

Butyric Acid-Induced Apoptosis of Murine Thymocytes, Splenic T Cells, and Human Jurkat T Cells

TOMOKO KURITA-OCHIAI,* KAZUO FUKUSHIMA, AND KUNIYASU OCHIAI

Department of Microbiology, Nihon University School of Dentistry
at Matsudo, Matsudo, Chiba 271, Japan

Received 11 June 1996/Returned for modification 14 August 1996/Accepted 23 October 1996

The purpose of this study was to examine the effect of butyric acid, an extracellular metabolite from periodontopathic bacteria, on apoptosis induction in murine thymocytes, splenic T cells, and human Jurkat T cells. Butyric acid significantly suppressed T-cell viability in both a concentration- and time-dependent fashion. The results of DNA fragmentation assay indicated that butyric acid rapidly induced apoptosis in thymocytes (with 1.25 mM butyric acid and 6 h after treatment) and in splenic T cells and Jurkat cells (with 2.5 mM butyric acid and 16 h after treatment). Incubation of thymocytes or Jurkat cells with 5 mM butyric acid for 21 h resulted in the typical ladder pattern of DNA fragmentation. Furthermore, Jurkat cells treated with 5 mM butyric acid showed the characteristic pattern of apoptotic cells such as chromatin condensation and hypodiploid nuclei. Experiments with fractionated subpopulations of splenic T cells revealed that DNA fragmentation was predominantly observed in CD4⁺ T cells. Butyric acid-induced apoptosis of thymocytes was decreased by the protein kinase inhibitors H7 and staurosporine. These inhibitors were less effective with similarly treated splenic T cells and Jurkat cells. These data suggest that butyric acid, one of the volatile fatty acids produced by periodontopathic bacteria and one that easily penetrates the oral mucosa, can modulate the immunoregulatory cell population in periodontal tissue by inducing T-cell death through apoptosis.

It is well-known that periodontal diseases are infectious and that periodontal tissue breakdown results from the interaction of specific anaerobic bacteria and host immune mechanisms. A recent study indicates that severe destructive adult periodontitis is a multibacterial infection and that certain combinations of periodontopathogens, namely, *Porphyromonas*, *Prevotella*, and *Fusobacterium* spp., seem to be important in the pathogenesis of the disease (44). These bacteria produce an elaborate variety of virulence factors such as proteases, lipopolysaccharides, and fimbriae (43).

The respective metabolisms of these bacteria are also characterized by the production of an identifiable fingerprint of short-chain fatty acids, which are major by-products of anaerobic metabolism that are released into the microenvironment at the infection site (16) and can diffuse across biological membranes (40). Previous studies have demonstrated that these fatty acids exert inhibitory effects on gingival fibroblast proliferation (41), colon cancer cell growth (15), and phagocytosis (13, 38). Our previous study (23) demonstrated that short-chain fatty acids, especially volatile fatty acids (VFA) present in the culture filtrates of *Porphyromonas gingivalis*, *Prevotella loescheii*, and *Fusobacterium nucleatum*, greatly inhibited murine T- and B-cell proliferation and cytokine production by concanavalin A-stimulated splenic T cells.

Apoptosis, a form of active cell death, plays important roles in the development and regulation of the immune system (7, 21). Morphological changes at the nuclear level and endonucleolysis are well characterized (7). Pursuing the inhibitory mechanism of VFA, we found that a representative VFA, butyric acid, induced cytotoxicity and apoptosis in murine thymocytes. In this study, we report that butyric acid induces apoptosis in murine thymocytes, splenic T cells, and human

Jurkat T cells. Furthermore, evidence is provided that protein kinase inhibitors reduce apoptosis induced by butyric acid. The observed differences in inhibition seen with thymocytes, splenic T cells, and Jurkat cells are discussed.

MATERIALS AND METHODS

Short-chain fatty acids. Highly purified butyric acid was purchased from Sigma Chemical Co. (St. Louis, Mo.). Solutions of butyric acid ranging in concentrations from 0.31 to 10 mM were diluted in RPMI 1640 (Gibco Laboratories, Grand Island, N.Y.) medium and adjusted to pH 7.2 with sodium hydroxide.

Mice. C3H/HeN mice (female, 8 to 9 weeks old) were obtained from Charles River Breeding Laboratories (Kanagawa, Japan). The mice were maintained in the Animal Facility of Nihon University School of Dentistry at Matsudo under standard care and given food and water ad libitum. Mice were used at 9 to 10 weeks of age and were matched by age within each experiment.

T-cell preparation. Spleens and thymocytes were aseptically removed, and single-cell suspensions were prepared by gently teasing the cells through sterile stainless steel screens. The thymocyte cell suspension was used directly. Preparations of T cells from mouse spleens were obtained as described previously (24). Briefly, T-cell-enriched fractions were obtained by passing spleen cells through a Sephadex G-10 (Pharmacia, Piscataway, N.J.) column followed by panning (45) cells on plastic petri dishes (15 by 100 mm) coated with rabbit anti-mouse F(ab')₂ immunoglobulin G (Organon Teknica Co., West Chester, Pa.). After incubation for 90 min at 4°C, the nonadherent T-cell-enriched population was recovered and was usually consisted of more than 95% Thy 1.2⁺ and less than 5% immunoglobulin-positive cells, as determined by immunofluorescence with a FACScan fluorescence-activated cell sorter (Becton Dickinson and Co., Sunnyvale, Calif.). Splenic T cells were further incubated with anti-CD4- and anti-CD8-coated magnetic beads (PerSeptive Diagnostics, Inc. Cambridge, Mass.) and passed on a magnet for several cycles. Recovered cells obtained by positive magnetic selection resulted in both enriched CD4⁺ (>98%) and CD8⁺ (>98%) populations by fluorescence-activated cell sorter analysis.

The human T lymphoma cell line Jurkat was kindly provided by FCCH (Fujisaki Cell Center-Hayashibara, Okayama, Japan). These cells were cultured at 37°C in a moist atmosphere of 5% CO₂ in complete medium consisting of RPMI 1640 supplemented with 10% heat-inactivated fetal calf serum, 2 mM L-glutamine, 100 U of penicillin per ml, 100 µg of streptomycin per ml, and 0.05 mM 2-mercaptoethanol.

Cell proliferation assay and cell viability. As a method of assessing cellular proliferation following the addition of butyric acid, the colorimetric MTT (3-[4,5-dimethyl-2-thiazolyl]-2,5-diphenyl tetrazolium bromide; Sigma Chemical Co.) assay was performed (20). In viable cells, the mitochondrial enzyme succinate dehydrogenase can metabolize MTT into a formazan dye that absorbs light at 550 nm. T cells were seeded at a density of 7.0 × 10⁵ cells per well for

* Corresponding author. Mailing address: Department of Microbiology, Nihon University School of Dentistry at Matsudo, Matsudo-shi, Chiba 271, Japan. Phone: 473-68-6111. Fax: 473-64-6295. Electronic mail address: tkurita@mascap.nihon-u.ac.jp.

thymocytes and splenic T cells and at a density of 2.0×10^5 cells per well for Jurkat cells in 0.1 ml of complete medium in flat-bottom 96-well plates. Butyric acid in RPMI 1640 was added to a final concentration of 0.15 to 5 mM, and each concentration of butyric acid was tested in quadruplicate. After incubation for 42 h, 20 μ l of MTT (5 mg/ml in phosphate-buffered saline [PBS], pH 7.2) was added to each well. Following 6 h of incubation, the supernatants were decanted, and the formazan precipitates were solubilized by the addition of 150 μ l of 100% dimethyl sulfoxide (Sigma) and placed on a plate shaker for 10 min. Absorbance at 550 nm was determined on a Corona MT32 spectrophotometric microplate reader (Corona Electric Co., Ibaraki, Japan). The absorbance of the untreated cultures was set at 100%. The mean relative absorbance and the standard error of the mean were calculated for every concentration of butyric acid tested. Cell viability was determined by light microscopy after staining with 0.02% trypan blue (Sigma), and the results are expressed as the percentage of surviving cells.

T-cell culture for apoptosis. T cells were suspended in complete medium. Cells (4.0×10^6 per well for thymocytes and splenic T cells and 1.0×10^6 per well for Jurkat cells) were cultured in 24-well tissue culture plates (Falcon, Becton-Dickinson Labware, Lincoln Park, N.Y.) in the presence or absence of various concentrations of butyric acid. At the times indicated in the figures, cells were harvested and centrifuged at $400 \times g$ for 5 min and washed twice with ice-cold PBS. Cells were resuspended in 400 μ l of hypotonic lysis buffer (0.2% Triton X-100, 10 mM Tris, 1 mM EDTA; pH 8.0) and centrifuged for 15 min at $13,800 \times g$ (32). Half the supernatant, containing small DNA fragments, was subjected to gel electrophoresis, while the other half, as well as the pellet containing large pieces of DNA and cell debris, was used for the diphenylamine (DPA) assay (see below).

Gel electrophoresis. One-half of the supernatant was treated with an equal volume of absolute isopropyl alcohol and 0.5 M NaCl to precipitate the DNA and stored at -20°C overnight. After centrifugation at $13,800 \times g$ for 15 min, the pellet was washed with 200 μ l of 70% ethanol and allowed to dry at room temperature. The DNA was resuspended in 12 μ l of TE solution (10 mM Tris-HCl, 1 mM EDTA; pH 7.4) and 3 μ l of loading buffer (50% glycerol, 1 \times TAE, 10% saturated bromophenol blue, 1% xylene cyanol), incubated at 37°C for 20 min, and then electrophoresed on 1.7% agarose gel containing 0.71 μ g of ethidium bromide per ml for 1 h. Gels were photographed by using UV transillumination.

DNA fragmentation assay. The DPA reaction was performed by the method of Perandones et al. (35). Perchloric acid (0.5 M) was added to the pellets containing uncut DNA (resuspended with 200 μ l of hypotonic lysis buffer) and to the other half of the supernatants containing DNA fragments, and then 2 volumes of a solution containing 0.088 M DPA, 98% (vol/vol) glacial acetic acid, 1.5% (vol/vol) sulfuric acid, and 0.5% (vol/vol) of 1.6% acetaldehyde solution was added. The samples were stored at 4°C for 48 h. The colorimetric reaction was quantitated spectrophotometrically at 575 nm, using a UV spectrophotometer (UV-160A; Shimadzu Co. Ltd., Tokyo, Japan). The percentage of fragmentation was calculated as the ratio of DNA in the supernatants to the total DNA.

Flow cytometric analysis. Nuclear DNA content was analyzed by flow cytometry (Becton Dickinson, Pont de Claix, France) after propidium iodide staining by the method described by Nicoletti et al. (33). Cells were pelleted, resuspended in hypotonic fluorochrome solution (50 μ g of propidium iodide per ml in 0.1% sodium citrate and 0.1% Triton X-100), and kept at 4°C in the dark overnight before the analysis.

Detection of morphological apoptosis. After treatment with reagents, cells were fixed with 2% glutaraldehyde solution (TA AB Lab., Aldermastone, England) for 1 h and stained with 0.2 mM Hoechst 33258 to visualize the location of DNA. Cells were examined with a fluorescence microscope (BHT-RFC; Olympus, Tokyo, Japan) for determination of fragmentation of nuclei and/or condensation of chromatin.

Reagents. Hoechst 33258 (2'-[4-hydroxyphenyl]-5-[4-methyl-1-piperazinyl]-2,5'-bi-1H-benzimidazole) and staurosporine were purchased from Sigma. H7 (1-[5-isoquinolinesulfonyl]-2-methylpiperazine dihydrochloride) was from Seikagaku Kogyo (Tokyo, Japan).

Statistics. The significance of differences between groups was determined by Student's *t* test.

RESULTS

Effects of butyric acid on cell proliferation and viability. We examined the effects of various concentrations of butyric acid on the proliferative activity and viability of murine thymocytes, splenic T cells, and human Jurkat T cells. After 21 h of incubation, butyric acid caused a reduction in cell proliferative activity, as assessed by the colorimetric MTT assay, and in cell viability, as assessed by trypan blue dye exclusion. These cells exhibited a marked, dose-dependent response to butyric acid (Fig. 1). With 5 mM butyric acid, the proliferative responses of thymocytes, splenic T cells, and Jurkat cells were significantly suppressed by 70.0, 78.7, and 76.9%, respectively, as assessed by the MTT assay (Fig. 1A); viability was reduced by 33.1, 41.3,

and 34.3%, respectively, as assessed by the trypan blue assay (Fig. 1B). These decreases in cell proliferation and viability prompted us to determine the type of cell death induced by butyric acid.

DNA fragmentation caused by butyric acid. The induction of apoptosis by butyric acid was indicated by the colorimetric DNA fragmentation assay, electrophoresis of the fragmented DNA, flow cytometric analysis of DNA contents, and nuclear morphology. When the three types of cells were cultured in the presence of 0.625 to 5.0 mM butyric acid for 21 h and quantitated by the DNA fragmentation assay, a dose-dependent increase in DNA fragmentation was seen (Fig. 2). Butyric acid induced a substantial and near-maximal (65% with 5 mM butyric acid) increase in DNA fragmentation (58.6%) for thymocytes at 1.25 mM ($P < 0.01$). For splenic T cells and Jurkat cells, 2.5 mM butyric acid increased the amount of DNA fragmentation to 35.0 and 46.0%, respectively (Fig. 2). These results indicate that different degrees of apoptosis induction by butyric acid depend on the differences in sensitivity of the cell populations.

In similar experiments, cells were cultured with 5 mM butyric acid and examined for DNA fragmentation at various times throughout a 21-h time period (Fig. 3). Treatment of thymocytes with butyric acid for 6 h resulted in a markedly increase in DNA fragmentation. Splenic T cells and Jurkat cells treated with butyric acid showed an increase in DNA fragmentation after 6 h of incubation and continued to increase throughout the experiment.

The induction of apoptosis by butyric acid in these cell populations was further confirmed by electrophoresis of fragmented DNA and nuclear morphology (Fig. 4). Low-molecular-weight DNA fragments extracted from the thymocytes and Jurkat cells cultured with 5 mM butyric acid for 21 h showed typical oligonucleosomal ladders (Fig. 4). Negligible cleavage of DNA into nucleosomal fragments was seen with untreated thymocytes and Jurkat cells.

When we examined the morphological changes in the nuclei of 5 mM butyric acid-treated Jurkat cells by DNA staining with Hoechst 33258, the characteristic features of apoptosis, including condensation and aggregation of chromatin near the nuclear membrane, were observed (Fig. 5B). The DNA content of butyric acid-treated Jurkat cells, stained by propidium iodide, was also analyzed with a flow cytometer. Apoptotic nuclei were distinguishable by the hypodiploid DNA contents compared with the diploid DNA contents of normal cells. A single peak of DNA, indicating diploid DNA content, characterized untreated Jurkat cells (Fig. 6A). In contrast, the percentage of Jurkat cells with hypodiploid DNA was increased by treatment with butyric acid (Fig. 6B). Cell cycle analysis of butyric acid-treated cells revealed a progressive decrease in the percentage of cells in the S and G_1 phases.

In order to investigate which T-cell subpopulation could be involved in the apoptosis of murine splenic T cells induced by butyric acid, the T cells were separated by positive selection into $CD4^+$ and $CD8^+$ T cells. As shown in Fig. 7, treatment with 5 mM butyric acid significantly ($P < 0.01$) increased DNA fragmentation on unseparated splenic ($43.2\% \pm 4.4\%$) and $CD4^+$ ($68.3\% \pm 1.2\%$) T cells compared to untreated splenic ($16.0\% \pm 1.4\%$) and $CD4^+$ ($19.9\% \pm 1.7\%$) T cells. In contrast, butyric acid-treated $CD8^+$ T cells showed no significant increase ($29.5\% \pm 1.5\%$) in DNA fragmentation compared to untreated $CD8^+$ T cells ($24.1\% \pm 1.8\%$).

Suppression of butyric acid-induced DNA fragmentation by endonuclease inhibitor. To characterize butyric acid-induced apoptosis in thymocytes, splenic T cells, and Jurkat cells, we treated these cells with various doses of $ZnCl_2$, a DNA endo-

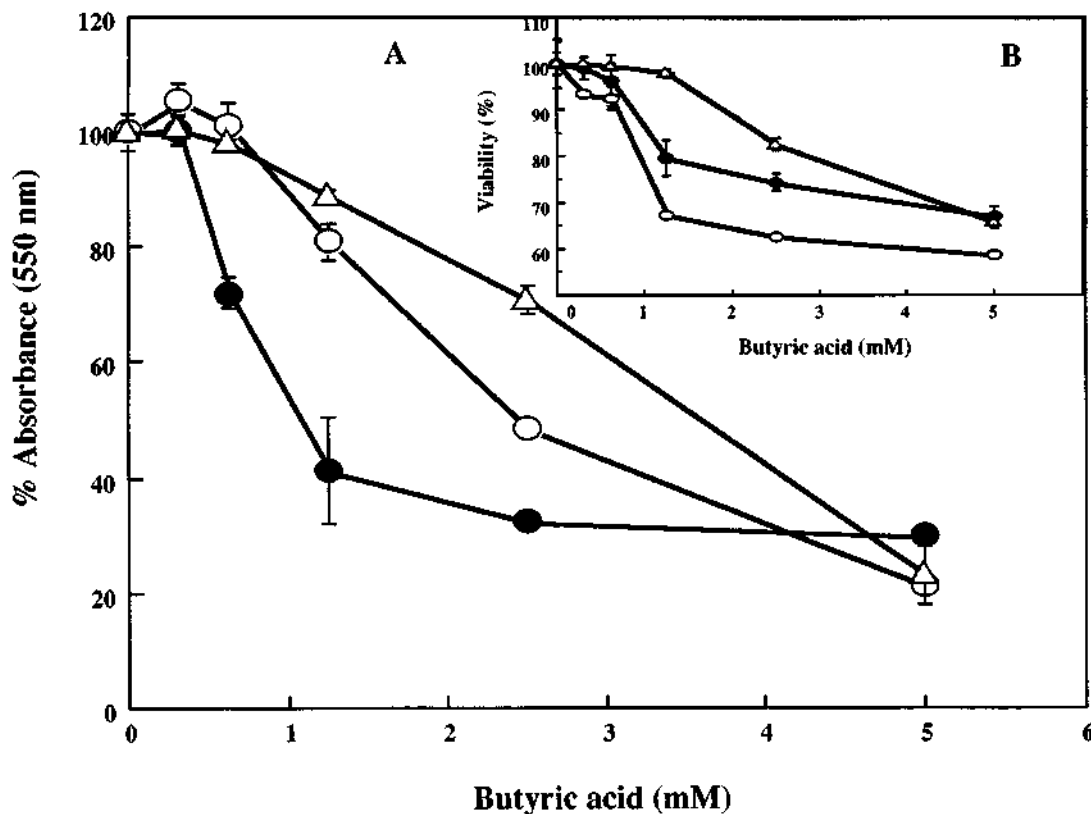


FIG. 1. Dose-dependent effects of butyric acid on cell proliferation and viability. Thymocytes (●), splenic T (○), and Jurkat cells (△) were cultured with the indicated concentration of butyric acid for 21 h. Cellular proliferation (A) was determined by MTT assay and expressed as the percentage of the absorbance value obtained without butyric acid. Cell viability (B) was determined by trypan blue dye exclusion. The data are expressed as the means \pm standard errors from three different experiments with triplicate cultures.

nuclease inhibitor. Addition of 1 mM $ZnCl_2$ completely inhibited butyric acid-induced DNA fragmentation in thymocytes, splenic T cells, and Jurkat cells, and these DNA fragmentations were comparable to that of untreated controls (Table 1). The addition of $ZnCl_2$ alone had no effect on the viability of these cells (data not shown).

Effect of protein kinase inhibitor on butyric acid-induced apoptosis. To further characterize apoptosis in thymocytes, splenic T cells, and Jurkat cells, we next examined the effect of protein kinase inhibition on butyric acid-induced apoptosis. Protein kinase inhibitors H7 (50 μ M) and staurosporin (2.5 nM) significantly ($P < 0.01$) inhibited DNA fragmentation of butyric acid-treated thymocytes (68.7 to 9.5% for H7 and 68.7 to 21.8% for staurosporine) (Fig. 8). In contrast, H7 was unable to modify the extent of butyric acid-induced apoptosis in splenic T cells (27.8 to 24.6%), although staurosporine effectively ($P < 0.05$) inhibited DNA fragmentation of butyric acid-treated splenic T cells (27.8 to 11.6%). With Jurkat cells, H7 and staurosporine slightly decreased DNA fragmentation of butyric acid-treated cells (54.5 to 43.4% for H7, [$P < 0.05$] and 54.5 to 32.8% for staurosporine [$P < 0.01$]).

DISCUSSION

A previous study (23) indicated that a low concentration (2.5 mM) of butyric acid, significantly suppressed T- and B-cell proliferation and production of cytokines by concanavalin A-stimulated murine splenic T helper cells. Since culture filtrates from *P. gingivalis*, *P. loescheii*, and *F. nucleatum* cells contain from 13.3 to 26.8 mM of butyric acid (23), the concentration of

butyric acid in subgingival plaque from periodontitis sites could reach 14.4 to 20 mM (26, 31), and its concentration in periodontal pockets has been shown to correlate with the severity of periodontal disease (5), it is likely that butyric acid is a most important virulent factor of these periodontopathogens. In attempting to determine the mechanism of butyric acid-induced suppression of T- and B-cell responses, we further examined the capacity of butyric acid to regulate proliferation and apoptosis of T cells. In the present study, we demonstrated a time- and dose-dependent suppression of butyric acid on the viability and induction of apoptosis of murine thymocytes and splenic T cells and of human Jurkat T cells. The main findings were as follows. (i) Butyric acid inhibits cell growth and induces apoptosis in mouse immature thymocytes, splenic T cells, and human Jurkat cells. (ii) Butyric acid-induced apoptosis is endonuclease dependent. (iii) Butyric acid-induced apoptosis in thymocytes is suppressed by protein kinase C (PKC) inhibitors, but these inhibitors are less effective on the butyric acid-induced apoptosis of splenic T cells and Jurkat cells.

We have shown that butyric acid-stimulated mouse thymocytes, splenic T cells, and Jurkat cells underwent apoptosis in vitro, a specific form of programmed cell death characterized by internucleosomal DNA digestion, revealed by colorimetric DNA fragmentation assay (Fig. 2 and 3) followed by gel electrophoresis (Fig. 4). Cell death was also associated with chromatin condensation (Fig. 5) and flow cytometric determination of the proportion of cells with hypodiploid DNA (Fig. 6). Thymocytes were more sensitive to apoptosis caused by butyric acid than splenic T cells and Jurkat cells (Fig. 2), suggesting

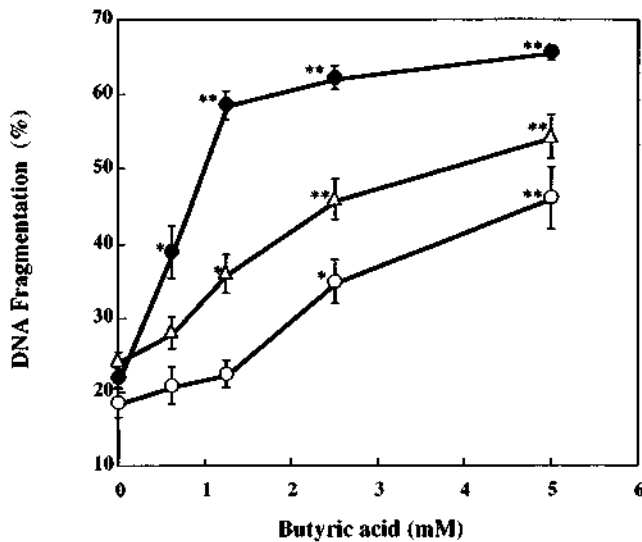


FIG. 2. Butyric acid dose-response curves for apoptosis. Thymocytes (●), splenic T (○), and Jurkat cells (△) were cultured with the indicated concentration of butyric acid for 21 h. Harvested cells were assayed by the DPA assay. The data are expressed as the means \pm standard errors from three different experiments with triplicate cultures. Values that are significantly different from the values for the respective controls at $P < 0.01$ (**) and $P < 0.05$ (*) are indicated.

that the effect of butyric acid was most pronounced on immature T cells. Glucocorticoids (9), heat (30), irradiation (39), dioxine (29), and human immunodeficiency virus (4) are known to induce thymic apoptosis. Immature T cells undergo intrathymic apoptosis when they encounter antigens, resulting in clonal deletion before they migrate to the periphery. After maturation, the same stimulus delivered through the T-cell receptor-CD3 complex leads to activation instead of apoptosis (1, 2, 42). Thus, the conventional wisdom is that mature T cells, having survived negative selection, should be relatively resistant to apoptosis.

Evidence for spontaneous apoptosis in T cells has been reported (8, 35). Peradone et al. (35) proposed that mature mouse splenic T cells succumbed to apoptosis when cultured without stimulus. Cohen and Duke (8) have suggested that the increase in DNA fragmentation when thymocytes are incubated in the absence of stimuli is probably due to the action of an endonuclease for which synthesis was induced by corticosterone in the animal before the removal of the thymus. Although in our study, unstimulated normal murine thymocytes, splenic T cells, and Jurkat cells underwent spontaneous apoptosis when cultured for long periods (Fig. 3), the number of spontaneously induced apoptotic cells was negligible compared to that seen after exposure of the cells to butyric acid.

Flow cytometric analysis revealed that maximal DNA degradation appeared to occur 21 h after butyric acid treatment (Fig. 3), as evidenced by the appearance of DNA with low fluorescence (Fig. 6). Kinetic studies of flow cytometric cell cycle analysis of propidium iodide-stained Jurkat cells treated with 5 mM butyric acid revealed the appearance of a distinct cell cycle region below the G_1 region. This extra "sub- G_1 " peak displaying reduced fluorescence of the DNA is likely due to a reduction in cell volume and nuclear condensation characteristics of apoptotic cells (12). However, as necrotic cells have a staining pattern similar to that of apoptotic cells with propidium iodide staining, this result may contain a certain amount of DNA from necrotic cells. Indeed, it is also the reason why the 82.1% value is so high compared to the per-

centage of apoptotic cells detected by DPA assay. Cell cycle distribution also changed during incubation: after 16 h of incubation, butyric acid treatment resulted in the degradation of the cells in the S and G_2/M phases of the cell cycle (data not shown), which means that butyric acid-induced inhibition of cell growth was correlated to an arrest in the G_1 phase of the cell cycle. However, alterations also were observed in other phases of the cycle with the passage of incubation time, that is, after 21 h of incubation, butyric acid induced the degradation of cells in all phases of the cell cycle. Gorczyca et al. (17), in an

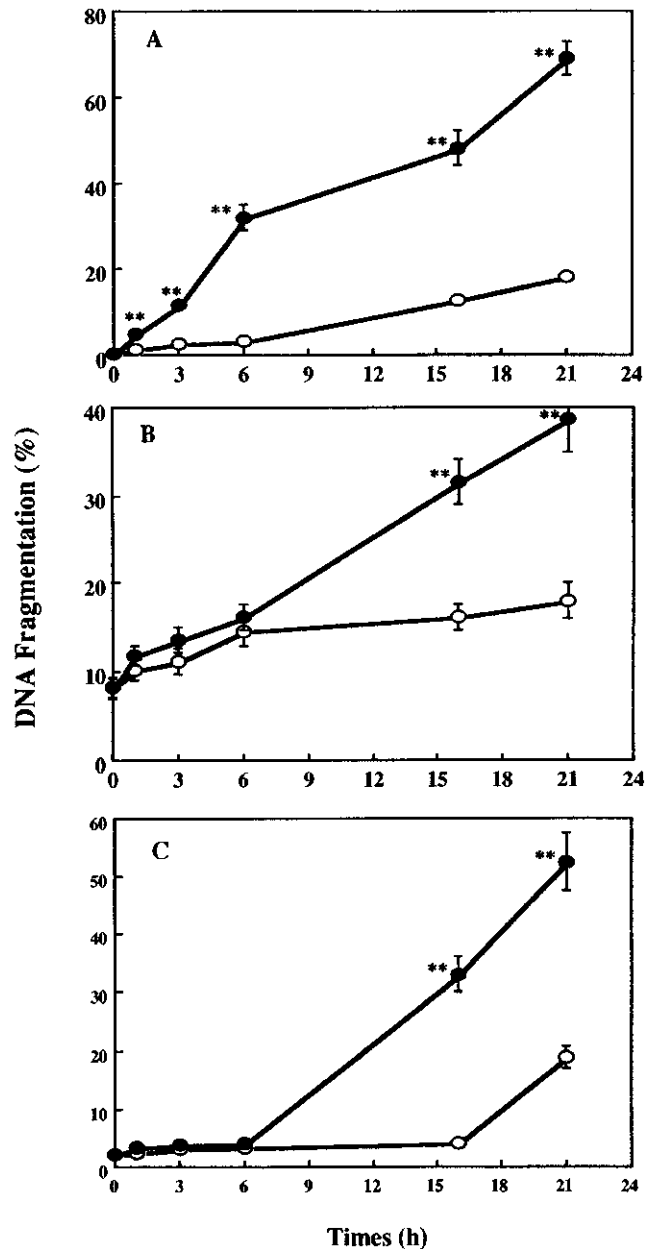


FIG. 3. Time course of butyric acid-induced apoptosis. Thymocytes (A), splenic T cells (B), and Jurkat cells (C) were cultured for the indicated times in the presence (●) or absence (○) of butyric acid (5 mM). Harvested cells were assayed by the DPA assay. The data are expressed as the means \pm standard errors from three different experiments with triplicate cultures. Values that are significantly different from the values for the respective controls at $P < 0.01$ (**) are indicated.

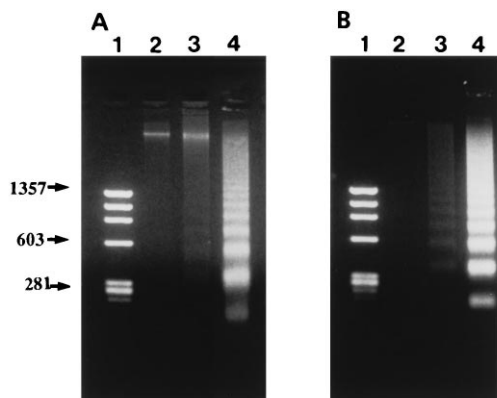


FIG. 4. Effect of butyric acid on DNA fragmentation. Jurkat cells (A) and thymocytes (B) were cultured for 0 h (lane 2) or 21 h in the absence (lane 3) or presence (lane 4) of butyric acid (5 mM). After incubation, DNA was collected and analyzed by electrophoresis on a 1.7% agarose gel. Lane 1 contains molecular weight markers (*Hae*III digested ϕ X174 DNA). Numbers at left indicate size in base pairs.

extensive survey of the susceptibility of the cell cycle phases to apoptosis by various agents such as cycloheximide, genistein, and gamma irradiation, showed that cell death induced by these agents occurred at different phases of the cell cycle. For example, cycloheximide and genistein exhibited no specific cell cycle preference, whereas gamma irradiation induced apoptosis was largely at the G_2/M phase. Other reports indicated that cells in the G_1 phase were preferentially affected by glucocorticoid (11), while the fraction of B cells in the S phase was reduced by lovastatin (36).

The induction of apoptosis by butyric acid has been described before for the human colonic tumor cell (18), Burkitt's lymphoma cell (14), and HL-60 myeloblastic cells (6). Although some studies have shown that inhibition of DNA synthesis by butyric sodium salt is associated with a hyperacetylation of histones H3 and H4 due to an *n*-butyrate-induced decrease in activity of some histone deacetylase (3, 37), the mechanism of butyric acid-induced apoptosis is still unknown. The data presented in this study with cultured thymocytes and splenic T cells provide the first evidence that butyric acid-

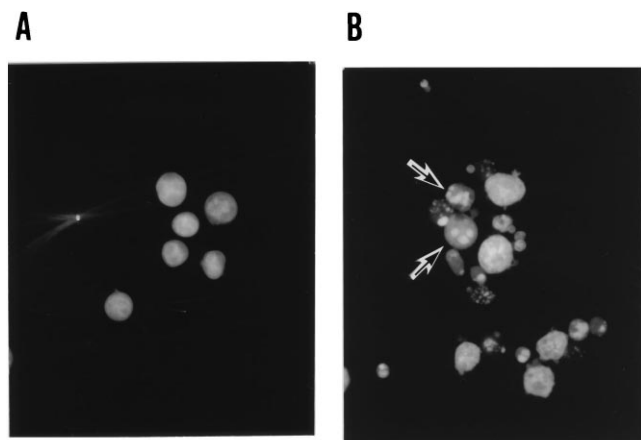


FIG. 5. Fluorescence microscopy appearance of butyric acid-treated Jurkat cells. Cells from untreated cultures (A) or cultures exposed to 5 mM of butyric acid (B) for 16 h were stained with Hoechst 33258. Arrows indicate the nuclei that show chromatin condensation.

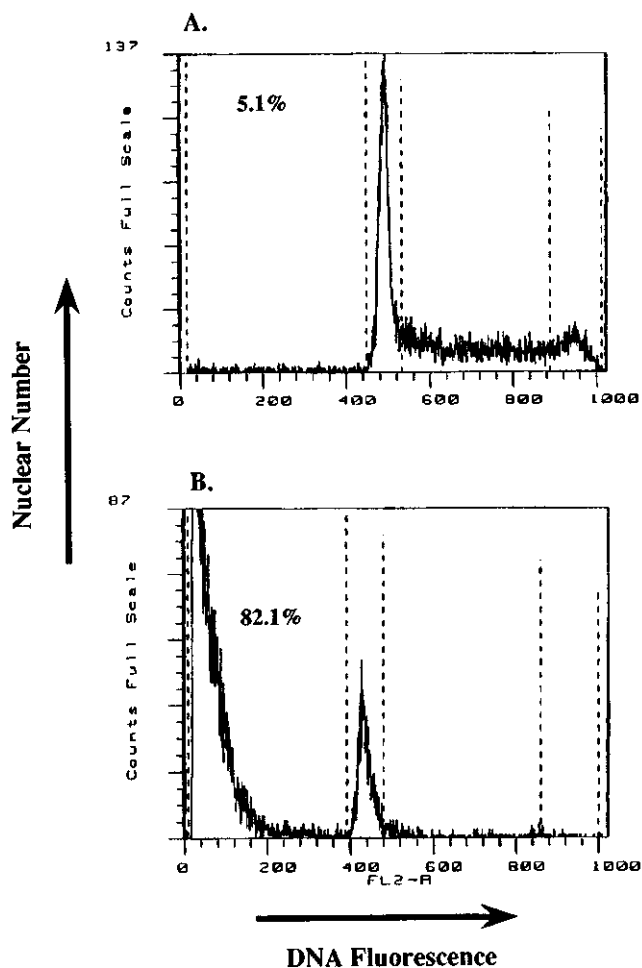


FIG. 6. Flow cytometric analysis of the DNA content of Jurkat cells. Jurkat cells were cultured in the absence (A) or presence (B) of butyric acid (5 mM). After 21 h of incubation, the number of cells undergoing apoptosis was measured by staining with propidium iodide and then analyzed by flow cytometry. The percentages of Jurkat cells with hypodiploid DNA are shown.

induced apoptosis is not limited to leukemic cells but also occurs in normal systemic cells.

Butyric acid-induced DNA fragmentation was inhibited in the presence of the endonuclease inhibitor $ZnCl_2$ (Table 1). The pattern of DNA fragments obtained from butyric acid-treated thymocytes and Jurkat cells (Fig. 4) also suggested that DNA was being hydrolyzed by an endonuclease with specificity for the linker regions between nucleosomes. It is possible that whenever cell death is a physiologically acceptable event, whatever the actual mediator that initiates the process, the final common pathway may be the activation of an endogenous endonuclease that breaks DNA down to nonfunctional subunits. In this study, butyric acid-induced apoptosis was predominantly observed in $CD4^+$ T cells rather than in $CD8^+$ T cells. The mechanism by which sensitization with butyric acid primes $CD4^+$ T cells for apoptosis is presently unknown. However, since infection with human immunodeficiency virus type 1 (28) and *Trypanosoma cruzi* (25) induce apoptosis in $CD4^+$ T cells specifically, it seems likely that $CD4^+$ T cells are more sensitive to apoptosis than $CD8^+$ T cells. Butyric acid-induced apoptosis in thymocytes also required protein synthesis as observed in other cell systems (19, 22). Signal transduction pathway of apoptosis is known to share common signal trans-

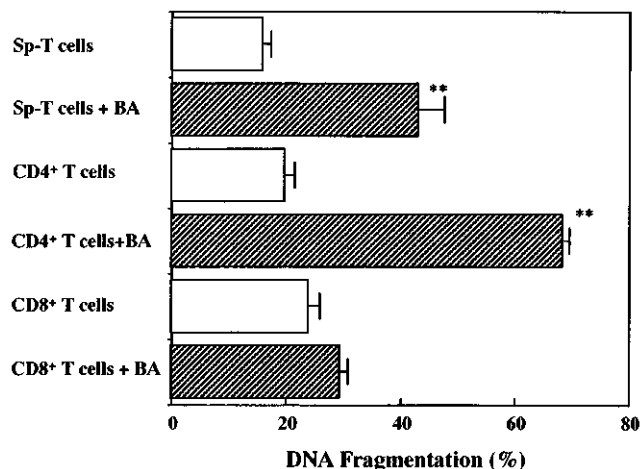


FIG. 7. Butyric acid-induced apoptosis in CD4⁺ T cells. Splenic T cells (Sp-T cells) were fractionated into CD4⁺ T and CD8⁺ T cells as described in Materials and Methods. Unseparated splenic T cells, CD4⁺ T cells, and CD8⁺ T cells were cultured in the absence or presence of butyric acid (BA) (5 mM) for 21 h. Harvested cells were assayed by the DPA assay. The data are expressed as the means \pm standard errors from three different experiments with triplicate cultures. Values that are significantly different from the values for the respective controls at $P < 0.01$ (**) are indicated.

duction pathway. Both PKA-PKC inhibitor, H7 or a PKC inhibitor, staurosporine, inhibited apoptotic changes in thymocytes. However, with splenic T cells and Jurkat cells, H7 or staurosporine was less effective against butyric acid-induced apoptosis. This discrepancy between the behavior of thymocytes and of splenic T and Jurkat cells might reflect the differences in the maturity between cells and in the mechanism of induction of apoptosis. Peradone et al. (35) have indicated that dexamethasone caused apoptosis in both thymus and spleen T cells but that only thymocytes revealed a requirement for protein synthesis in dexamethasone-induced death. From these findings, they have proposed that thymocytes and mature spleen T cells differ radically in their apoptosis control mechanisms, indicating that a transition in apoptosis regulation must occur during T-cell development, perhaps as the T cell evolves from dominant susceptibility to negative selection to dominant susceptibility to activation. Furthermore, previous studies indicate that in contrast to what has been observed in mature thymocytes (34, 46), no requirement for RNA or protein synthesis seems necessary for the apoptotic process in established cell lines (10, 27). A previous report indicated that

TABLE 1. Effect of endonuclease inhibitor on butyric acid-induced apoptosis

Treatment	% DNA fragmentation ^a		
	Thy	Sp-T	Jurkat
None	8.2 \pm 1.1	7.2 \pm 0.8	2.4 \pm 0.3
Butyric acid alone	70.7 \pm 2.6	34.0 \pm 2.8	53.2 \pm 2.2
Butyric acid with:			
1 mM ZnCl ₂	4.8 \pm 0.6	1.6 \pm 0.2	2.7 \pm 0.4
0.5 mM ZnCl ₂	12.0 \pm 1.0	9.5 \pm 0.4	17.6 \pm 2.0
0.1 mM ZnCl ₂	60.7 \pm 3.2	28.4 \pm 2.2	48.7 \pm 3.8

^a Thymocytes (Thy), splenic T cells (Sp-T), and Jurkat cells were treated with various concentrations of ZnCl₂ in the presence of butyric acid (5 mM) for 21 h. Harvested cells were assayed by DPA assay as described in Materials and Methods. The data are expressed as the means \pm standard errors from three different experiments with triplicate cultures.

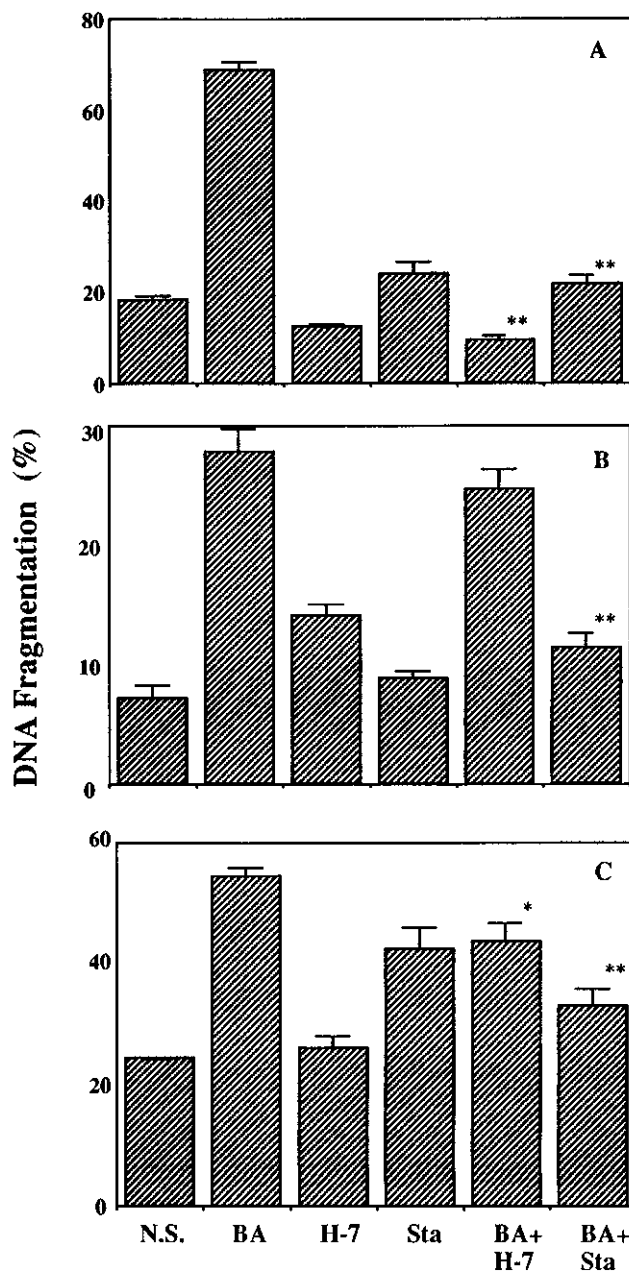


FIG. 8. Effects of PKC inhibitors on butyric acid-induced apoptosis. Thymocytes (A), splenic T cells (B), and Jurkat cells (C) were treated with no stimulus (N.S.), 5 mM butyric acid (BA), 50 μ M H7, 2.5 nM staurosporine (Sta), BA and H7, and BA and Sta for 21 h. Harvested cells were assayed by the DPA assay. The data are expressed as the means \pm standard errors from three different experiments with triplicate cultures. Values that are significantly different from the values for the respective controls at $P < 0.01$ (**) and $P < 0.05$ (*) are indicated.

spontaneous apoptosis was increased in splenic T cells by H7 or staurosporine (35). Our data showing that spontaneous apoptosis of splenic T cells increased 7.0 and 1.7% by addition of H7 and staurosporine, respectively, and that similarly spontaneous apoptosis of Jurkat cells increased 2.0 and 18.0% by addition of these reagents suggests that the difference in sensitivity of each cell type to the inhibitors also reflects the effect of PKC inhibitor on butyric acid-induced apoptosis.

In summary, we have reported here that butyric acid induces

apoptosis in murine thymocytes, splenic T cells, and human Jurkat cells. This specific form of programmed cell death was characterized by DNA fragmentation assay, gel electrophoresis, chromatin condensation, and flow cytometric analysis. These data support the hypothesis that activation of apoptosis is at least one essential step in the butyric acid-induced immunosuppressive pathway and that butyric acid can modulate the immunoregulatory cell population in the periodontal tissue by inducing T-cell death through apoptosis.

ACKNOWLEDGMENTS

We thank Suzanne Michalek, University of Alabama, for critical review of the manuscript.

This work was supported in part by grants-in-aid (07671992) for scientific research from the Ministry of Education, Science, and Culture of Japan and by a Suzuki research grant from Nihon University School of Dentistry at Matsudo.

REFERENCES

- Ashwell, J. D., and R. D. Klausner. 1990. Genetic and mutational analysis of the T-cell antigen receptor. *Annu. Rev. Immunol.* **8**:139-167.
- Bluestone, J. A., D. Pardoll, S. O. Sharrow, and B. J. Fowlkes. 1987. Characterization of murine thymocytes with CD3-associated T-cell receptor structures. *Nature* **326**:82-84.
- Boffa, L. C., G. Vidali, R. S. Mann, and V. G. Allfrey. 1978. Suppression of histone deacetylation *in vivo* and *in vitro* by sodium butyrate. *J. Biol. Chem.* **253**:3364-3366.
- Bonyhadl, M. L., L. Rabin, S. Salimi, D. A. Brown, J. Kosek, J. M. McCune, and H. Kaneshima. 1993. HIV induces thymus depletion *in vivo*. *Nature* **363**:728-732.
- Botta, G. A., L. Radin, A. Costa, G. Schito, and G. Blasi. 1985. Gas-liquid chromatography of the gingival fluid as an aid in periodontal diagnosis. *J. Periodont. Res.* **20**:450-457.
- Calabresse, C., L. Venturini, G. Ronco, P. Villa, C. Chomienne, and D. Belpomme. 1993. Butyric acid and its monosaccharide ester induce apoptosis in the HL-60 cell line. *Biochem. Biophys. Res. Commun.* **195**:31-38.
- Cohen, J. J. 1993. Apoptosis. *Immunol. Today* **14**:126-130.
- Cohen, J. J., and R. C. Duke. 1984. Glucocorticoid activation of a calcium-dependent endonuclease in thymocyte nuclei leads to cell death. *J. Immunol.* **132**:38-42.
- Cohen, J. J., M. Fschbach, and H. N. Claman. 1970. Hydrocortisone-resistance of graft-vs-host activity in mouse thymus, spleen, and bone marrow. *J. Immunol.* **105**:1146-1150.
- Collins, R. J., B. V. Harmon, T. Souvlis, J. H. Pope, and J. F. R. Kerr. 1991. Effect of cycloheximide on B-chronic lymphocytic leukemic and normal lymphocytes *in vitro*: induction of apoptosis. *Br. J. Cancer* **64**:518-522.
- Cutts, J. L., and G. Melnykovich. 1987. The role of cholesterol in the glucocorticoid-mediated inhibition of cell cycle progression in human acute lymphoblastic leukemia cells. *Exp. Cell Res.* **168**:95-104.
- Darzynkiewicz, Z., S. Bruno, G. D. Bino, W. Gorczyca, M. A. Hotz, P. Lassota, and P. Traganos. 1992. Features of apoptotic cells measured by flow cytometry. *Cytometry* **13**:795-808.
- Eftimiadi, C., M. Tonetti, A. Cavallero, O. Sacco, and G. A. Rossi. 1990. Short chain fatty acid produced by anaerobic bacteria inhibit phagocytosis by human lung phagocytes. *J. Infect. Dis.* **161**:138-142.
- Filippovich, I., N. Sorokina, K. K. Khanna, and M. F. Lavin. 1994. Butyrate induced apoptosis in lymphoid cells preceded by transient over-expression of HSP 70 mRNA. *Biochem. Biophys. Res. Commun.* **198**:257-265.
- Gamet, L., D. Daviaud, C. Denis-Pouxviel, C. Remesy, and J. C. Murat. 1992. Effect of short-chain fatty acids on growth and differentiation of the human colon-cancer cell line HT 29. *Int. J. Cancer* **52**:286-289.
- Gorbach, S., J. Mayhew, J. Bartlett, H. Thadepalli, and A. Onderdonk. 1976. Rapid diagnosis of anaerobic infections by direct gas liquid chromatography of clinical specimens. *J. Clin. Invest.* **57**:478-484.
- Gorczyca, W., J. Gong, B. Ardel, F. Traganos, and Z. Darzynkiewicz. 1993. The cell cycle related differences in susceptibility of HL-60 cells to apoptosis induced by various antitumor agents. *Cancer Res.* **53**:3186-3192.
- Hague, A., A. M. Manning, K. A. Hanlon, L. I. Huschtscha, D. Hart, and C. Paraskeva. 1993. Sodium butyrate induces apoptosis in human colonic tumor cell lines in a p53-independent pathway: implications for the possible role of dietary fibre in the prevention of large-bowel cancer. *Int. J. Cancer* **55**:498-505.
- Ji, L., G. Zang, and Y. Hirabayashi. 1995. Inhibition of tumor necrosis factor α - and ceramide-induced internucleosomal DNA fragmentation by herbimycin A in U937 cells. *Biochem. Biophys. Res. Commun.* **212**:640-647.
- Jordan, J. P., C. M. Hand, R. S. Markowitz, and P. Black. 1992. Test for chemotherapeutic sensitivity of cerebral gliomas: use of colorimetric MTT assay. *J. Neurooncol.* **14**:19-35.
- Kabelitz, D., T. Pohl, and K. Pechhold. 1993. Activation-induced cell death (apoptosis) of mature peripheral T lymphocytes. *Immunol. Today* **14**:338-339.
- Kameda, T., H. Ishikawa, and T. Tsutsui. 1995. Detection and characterization of apoptosis in osteoclasts *in vitro*. *Biochem. Biophys. Res. Commun.* **207**:753-760.
- Kurita-Ochiai, T., K. Fukushima, and K. Ochiai. 1995. Volatile fatty acids, metabolic by-products of periodontopathic bacteria, inhibit lymphocyte proliferation and cytokine production. *J. Dent. Res.* **74**:1367-1373.
- Kurita-Ochiai, T., K. Ochiai, N. Saito, and T. Ikeda. 1994. Adoptive transfer of suppressor T cells induced by *Actinobacillus actinomycetemcomitans* regulates immune response. *J. Periodont. Res.* **29**:1-8.
- Lopes, M. F., V. F. de Veiga, A. R. Santos, M. E. F. Fonseca, and G. A. DosReis. 1995. Activation-induced CD4⁺ T cell death by apoptosis in experimental Chagas' disease. *J. Immunol.* **154**:744-752.
- Margolis, H. C., J. H. Duckworth, and E. C. Moreno. 1988. Composition and buffer capacity of pooled starved plaque fluid from caries-free and caries-susceptible individuals. *J. Dent. Res.* **67**:1476-1482.
- Martin, S. J., S. V. Lennon, A. M. Bonham, and T. G. Cotter. 1990. Induction of apoptosis (programmed cell death) in human leukemic HL-60 cells by inhibition of RNA or protein synthesis. *J. Immunol.* **145**:1859-1867.
- Martin, S. J., P. M. Mearar, and A. Vyakarham. 1994. HIV-1 infection of human CD4⁺ T cells *in vitro*. *J. Immunol.* **152**:330-342.
- McConkey, D. J., P. Hartzell, S. K. Duddy, H. Hakensson, and S. Orrenius. 1988. 2,3,7,8-Tetrachlorodibenzo-p-dioxine kills immature thymocytes by Ca²⁺-mediated endonuclease activation. *Science* **242**:256-259.
- Migliorati, G., I. Nicoletti, F. Crocicchio, C. Pagliacci, F. D. Adamio, and C. Riccardi. 1992. Heat shock induces apoptosis in mouse thymocytes and protects them from glucocorticoid-induced cell death. *Cell. Immunol.* **143**:348-356.
- Naleway, C., H. Chou, T. Manos, C. Goodman, P. Robinson, and R. Singer. 1989. Assessment of the potential relationship between levels of SCFA found in subgingival plaque and periodontal health. *J. Dent. Res.* **68**(Suppl.):121. (Abstract.)
- Newell, M. K., L. J. Haughn, C. R. Maroun, and M. H. Julius. 1990. Death of mature T cells by separate ligation of CD4 and the T-cell receptor for antigen. *Nature* **347**:286-289.
- Nicoletti, L., G. Migliorati, M. C. Pagliacci, F. Grignani, and C. Riccardi. 1991. A rapid and simple method for measuring thymocyte apoptosis by propidium iodide staining and flow cytometry. *J. Immunol. Methods* **139**:271-279.
- Owens, G. P., W. E. Hahn, and J. J. Cohen. 1991. Identification of mRNAs associated with programmed cell death in immature thymocytes. *Mol. Cell. Biol.* **11**:4177-4188.
- Peradones, C. E., V. A. Illera, D. Peckham, L. L. Stunz, and R. F. Ashman. 1993. Regulation of apoptosis *in vitro* in mature spleen T cells. *J. Immunol.* **151**:3521-3529.
- Reedquist, K. A., T. K. Pope, and D. A. Roess. 1995. Lovastatin inhibits proliferation and differentiation and causes apoptosis in lipopolysaccharide-stimulated murine B cells. *Biochem. Biophys. Res. Commun.* **211**:665-670.
- Riggs, M. G., R. G. Whittaker, J. R. Neumann, and V. M. Ingram. 1977. *n*-Butyrate causes histone modification in HeLa and Friend erythroleukemia cells. *Nature* **268**:462-464.
- Rotstein, O. D., T. Vittorini, J. Kao, M. E. McBurney, P. E. Nasmith, and S. Grinstein. 1989. A soluble *Bacteroides* by-product impairs phagocytic killing of *Escherichia coli* by neutrophils. *Infect. Immun.* **57**:745-753.
- Sellins, K. S., and J. J. Cohen. 1987. Gene induction by γ -irradiation leads to DNA fragmentation in lymphocytes. *J. Immunol.* **139**:3199-3206.
- Siegel, I. 1977. Permeability of oral mucosa to organic acid. *J. Dent. Res.* **56**(Suppl.):52. (Abstract.)
- Singer, R., and B. Bucker. 1981. Butyrate and propionate: important components of toxic dental plaque extracts. *Infect. Immun.* **32**:458-463.
- Smith, C. A., G. T. Williams, R. Kingstone, E. J. Jenkinson, and J. J. T. Owen. 1989. Antibodies to CD3/T-cell receptor complex induce death by apoptosis in immature T cells in thymic cultures. *Nature* **337**:181-184.
- Socransky, S., and A. Haffajee. 1991. Microbiological mechanisms in the pathogenesis of destructive periodontal diseases: a critical assessment. *J. Periodont. Res.* **26**:195-212.
- Soder, P. O., L. J. Jin, and B. Soder. 1993. DNA probe detection of periodontopathogens in advanced periodontitis. *Scand. J. Dent. Res.* **101**:363-370.
- Wysocki, L. J., and V. L. Sato. 1978. "Panning" for lymphocytes: a method for cell selection. *Proc. Natl. Acad. Sci. USA* **75**:2844-2848.
- Yamada, T., and H. Ohyama. 1988. Radiation-induced interphase death of rat thymocytes is internally programmed (apoptosis). *Int. J. Radiat. Biol.* **53**:65-75.