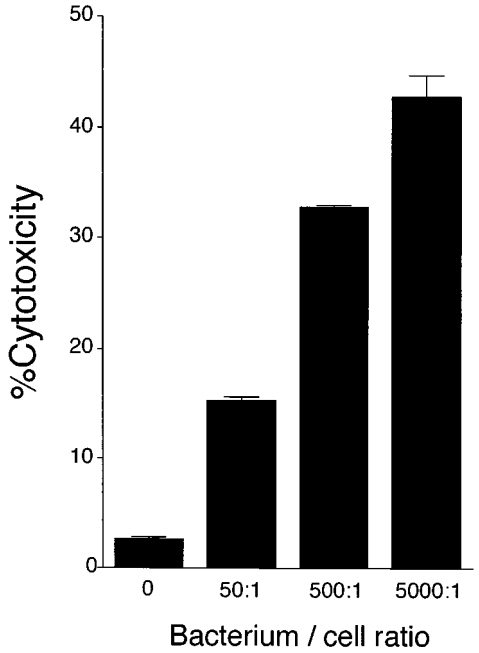

FIG. 3. Cell death of J774.1 cells induced by *A. actinomycetemcomitans* Y4 by LDH assay. J774.1 cells (2×10^4) were infected with various amounts of *A. actinomycetemcomitans* Y4. After being cultured for 48 h, the culture supernatants were collected. LDH activity was measured in cell lysates and culture supernatants with a colorimetric kit, the CytoTox 96 assay. The percent cytotoxicity was calculated according to the manufacturer's instructions. Data are expressed as the means ± standard deviations of five cultures. The experiment was performed two times, and similar results were obtained from each experiment.

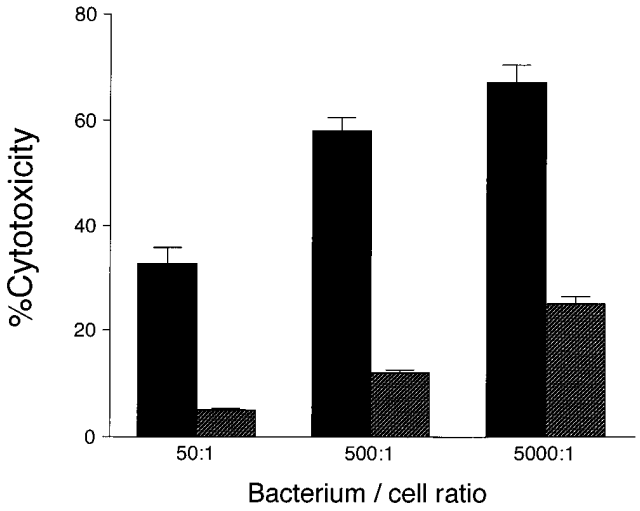

FIG. 4. Effect of cytochalasin D on cytotoxicity induced by *A. actinomycetemcomitans* Y4. J774.1 cells (2×10^4) were infected with *A. actinomycetemcomitans* Y4 at bacterium/cell ratios of 50:1, 500:1, and 5,000:1. The cytotoxicity of *A. actinomycetemcomitans* Y4 was determined in the absence (■) or presence (▨) of cytochalasin D (1 μg/ml) by the MTT viability assay. The percent cytotoxicity was determined as described in Materials and Methods. Data are expressed as the means ± standard deviations of six cultures. The experiment was performed three times, and similar results were obtained from each experiment.

comitans Y4 promoted apoptosis, we examined the induction of DNA fragmentation of J774.1 cells. As shown in Fig. 5, a nucleosome ladder pattern of DNA degradation was observed in J774.1 cells infected with *A. actinomycetemcomitans* Y4. This effect was not obtained with untreated J774.1 cells. DNA degradation was also determined by photometric quantitation of fragmented DNA. The extent of DNA fragmentation observed in J774.1 cells infected with *A. actinomycetemcomitans* increased at bacterium/cell ratios of 500:1 and 5,000:1. The percent DNA fragmentation in J774.1 cells infected with *A. actinomycetemcomitans* Y4 at bacterium/cell ratios of 500:1 and 5,000:1 were 45.5% ± 1.1% and 49.3% ± 1.0%, respectively (Fig. 6). We investigated the morphology of J774.1 cells infected with *A. actinomycetemcomitans* Y4. After 24 h of incubation, J774.1 cells infected with *A. actinomycetemcomitans* Y4 began to become round and to detach from the surface, which is associated with macrophage cell death (Fig. 7B and C), and revealed chromatin condensation, which is associated with the cells undergoing apoptosis (Fig. 7E and F).

Role of protein kinase C in the mechanism of *A. actinomycetemcomitans* Y4-induced apoptosis. We explored a potential role for protein kinase signal transduction in *A. actinomycetemcomitans* Y4-induced macrophage apoptosis. We chose to examine the effects of the two protein kinase inhibitors, H7 and HA1004. H7 (an inhibitor of protein kinase C and cyclic AMP [cAMP]-dependent protein kinases) suppressed *A. actinomycetemcomitans* Y4-induced cytotoxicity of J774.1 cells in a dose-related manner as determined by the MTT viability assay. In contrast, concentrations up to 20 μM HA1004 (a more specific inhibitor of cAMP-dependent protein kinases) showed no effect on *A. actinomycetemcomitans* Y4-induced cytotoxicity (Fig. 8).

DISCUSSION

Many bacterial species have the capacity to invade eukaryotic cells. It has been believed that the bacteria gain entry into the cells through the mechanism of phagocytosis (5). It has recently been shown that *S. flexneri*, the etiological agent of dysentery, induces apoptosis in infected macrophages (32). Khelef et al. also reported that *Bordetella pertussis*, the etiological agent of whooping cough in humans, induces apoptosis in a murine macrophage cell line and in alveolar macrophages in primary culture (11). In this study, the murine macrophage cell line J774.1 was used to assess the cytotoxic properties of *A. actinomycetemcomitans* (4). We report on macrophage cell death induced by the periodontopathic bacterium *A. actinomycetemcomitans*. Cytochalasin D, a potent microfilament inhibitor, completely inhibited the cytotoxicity of *A. actinomycetemcomitans* Y4 on J774.1 cells (Fig. 4), indicating that *A. actinomycetemcomitans* Y4 cytotoxicity occurs through a microfilament-dependent process. This finding demonstrates that *A. actinomycetemcomitans* is cytotoxic only when it is inside the cytoplasm. Furthermore, we suggest that *A. actinomycetemcomitans* induces cell death by apoptosis.

TABLE 1. ATA inhibition of cell death induced by *A. actinomycetemcomitans* Y4[a]

ATA (μM)	% Cytotoxicity (mean ± SD) of *A. actinomycetemcomitans* Y4 at bacterium/cell ratio:		
	50:1	500:1	5,000:1
0	34.2 ± 2.5	47.5 ± 3.5	63.4 ± 7.1
50	23.9 ± 1.5	40.1 ± 3.4	50.0 ± 2.6
100	14.6 ± 0.5	28.8 ± 0.9	41.8 ± 1.0
200	2.5 ± 0.2	19.2 ± 0.5	32.7 ± 3.7

[a] J774.1 cells (2×10^4) infected with *A. actinomycetemcomitans* Y4 were cultured with various concentrations of ATA. The percent cytotoxicity was calculated as described in Materials and Methods. Data shown are the means ± standard deviations of six cultures. The experiment was performed two times, and similar results were obtained from each experiment.

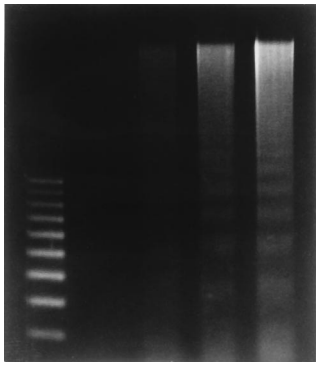

FIG. 5. *A. actinomycetemcomitans* Y4 infection induces internucleosomal DNA cleavage. The cellular DNA was isolated at 24 h postinfection from J774.1 cells infected with *A. actinomycetemcomitans* Y4 at bacterium/cell ratios of 50:1 (lane 2), 500:1 (lane 3), and 5,000:1 (lane 4). The DNA samples were analyzed by electrophoresis in a 2% agarose gel. Lane 1, the cellular DNA from untreated J774.1 cells; lane M, Superladder low double-stranded DNA marker kit (Gen Sura Laboratories, Inc., Del Mar, Calif.).

Recent work has shown that leukotoxin from *A. actinomycetemcomitans* kills up to 70% of human T lymphocytes in a time- and dose-dependent manner and induces increased cleavage of chromosomal DNA into nucleosome-sized fragments. These results suggest that leukotoxin kills T lymphocytes by the pathways resembling necrosis and apoptosis (15). Among the five strains of *A. actinomycetemcomitans* used in this study, *A. actinomycetemcomitans* ATCC 29523 is known to be a leukotoxin production-variable strain (21). *A. actinomycetemcomitans* TN-1, clinically isolated from a patient with juvenile periodontitis, proved to be a nonleukotoxic strain (data not shown). Molecular genetic studies have demonstrated that the leukotoxin of *A. actinomycetemcomitans* is a member of the repeats in the toxin family of bacterial cytolysin, including the alpha-hemolysin of *Pasteurella haemolytica* (12, 13, 14). In spite of the sequence similarities among these toxins, leukotoxin and hemolysin have distinctly different target-cell specificities (12). Taichman et al. demonstrated that the sonic extracts of leukotoxic *A. actinomycetemcomitans* strains yield a soluble, heat-labile substance (i.e., leukotoxin) which destroys human blood polymorphonuclear neutrophils, monocytes, and gingival crevice polymorphonuclear neutrophils (29). However, the cells derived from rabbits, rats, mice, and guinea pigs have been shown not to be susceptible to leukotoxin (29). These findings indicate that contamination with leukotoxin would not explain the ability of *A. actinomycetemcomitans* to induce cytotoxic effects on mouse macrophage cell line J774.1 cells.

The results presented here show that *A. actinomycetemcomitans* Y4-infected macrophages exhibit the characteristics of apoptosis linked to the activation of an internucleosomal nuclease. It was found that ATA, a cDNA endonuclease inhibitor, could efficiently increase the percentage of viable J774.1 cells infected with *A. actinomycetemcomitans* Y4 (Table 1). An ATA concentration of 200 μM achieved the strongest inhibitory effect on cytotoxicity induced by *A. actinomycetemcomitans* Y4 (Table 1). ATA (200 μM) did not affect normal cell growth of untreated J774.1 cells (data not shown). These findings suggest that cell death of J774.1 cells infected with *A. actinomycetemcomitans* Y4 occurs through apoptosis mediated by a cDNA endonuclease. We demonstrated that J774.1 cells infected with *A. actinomycetemcomitans* Y4 exhibit the typical ladder pattern of DNA fragmentation of apoptotic cells (Fig. 5). Apoptotic cell death induced by *A. actinomycetemcomitans* Y4 was also detectable by quantitative cell death detection ELISA (Fig. 6). In addition, staining the nuclei of J774.1 cells infected with *A. actinomycetemcomitans* Y4 for DNA showed chromatin condensation and other morphologic indications of apoptotic cell death (Fig. 7). It has been reported that visualization of focal in situ staining inside intact apoptotic nuclei correlates with the typical biochemical and morphological characteristics of apoptosis (6). Taken together, these findings indicate that cell death of *A. actinomycetemcomitans* Y4-infected J774.1 cells occurs through apoptosis. This is the first evidence that an infection with periodontopathic bacteria can induce apoptosis in its host cells.

Apoptosis is essential for normal development and the control of the immune and hematopoietic systems (23). This programmed cell death is distinct from accidental cell death or necrosis. Recently, Terai et al. have reported that acute human immunodeficiency virus type 1 infection of lymphoblasts and activated normal peripheral blood mononuclear cells induces apoptosis (30). Apoptosis has been proposed to combat AIDS in combination with several treatments, including antiviral agents, antibiotics, and antiapoptotic drugs (7). The ability of phagocytic macrophages to ingest and kill microorganisms is one of the major mechanisms whereby microbial invasion is thwarted by the host. The process of phagosomal acidification following infection by a microorganism appears to be a crucial step in the normal degradative process characteristic of macrophages (9). Recently, Barry and Eastman indicated that intracellular acidification is consistent with the involvement of endonuclease activation in apoptosis (1). In light of such proposals, the mechanism by which *A. actinomycetemcomitans* induces apoptosis remains an important area for future investigation.

To study the effects of protein kinase C activity, *A. actino-*

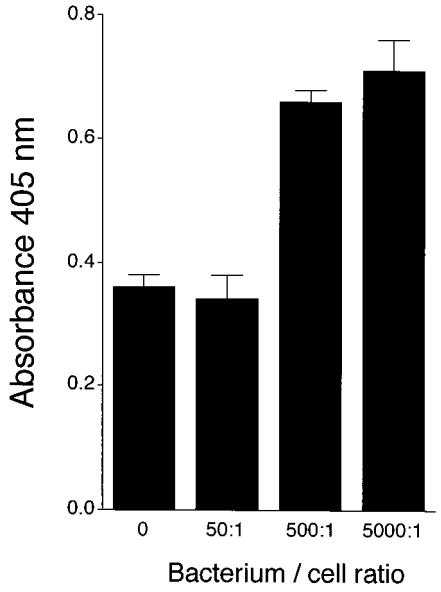

FIG. 6. DNA fragmentation of J774.1 cells infected with *A. actinomycetemcomitans* Y4. J774.1 cells (2×10^4) were infected with various amounts of *A. actinomycetemcomitans* Y4. After being cultured for 10 h, the cells were lysed in hypotonic lysing buffer. Cell death detection ELISA was used to identify the cytoplasmic histone-associated DNA fragments in the cell lysates. Data are expressed as the means ± standard deviations of five cultures. The experiment was performed two times, and similar results were obtained from each experiment.

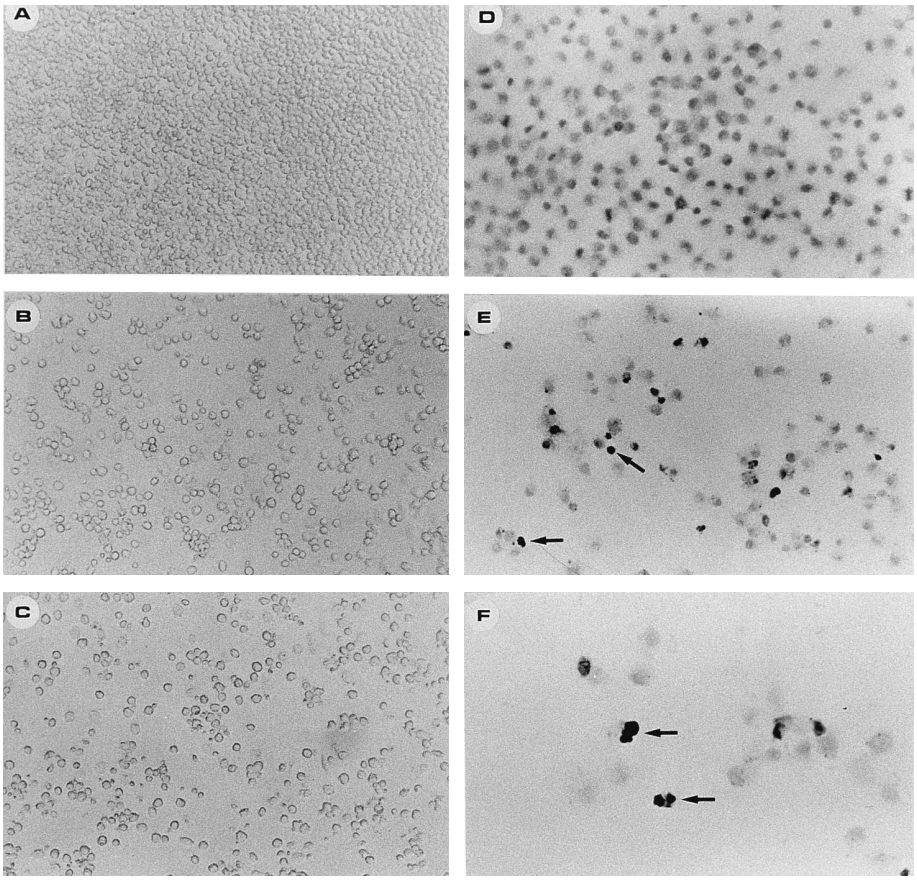

FIG. 7. Apoptotic morphology induced by *A. actinomycetemcomitans* Y4 infection. Light micrographs of J774.1 cells infected with *A. actinomycetemcomitans* Y4 ([A] without bacteria; [B] a bacterium/cell ratio of 500:1; [C] a bacterium/cell ratio of 5,000:1). Cytochemical staining of J774.1 cells infected with *A. actinomycetemcomitans* Y4 ([D] without bacteria; [E and F] a bacterium/cell ratio of 500:1) with Apop Tag, an in situ apoptosis detection kit. Arrows indicate the apoptotic nuclei of J774.1 cells infected with *A. actinomycetemcomitans* Y4. Incubation times postinfection were 48 h (A, B, and C) and 24 h (D, E, and F). Magnification, A, B, and C, ×40; D and E, ×100; F, ×200.

mycetemcomitans Y4-infected J774.1 cells were cultured in the presence or absence of protein kinase inhibitors. Perandones et al. (22) have examined the mechanism of the regulation of apoptosis in vitro in mature murine spleen T cells with protein kinase inhibitors. Dexamethasone-induced DNA fragmentation can be prevented by the protein kinase C inhibitor H7, and the protein kinase A and protein kinase G inhibitor HA1004 shows no effect on the process of apoptosis. H7 has an inhibition constant (K_i) of 6 μM for protein kinase C and 3 μM for cAMP-dependent protein kinases, whereas HA1004 has a K_i of 40 μM for protein kinase C and 2.3 μM for cAMP-dependent protein kinases (8, 10). In this study, H7, an inhibitor of protein kinase C, significantly and dose-dependently reduced *A. actinomycetemcomitans* Y4-induced cytotoxicity of J774.1 cells (Fig. 8). However, HA1004, a inhibitor of protein kinase A and protein kinase G, showed no effect on the processes of J774.1 cells infected with *A. actinomycetemcomitans* Y4. These results suggest that *A. actinomycetemcomitans* might stimulate the protein kinase C pathway to induce DNA fragmentation.

In conclusion, the results presented here support the thesis that *A. actinomycetemcomitans* infection induces an increase in the proportion of fragmented DNA, which correlates with cell death of the murine macrophage cell line J774.1 cells. The ability of *A. actinomycetemcomitans* to promote apoptosis might be important in the initiation and progression of periodontitis. Further work is needed to determine the roles of the sub-

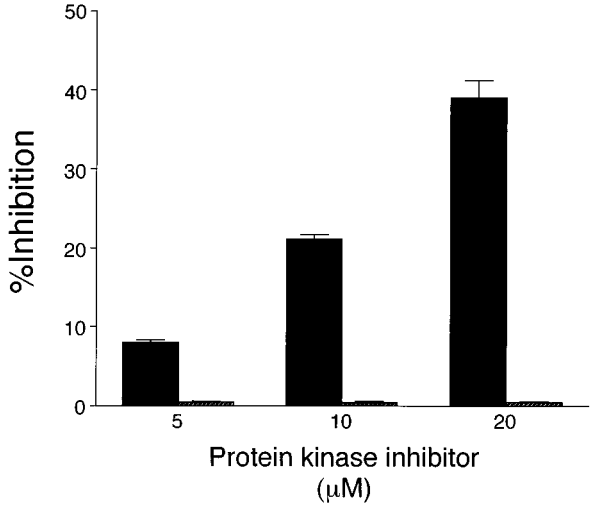

FIG. 8. Effect of protein kinase inhibitors on cytotoxicity of J774.1 cells infected with *A. actinomycetemcomitans* Y4. J774.1 cells were infected with *A. actinomycetemcomitans* Y4 at a bacterium/cell ratio of 500:1. After being cultured for 48 h in the presence of H7 (■) or HA1004 (▨), the percent cytotoxicity was determined by the MTT viability assay as described in Materials and Methods. The percent inhibition was calculated by the following formula: percent inhibition = 100 × (1 − percent cytotoxicity with protein kinase inhibitors/percent cytotoxicity without protein kinase inhibitors). Data are expressed as the means ± standard deviations of six cultures. The experiment was performed three times, and similar results were obtained from each experiment.

stances from *A. actinomycetemcomitans* within the cytosome for subsequent expression of cytotoxic activity.

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