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Human epithelial cell death caused by *Actinobacillus actinomycetemcomitans* infection

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The gingival sulcus is the shallow crevice around the tooth, and its epithelium is a gateway for initial bacterial infection in periodontal disease. Recent studies have shown that *Actinobacillus actinomycetemcomitans* invades an epithelial cell line, KB cells, *in vitro*. The aim of the present study was to clarify the changes in KB cells after *A. actinomycetemcomitans* infection. The cytotoxic effects of *A. actinomycetemcomitans* on KB cells were determined at 72, 96 and 120 h after infection by an MTT assay. Nuclear morphological changes were observed by staining with Hoechst 33258. Cytoplasmic histone-associated DNA fragmentation in the infected KB cells was determined by ELISA. *A. actinomycetemcomitans* was cytotoxic on KB cells, and condensation and degradation of the nuclei were observed. DNA fragmentation was increased after the infection. In addition, *A. actinomycetemcomitans* showed similar cytotoxic effects on human gingival epithelial cells. The present study demonstrated that *A. actinomycetemcomitans* induces apoptotic cell death of oral epithelial cells in an in-vitro culture system. This induced apoptosis might be involved in the initiation and progression of periodontitis.

Introduction

The initial event in most bacterial diseases is microbial invasion of host cells and tissues. Epithelial cells act as the first barrier against this invasion. Invasion of the cells of the intestinal mucosa is an early step in the establishment of infection by enteric bacteria, including *Shigella* spp., *Salmonella* spp., *Yersinia* spp. and *Escherichia coli* [1, 2].

Actinobacillus actinomycetemcomitans is a gram-negative, capnophilic, fermentative coccobacillus which has been implicated in the pathogenesis of several forms of periodontal disease [3]. Christersson *et al.* detected the presence of antigens from *A. actinomycetemcomitans* in gingival tissues, indicating that this bacterium could invade the tissue of the periodontium in severe periodontal diseases [4]. Fives-Taylor *et al.* demonstrated that adhesion of the bacterium to KB cells is a rapid process which occurs within 15 min and that internalisation occurs through a cytochalasin D-sensi-

tive process in KB cells. Furthermore, the adhesion was found to involve multiple determinants and to be influenced by both bacterial and host environmental conditions [5, 6]. However, these studies did not focus on changes in the infected KB cells.

An earlier study developed an in-vitro infection model for *A. actinomycetemcomitans* to provide evidence for the apoptotic cell death of murine macrophages after infection [7–9]. Therefore, the present study explored changes in KB cells after *A. actinomycetemcomitans* infection.

Materials and methods

Bacterial strains and growth conditions

Five strains of *A. actinomycetemcomitans* (ATCC 29523, Y4, JP2, NCTC 9710 and IDH 781) were used in this study. The test strains were grown in Todd-Hewitt Broth (Difco) supplemented with yeast extract 1% w/v at 37°C for 1 day in an atmosphere of CO₂ 5% in air. *Streptococcus mutans* 33477 and *S. salivarius* 9227 were also grown in the same conditions as the *A. actinomycetemcomitans* strains.

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Epithelial cells

KB cells (ATCC CCL 17), an epithelial-like cell line originally isolated from a human oral epithelioid carcinoma, were maintained in Dulbecco's Modified Eagle's Medium (DMEM; Gibco BRL, Grand Island, NY, USA) supplemented with heat-inactivated fetal bovine serum (FBS; Gibco BRL) 10%, penicillin G 100 U/ml and streptomycin 100 µg/ml at 37°C in CO₂ 5% in air.

Human gingival epithelial cells (HGE cells) were also used in certain experiments. Healthy gingival tissue was obtained from patients undergoing surgery for removal of impacted teeth, after informed consent had been obtained. Specimens were cut into small pieces and incubated with Dispase (Godo Shusei, Tokyo, Japan) 0.4% overnight at room temperature. The surface epithelium was separated and placed in sterile phosphate-buffered saline (PBS) containing trypsin 0.05% and 0.53 mM EDTA. Cells were collected by centrifugation, suspended in DMEM containing FBS 10% and incubated at 37°C in CO₂ 5% in air. At confluence, the cells were trypsinised and re-seeded at 2×10^4 cells/well in a 96-well plate [10].

Cytotoxicity assay

KB cells were plated in a 96-well plate at a concentration of 2×10^4 cells/well 24 h before the experiment. *A. actinomycetemcomitans* Y4 was harvested by centrifugation and resuspended in DMEM without antibiotics. Bacterial suspension was added to the wells and the plates were centrifuged at 1000 g for 10 min at 4°C before incubation at 37°C for 1 h in an atmosphere of CO₂ 5% in air. Cells infected at bacterium:cell ratios of 500:1, 5000:1 and 50 000:1 were washed three times with DMEM containing penicillin G, streptomycin and gentamicin 200 µg/ml to remove extracellular bacteria. The infected cells were cultured with DMEM containing FBS 5% and antibiotics for 72, 96 and 120 h. Stock MTT solution, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (Sigma) 2.5 mg/ml, was added to the wells (20 µl/well), and the plates were incubated for 4 h. After the addition of acid-isopropanol (100 µl of 0.04 N HCl in isopropanol) and thorough mixing, the plates were read on a microplate reader (TOSOH Microplate reader MRP A4i, Tokyo, Japan), with a test wavelength of 570 nm and a reference wavelength of 620 nm. The percentage cytotoxicity was calculated by the following formula: $100 \times (1 - \text{optical density with infection} / \text{optical density without infection})$. Data were expressed as the mean and SD of tests performed in triplicate.

Microscopic study

KB cells were plated in a 24-well plate (1×10^5 cells/well) for 24 h before the experiment and then infected with *A. actinomycetemcomitans* Y4 at a

bacterium:cell ratio of 50 000:1. After culture for 96 h, infected KB cells were examined with a phase-contrast microscope (Olympus CK2, Tokyo, Japan). Nuclear morphological changes in the KB cells were also observed. The infected KB cells were fixed in a culture dish with methanol:acetic acid (3:1 v:v) at room temperature for 10 min. After washing with PBS, the samples were incubated in Hoechst 33258 (Sigma) 0.1 mg/ml at 37°C for 10 min and observed by fluorescence microscopy (365 nm; Nikon UFX-DX, Tokyo, Japan).

DNA fragmentation

The infected KB cells (2×10^4 cells/well) were cultured in 96-well culture plates in an atmosphere of CO₂ 5% in air. After culture for 24 h, the culture supernate was removed and measured for DNA fragmentation. Cell death detection by an enzyme-linked immunosorbent assay (Cell death detection ELISA plus; Boehringer GmbH, Mannheim, Germany) was used for the quantitative determination of cytoplasmic histone-associated DNA fragments, as described in the manufacturer's instructions. Data were expressed as the mean and SD of tests performed in triplicate.

Results

Cytotoxic effects of *A. actinomycetemcomitans*

A. actinomycetemcomitans cytotoxicity on KB cells was tested by an MTT assay. KB cells infected at a bacterium:cell ratio of 50 000:1 showed 15.9 (SD 0.8)%, 34.9 (SD 2.5)% and 47.6 (SD 1.5)% cytotoxicity after incubation for 72, 96 and 120 h, respectively (Fig. 1). The cytotoxic effects of five *A. actinomycetemcomitans* strains (ATCC 29523, Y4, JP2, NCTC 9710 and IDH 781) and *S. mutans* and *S. salivarius* were compared after incubation for 96 h. All *A. actinomycetemcomitans* strains showed cytotoxic effects on the KB cells. However, *S. mutans* and *S. salivarius* showed only weak cytotoxic effects when compared with *A. actinomycetemcomitans* strains at the same bacterium:cell ratios (Fig. 2). The cytotoxic effects of *A. actinomycetemcomitans* Y4 on KB cells were further examined in the presence or absence of cytochalasin D (an inhibitor of actin polymerisation; 1 µg/ml) and aurintricarboxylic acid (ATA; a DNA endonuclease inhibitor; 100 µM). After incubation for 96 h, the cytotoxicity for control KB cells was 39.5 (SD 7.6)%, and then decreased to 13.2 (SD 1.3)% and 6.2 (SD 0.2)% with the addition of cytochalasin D and ATA, respectively (Fig. 3).

DNA fragmentation in infected KB cells

Control KB cells showed absorbance values of 0.36 (SD 0.03). The KB cells infected at bacterium:cell

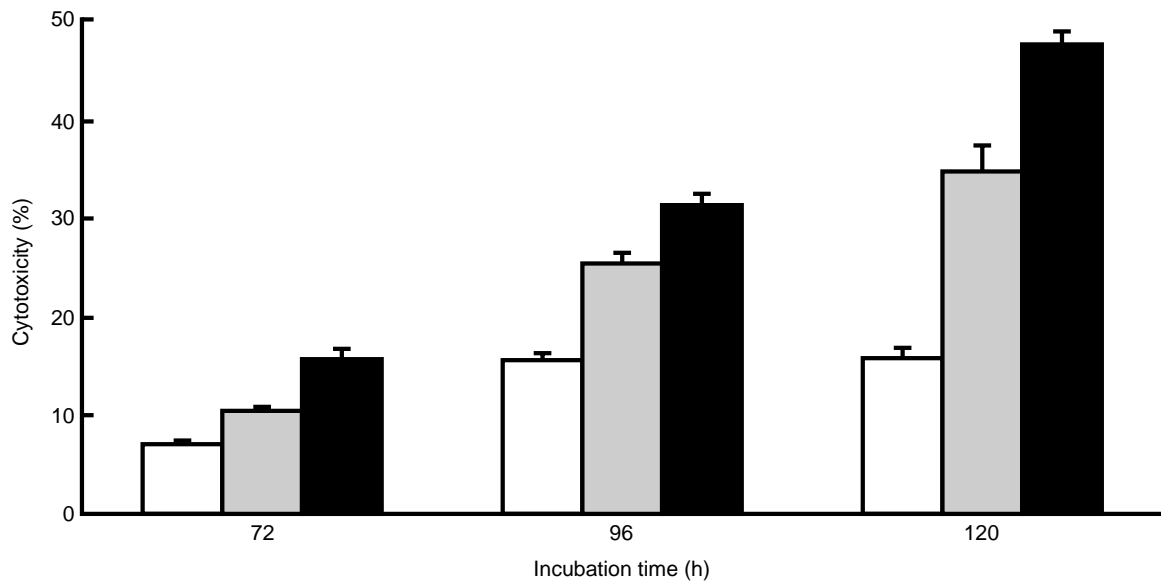


Fig. 1. Cytotoxic effect of *A. actinomycetemcomitans* Y4 on KB cells infected with *A. actinomycetemcomitans* Y4 at bacterium:cell ratios of 500:1 (□), 5000:1 (▨) and 50 000:1 (■).

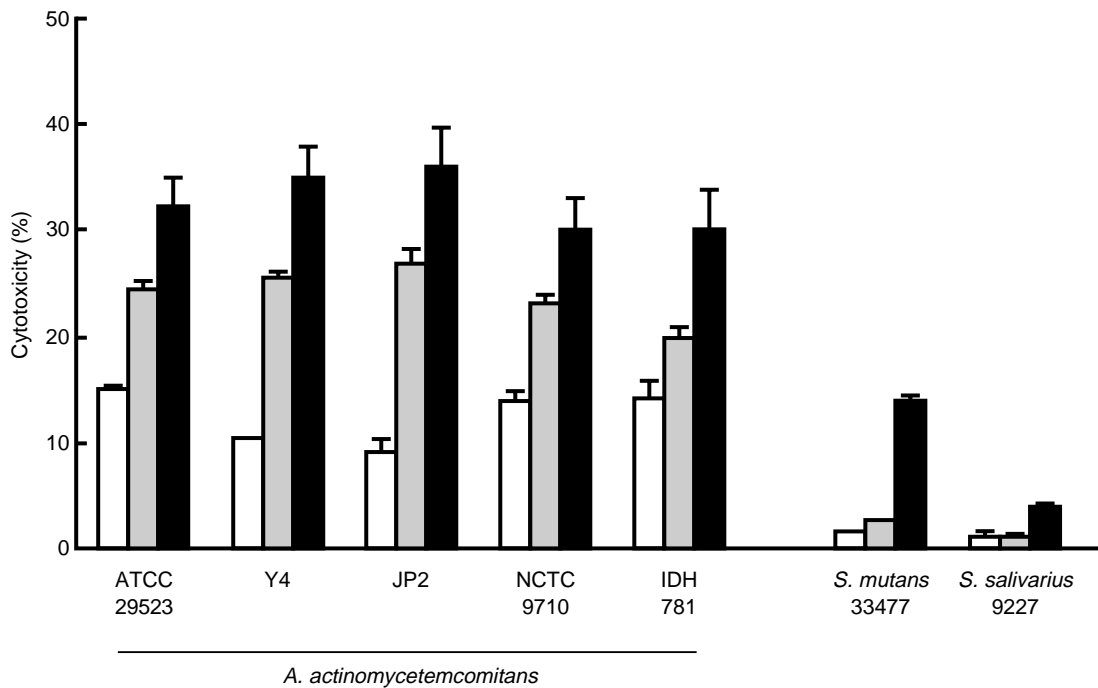


Fig. 2. Cell death of KB cells induced by several strains of *A. actinomycetemcomitans* and streptococci. KB cells were infected with *A. actinomycetemcomitans* ATCC 29523, Y4, JP2, NCTC 9710, IDH 781, *S. mutans* 33477 and *S. salivarius* 9227 at bacterium:cell ratios of 500:1 (□), 5000:1 (▨) and 50 000:1 (■) for 96 h.

ratios of 500:1, 5000:1 and 50 000:1 showed values of 0.54 (SD 0.04), 0.61 (SD 0.05) and 0.89 (SD 0.06), respectively (Fig. 4).

Morphological changes in infected KB cells

Control KB cells grew as discrete cells and then coalesced to form confluent monolayers after incubation for 96 h. The cells exhibited a ‘cobble-stone’ morphology, which is characteristic of homogeneous confluent epithelial monolayers (Fig. 5a). Infected KB

cells were devoid of the characteristic features of control KB cells, as the infected cells were comparatively large, oddly shaped and had a multinucleate appearance (Fig. 5b). Infected KB cells were observed by fluorescence microscopy with Hoechst dye staining. In the control KB cells, Hoechst 33258 fluorescence was detected in chromatin in the nucleosomal structure of the nuclei (Fig. 5c). The apoptotic cells were identified according to characteristic cell morphologies, such as condensation and degradation of the nuclei (Fig. 5d).

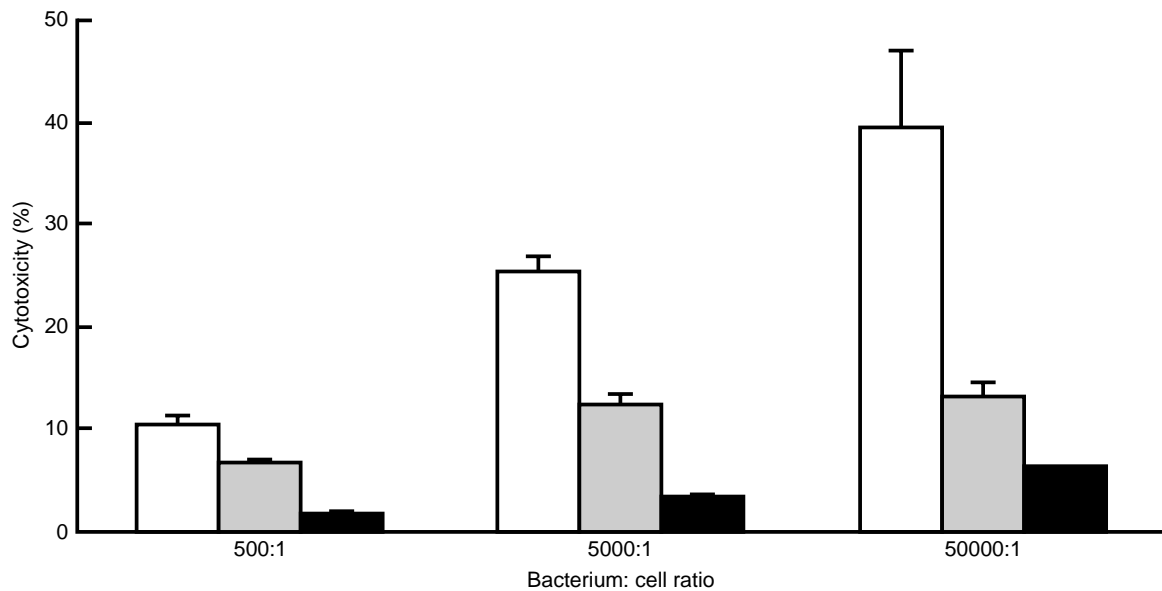


Fig. 3. Effects of cytochalasin D and ATA on the cytotoxicity of *A. actinomycetemcomitans* Y4 on KB cells infected at bacterium:cell ratios of 500:1, 5000:1 and 50 000:1. The *A. actinomycetemcomitans* Y4-infected KB cells were cultured for 96 h: control (□), cytochalasin D (1 µg/ml; ▒) and ATA (100 µM; ■).

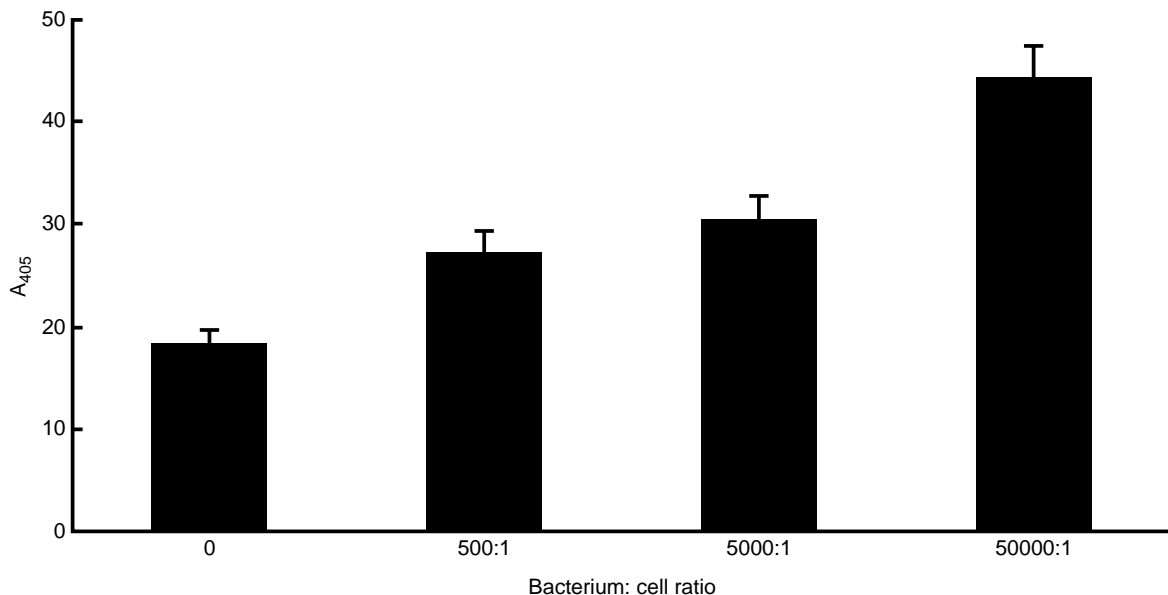


Fig. 4. DNA fragmentation of KB cells infected with *A. actinomycetemcomitans* Y4.

Cytotoxic effects on human gingival epithelial cells

A. actinomycetemcomitans Y4 showed 32.5 (SD 1.4)% and 22.9 (SD 2.4)% cytotoxicity for HGE cells (patients 1 and 2), respectively, at a bacterium:cell ratio of 50 000:1, while this strain showed 31.2 (SD 1.3)% cytotoxicity for KB cells (Fig. 6).

Discussion

Pathogenic microbes, including bacteria, bind to host cell surfaces and become internalised, forming the process called microbial invasion. Epithelial cell

invasion is a strategy adopted by various pathogenic bacteria [11]. *A. actinomycetemcomitans* can invade periodontal tissue in periodontal diseases once colonisation by the bacterium has taken place [12, 13]. Furthermore, *A. actinomycetemcomitans* can invade human oral epithelial cells [6].

The present study demonstrated death of KB cells after infection with *A. actinomycetemcomitans* Y4 (Fig. 1). An earlier study demonstrated that *A. actinomycetemcomitans* infection induced apoptotic cell death of the macrophage cell line J774.1 and that invasion by the bacterium was essential for the induction of apoptosis. The numbers of bacteria required to induce death of the KB cells were 10 times higher in the present study

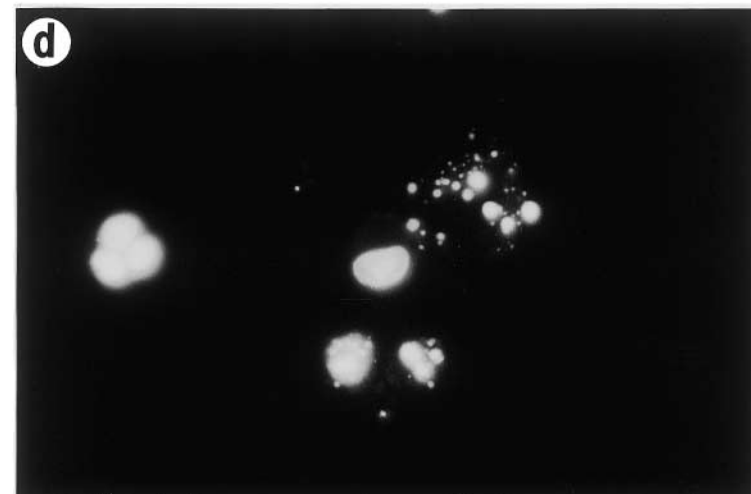
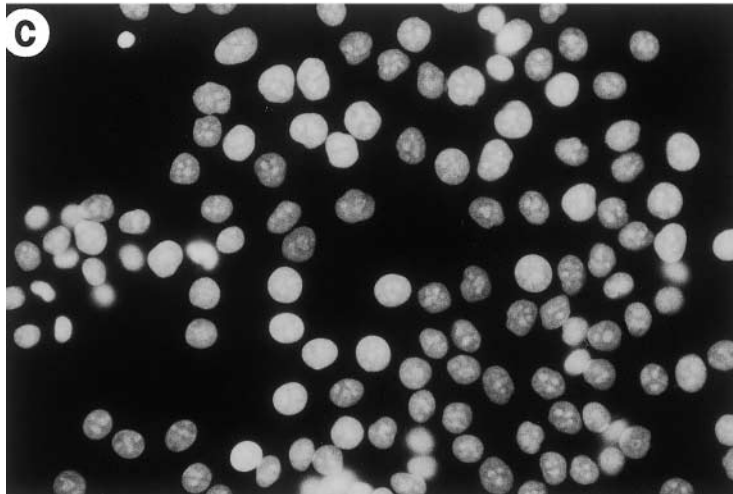
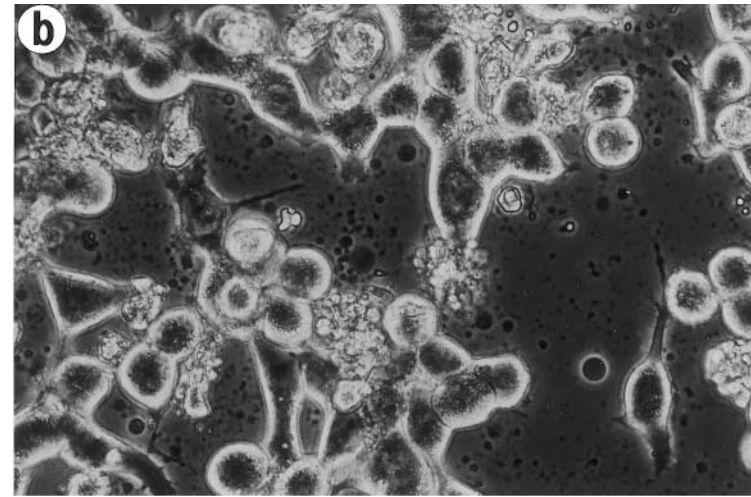
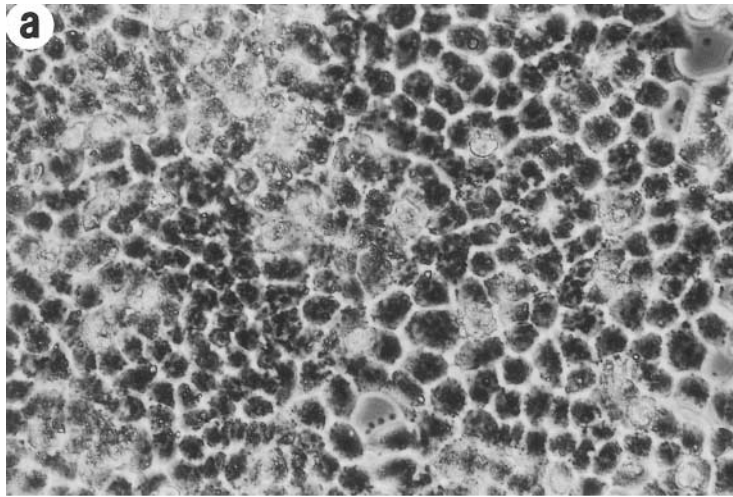


Fig. 5. Apoptotic morphology induced by *A. actinomycetemcomitans* Y4 infection. Phase-contrast micrographs of KB cells infected with *A. actinomycetemcomitans* Y4 (**a**, control KB cells; **b**, KB cells infected at a bacterium:cell ratio of 50 000:1) and cytochemical staining of KB cells infected with *A. actinomycetemcomitans* Y4 (**c**, control KB cells; **d**, KB cells infected at a bacterium:cell ratio of 50 000:1) with the DNA-specific fluorochrome Hoechst 33258. Magnification: $\times 160$ (a,b), $\times 80$ (c,d).

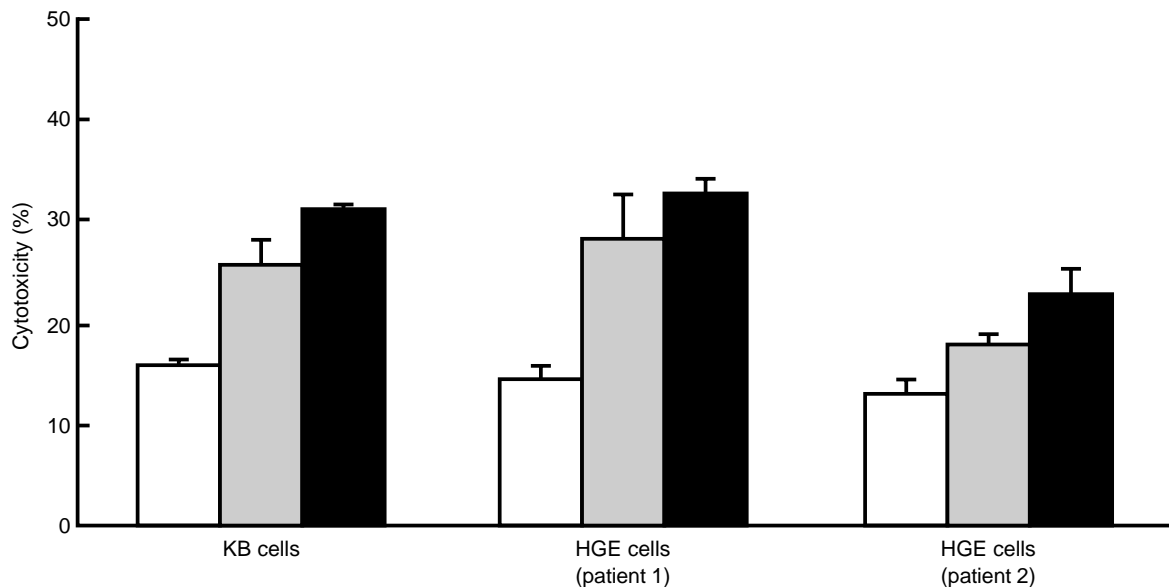


Fig. 6. Cytotoxic effects of *A. actinomycetemcomitans* Y4 on human gingival epithelial (HGE) cells infected with *A. actinomycetemcomitans* Y4 at bacterium:cell ratios of 500:1 (□), 5000:1 (◻) and 50 000:1 (■).

than those for J774.1 cells. Furthermore, the cytotoxic effects of *A. actinomycetemcomitans* Y4 on J774.1 cells appeared at 24 h after infection, whereas those on KB cells appeared at 72 h. These data may indicate a greater resistance of epithelial cells to bacterial infections. Recent studies suggest that leucotoxin kills human immune cells by a pathway resembling apoptosis [14, 15]. Two leucotoxic strains, *A. actinomycetemcomitans* ATCC 29523 and JP2, were used in this study. However, these two strains produced similar cytotoxic effects on KB cells to Y4, a non-leucotoxic strain (Fig. 2). Therefore, leucotoxin does not explain the ability of *A. actinomycetemcomitans* to induce cytotoxic effects on KB cells. Cytochalasin D significantly inhibited the cytotoxicity of *A. actinomycetemcomitans* Y4 on KB cells (Fig. 4), indicating that the cytotoxicity occurs through a microfilament-dependent process and that *A. actinomycetemcomitans* is cytotoxic only when it is inside the cytoplasm. Scanning electron microscopy demonstrated that the bacterium can adhere to KB cells and seems to invade them (data not shown). Fives-Taylor *et al.* also provided similar evidence for the invasion of KB cells by *A. actinomycetemcomitans* and determined that its invasion occurs through cytochalasin D-sensitive processes [16].

In the present study, *A. actinomycetemcomitans* Y4-infected KB cells exhibited the characteristics of apoptosis linked to the activation of an internucleosomal nuclease. ATA efficiently increased the percentage of viable KB cells infected with *A. actinomycetemcomitans* Y4 (Fig. 3). The nuclei of the infected KB cells showed chromatin condensation and other morphological alterations seen in apoptotic cells (Fig. 5d). Similar findings were observed for those cells infected at a bacterium:cell ratio of 500:1 (data not shown). Apoptotic cell death of the infected KB cells was also

confirmed by quantitative cell death detection ELISA (Fig. 4). These findings suggest that the death of KB cells infected with *A. actinomycetemcomitans* Y4 occurs through apoptosis mediated by a cDNA endonuclease. Furthermore, HGE cells infected with *A. actinomycetemcomitans* also showed cytotoxicity similar to that seen in KB cells (Fig. 6).

A. actinomycetemcomitans has been considered to be an aetiological agent of periodontal disease, especially in the localised form of juvenile periodontitis (LJP). The most striking feature of LJP is the lack of clinical inflammation, despite the presence of deep periodontal pockets and vertical loss of alveolar bone around the first molars and incisors. The results of the present study suggest that the periodontal pathogen *A. actinomycetemcomitans* can invade gingival epithelium by stealth without inflammation, by inducing apoptotic cell death of the epithelial cells. Destruction of the first-step defensive structure of living gingival epithelial cells could lead to an initiation of infection and further the development of periodontal inflammation. The detailed pathways of apoptosis mediated by cDNA endonuclease, as well as the adhesion of bacteria to epithelial cell surfaces, destruction of the cell membrane and infiltration into epithelial cell cytoplasm, should be investigated further.

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