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CELLULAR AND MOLECULAR MECHANISMS
OF T LYMPHOCYTES APOPTOSIS
BY *ACTINOBACILLUS ACTINOMYCETEMCOMITANS*

by

Ayten Nalbant

A Dissertation Presented to the
FACULTY OF THE GRADUATE SCHOOL
UNIVERSITY OF SOUTHERN CALIFORNIA
In Partial Fulfillment of the
Requirements for the Degree
DOCTOR OF PHILOSOPHY
(CRANIOFACIAL BIOLOGY)

December 2003

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requirements for the degree of*

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DEDICATION

I dedicate this dissertation
to my sons, **ATAOL BEHRAM** and **BERK TROY**
who are love of my life and my reasons to go on everyday
and
to my parents, my father **Recep Nalbant** and my mother **Sabriye Nalbant**
who gave life to me and made me the person who I am today.

I am forever grateful to my parents especially to my mother. My father died, when I was ten years old. It was a very bad car accident. It changed our lives forever. Since that day, my mother incredibly dedicates her life to us, myself and my sisters. Without her promise of a lifetime love, trust, courage and commitment to me and my education, I wouldn't be able to accomplish what I have so far. I am flattered to have her as an extraordinary human being in my life.

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ABSTRACT

Actinobacillus actinomycetemcomitans is a major pathogen implicated in several forms of periodontal diseases, as well as nonoral infections. Despite extensive investigation, the exact mechanism of pathogenicity of this microorganism remains unknown. There is evidence that this bacterium uses a number of mechanisms to suppress the host response. One such mechanism is the killing of host cells by apoptosis. The cytotoxicity of *A. actinomycetemcomitans* toward a number of cell types has been demonstrated, however comparatively little information is available on *A. actinomycetemcomitans* -induced apoptosis of T cells. Therefore, the aim of the present study was to determine the magnitude and mechanism of T cell apoptosis in response to *A. actinomycetemcomitans*. To that end, an *in vitro* culture system was used, where peripheral blood mononuclear cells (PBMC) were cultured at different time points in the presence of different preparations of *A. actinomycetemcomitans*. Cells were immunofluorescently labeled with monoclonal antibodies or reagents specific for markers associated with apoptosis, in concert with lineage and subset-specific markers. Flow cytometric analysis was then performed to determine the proportion of cells with a specific marker. Results demonstrated that following exposure of T cells to preparations of *A. actinomycetemcomitans*, progressively, plasma membrane alterations associated with apoptosis were detected. These included externalization of phosphatidylserine, which was detected in approximately half

of all T cells by 96 hours. This was followed by loss of membrane integrity. The data presented here provides evidence for the induction of apoptosis among the majority of the T cells responding to *A. actinomycetemcomitans*.

To investigate the mechanism(s) of *A. actinomycetemcomitans* induced T cell apoptosis, the involvement of the Fas-mediated apoptotic pathway was investigated. Results demonstrated upregulation of Fas and activation of caspase-3 in T cells in response to *A. actinomycetemcomitans*. Monocytes were the only cells analyzed to constitutively express FasL, which was further upregulated in response to *A. actinomycetemcomitans*, while T cells expressed FasL only after this stimulation. Depletion of monocytes prior to stimulation with *A. actinomycetemcomitans* led to a marked decline in apoptosis. Blocking Fas-FasL interactions lead to a significant decline, but not abolition of T cell apoptosis. Nearly all T cells expressed Bcl-2 at the outset of culture and Bcl-2 expression declined in T cells stimulated with *A. actinomycetemcomitans*. Collectively, these data provide evidence for the induction of T cell apoptosis by *A. actinomycetemcomitans* via Fas-mediated pathway.

A. actinomycetemcomitans expresses two known cytotoxin, namely leukotoxin (Ltx) and cytolethal distending toxin (CDT). CDT is also capable of inducing a cell cycle arrest in T cells in the G2 phase. However the extent of contribution of each of these toxins, as well as the existence of other possible molecules with

cytolytic and cytostatic activities is not known. To that end, a series of isogenic deletion mutants were generated. The cytolytic and cytostatic activities of wild type and isogenic deletion mutants were compared. Results showed that the leukotoxin and CDT partially contributed to the cytotoxicity of *A. actinomycetemcomitans*, since each of the isogenic mutants exhibited reduced apoptosis for T cells. The expression of *ltx* and each of the *cdt* gene loci partially contributed to the cytotoxicity of *A. actinomycetemcomitans*, since each of the isogenic mutants exhibited reduced cytotoxicity for T cells. On the other hand, the ability to induce a cell cycle block was abolished in each of the *cdt* isogenic mutants. Double mutants with deletion of *ltx*, as well as all 3 *cdt* gene loci retained significant ability to induce T cell apoptosis. Collectively, these data provide evidence for the expression of other cytolytic molecule(s) of *A. actinomycetemcomitans* distinct from CDT and leukotoxin, while suggesting that the cytostatic function is dependent on the expression of all 3 *cdt* gene loci.

CHAPTER I

INTRODUCTION

Periodontal Diseases

Periodontal diseases are a group of inflammatory diseases of the supporting structures of the periodontium. The periodontium is composed of the alveolar bone, cementum, periodontal ligament, and gingiva. They are most common of the oral inflammatory diseases and are described as the bacterially initiated conversion of a healthy gingival region to one characterized by inflammation (gingivitis) and the destruction of the supporting structures of the teeth (periodontitis).

Gingivitis is inflammation of gingiva in response to plaque bacteria and is characterized by erythema and edema and loss of the extracellular matrix in the periodontium. Gingivitis occurs as the microflora of dental plaque progresses from gram-positive bacteria to a more complex microflora of gram-negative bacteria and spirochetes [Kornman KS, 1996]. Some forms of gingivitis are brought on by systemic diseases or hormonal changes such as diabetes mellitus or pregnancy. Most individuals exhibit some degree of gingivitis and the condition can often be reversed with improved oral hygiene and debridement of the hard and soft deposits on teeth.

Periodontal disease is unusual in that it affects otherwise healthy people. Bacterial plaque accumulation leads to a chronic inflammation of gingiva. The gingiva begins to detach and migrate apically on the tooth surface. A distinct gap between the tooth surface and the gingiva forms a pocket that is caused by migration of the junctional epithelium along the root [Nisengard RJ et al, 1994]. Finally, the underlying alveolar bone is resorbed, possibly leading to loss of the tooth.

Periodontal diseases are polymicrobial in etiology. The episodic nature of periodontitis and host inflammatory response to plaque bacteria add to the puzzle of what exactly causes the tissue damage and destruction. Persistent infection and inadequate repair of damaged gingiva lead to a frustrated immune response in the host characterized by the excessive release of proinflammatory cytokines (Nisengard RJ et al, 1994). Attracted neutrophils flood the area with interleukins, tumor necrosis factor (TNF)- α , oxygen radicals and other molecules deleterious to microorganisms. Such a massive attack by the host immune system can lead to even more tissue damage (Lamster IB, 1996). This amplification of the immune response makes the person more susceptible to an increased severity of periodontitis (Kornman KS and di Giovine FS, 1998).

While a large number of different species have been recognized as members of the periodontal environment, it is now recognized that it is not these large numbers of bacteria that result in the progression from health to periodontal

disease, but it appears to be the establishment and growth of a very few bacterial species among the 300 or more proposed different bacterial species resident in the subgingival niche that are periodontopathic. One the most import bacteria among these putative periodontal pathogens is *Actinobacillus actinomycetemcomitans*.

Actinobacillus actinomycetemcomitans

A. actinomycetemcomitans is a gram negative coccobacillus. Microscopically, cultures appear predominantly bacillary with a few coccal forms. *A. actinomycetemcomitans* is capnophilic, requiring an atmosphere containing 5-10% CO₂ for good growth (Slots J, 1982). It is microaerophilic and a facultative anaerobe and can grow under anaerobic conditions (Pulverer G and Ko HL, 1972). The colonial morphology of fresh isolates is distinctive with the internal star shaped or crossed cigar morphology form embedding in the agar that gives *A. actinomycetemcomitans* its name (Zambon JJ, 1985). *A. actinomycetemcomitans* is an important pathogen in severe and recurrent forms of periodontitis. The prevalence of *A. actinomycetemcomitans* is nearly 90% in localized juvenile periodontitis and 30-50% in severe adult periodontitis (Asikainen S and Alaluusua S, 1993). It is frequently associated with rapidly progressive periodontitis (Slots J Bragd L et al., 1986; Zambon JJ, 1985). Among the five currently recognized serotypes of *A. actinomycetemcomitans* (Gmur R et al., 1993), serotype b strains often predominate in periodontal lesions of localized juvenile periodontitis

patients (Zambon JJ, 1985), although there is considerable overlapping in the serotype distribution (Asikainen S, Lai CH et al., 1991).

Virulence factors of *A. actinomycetemcomitans*

Virulence factors are attributes of microorganism that enable it to colonize a particular niche in its host, overcome the host defense and initiate a disease process. These factors frequently involve the ability to be transmitted to susceptible hosts. Organisms with many virulence factors are more pathogenic. Numerous studies have focused on the virulence of *A. actinomycetemcomitans* because of its strong association with periodontal diseases and related extraoral infections.

A. actinomycetemcomitans has been shown to possess a number of virulence factors that enhance its survival in the oral cavity and enable it to avoid the host's protective strategies. Many of this virulence factors may be involved in the pathogenesis of periodontitis. These factors include: leukotoxin (Taichman et al. 1980; Tsai et al. 1984; Lally et al. 1989; Lally et al. 1989), collagenase (Robertson et al. 1982), lipopolysaccharide (LPS) (Kiley et al. 1980), fibroblast-inhibitory factor (Meghji et al. 1992), bone resorption-inducing toxin (Slots et al. 1984), polymorphonuclear leukocyte chemotaxis inhibitory factor (Van Dyke et al. 1982), and lymphocyte suppressive factor (Shenker et al. 1982; Shenker et al.

1990; Kurita-Ochiai et al. 1992) and cytolethal distending toxin (Shenker et al. 1999; Shenker et al. 2000; Shenker et al. 2001).

The role of apoptosis in maintaining immunological balance

It is well established that apoptosis plays a major role in maintaining immunological homeostasis. Apoptosis is an active process by which the target cell activates a cascade of events leading to its demise. Apoptosis may be induced through several pathways. The best-defined pathway initiates when Fas ligand (FasL) binds to Fas on target cell. This cross-links Fas and its associated death domains, allowing can then bind to adaptor proteins with death domains to bind to Fas death domains. This activates caspases, which are a family of cysteine proteases with a critical role in apoptosis. The caspase cascade culminates in the activation of DNase, which cleaves genomic DNA into 200 base pair fragments, a hallmark of apoptosis. Another pathway of apoptosis involves tumor necrosis factor (TNF) and its cellular receptors. One of the earliest cellular changes occurring during apoptosis is the externalization of phosphatidylserine (PS). PS is normally present on the inner aspect of the plasma membrane and is externalized early during apoptosis. Annexin V is a phospholipid-binding protein that binds to externalized PS on plasma membrane of apoptotic cells (Koopman, 1994; Martin, 1995).

A number of regulatory molecules are involved in apoptosis regulation. Bcl are a family of proteins that are important regulators of apoptosis. Some of the members of this family, (Bcl-2 and bcl-X_L) inhibit apoptosis, while others (Bax and Bad) promote apoptosis. During apoptosis, mitochondria swell, releasing Cytochrome C, which can contribute to the activation of caspases. Bcl-2 binds to mitochondrial surfaces, blocking its swelling and Cytochrome C release, thereby inhibiting apoptosis. Normal homeostasis requires a delicate balance between the induction of apoptosis and its inhibition, maintaining a constant number of lymphocytes.

Apoptosis as a virulence mechanism of bacteria

The presence of a mechanism for the host to participate in its own destruction has made an obvious target for the virulence factors of some microorganisms. Activation or prevention of cell death could be a critical factor in the outcome of an infection. Apoptosis has been observed as a response to infection by a wide range of pathogens and is mediated by an array of pathogen-encoded virulence determinants (Weinrauch, 1999). Pathogen-induced modulation of the host cell-death pathway may serve to eliminate key immune cells or evade host defenses that can act to limit the infection. Alternatively, suppression of the death pathway may facilitate the proliferation of intracellular pathogens.

Apoptosis and *A. actinomycetemcomitans*

A. actinomycetemcomitans expresses two known toxins with cytolytic properties against human cells, namely leukotoxin and CDT. Leukotoxin is a member of the repeats-in toxins (RTX) family of pore-forming proteins, whose members include hemolysins and leukotoxins (Lally, 1989). Leukotoxin is variably expressed by different strains, enabling them to kill both lymphoid and myeloid leukocytes (Lally, 1989; Lally, 1994; Kolodrubetz, 1996; Ohta, 1993; Mangan, 1991; Taichman, 1980; Tsai, 1984). Leukotoxin has been shown to kill human T cells by apoptosis (Mangan, 1991; Nalbant, 2000; Shenker, 2000; Shenker, 2001).

CDT is a soluble protein encoded by a cluster of three genes designated *cdtABC* (Pickett, 1999). The molecular mass of polypeptide products of these three genes is approximately 25-35 kDa. CDT is expressed by a number of gram negative organisms such as *A. actinomycetemcomitans* (Sugai, 1998; Shenker, 1999; Shenker, 2000), *Haemophilus ducreyi* (Cope, 1997), *Escherichia coli* (Scott, 1994; Perez, 1997; Pickett, 1994), *Shigella* (Okuda, 1995) and *Campylobacter jejuni* (Whitehouse, 1998; Pickett, 1996). CDT causes irreversible G2 arrest and subsequent cell death (Pickett, 1999). It has been shown that *A. actinomycetemcomitans* and *H. ducreyi* CDT induce apoptosis of T cells (Gelfanova, 1999; Shenker, 2001). Moreover, the *cdtB* gene has been shown to encode the biologically active component of *A. actinomycetemcomitans* responsible for cell cycle arrest of human lymphocytes (Shenker, 2000; Shenker,

2001). In contrast, *cdtC* encodes the structural toxin of *H. ducreyi* (Stevens, 1999) and antibodies against *H. ducreyi* CdtC neutralize *H. ducreyi* CDT activity (Stevens, 1999). On the other hand, CdtC encoded by *A. actinomycetemcomitans* neutralizes cytotoxic activity for epithelial cells but serum raised to *A. actinomycetemcomitans* CdtB lacks neutralizing activity (Sugai, 1998). Therefore, the biologic activity of different *cdt* gene products needs to be clarified.

Gaps in our knowledge regarding T cell apoptosis response to *A. actinomycetemcomitans*:

A number of questions remain unanswered regarding the cytotoxicity of *A. actinomycetemcomitans* toward T cells. These include:

- What is the magnitude of T cell apoptosis response by *A. actinomycetemcomitans*
- What are the cellular and molecular events that occur in T cells undergoing apoptosis in response to *A. actinomycetemcomitans*
- Which *A. actinomycetemcomitans* molecules induce T cell apoptosis

CHAPTER II

Evidence for apoptosis of the majority of T cells activated *in vitro* with *Actinobacillus actinomycetemcomitans*

ABSTRACT

Our previous studies had demonstrated that nearly half of all T cells stimulated with *A. actinomycetemcomitans* are activated within a few hours. However, it was not known whether all of these T cells survive. The aim of the present study was to determine whether the T cells activated in response to *A. actinomycetemcomitans* undergo apoptosis. To that end, peripheral blood mononuclear cells (PBMC) were cultured at different time points in the presence of *A. actinomycetemcomitans*. Flow cytometric analysis demonstrated that following exposure to a preparation of *A. actinomycetemcomitans*, T cells progressively externalized their plasma membrane phosphatidylserine (PS), as measured by Annexin V binding. Approximately half of all T cells bound Annexin V by 96 hours. During this period, Annexin V⁺ T cells also incorporated propidium iodide (PI) suggesting loss of membrane integrity. The externalization of PS occurred at a higher rate among activated (CD69⁺) T cells, where roughly two-third of became Annexin V⁺. Flow cytometric analysis also demonstrated shrinkage of the Annexin V⁺PI⁺ T cells. The data presented here provides

evidence for the induction of apoptosis among the majority of the T cells responding to *A. actinomycetemcomitans*.

INTRODUCTION

It is widely believed that specific gram-negative bacteria are involved in the initiation of inflammatory periodontal diseases. However, both microbial and host factors are likely to contribute to the tissue destruction. One of the major microorganisms implicated in some forms of periodontal disease is *Actinobacillus actinomycetemcomitans*. Though the exact mechanism of the pathogenicity of *A. actinomycetemcomitans* is not clear, a number of virulence factors have been characterized. These factors include: leukotoxin (Taichman, Dean et al. 1980; Tsai, Shenker et al. 1984; Lally, Golub et al. 1989; Lally, Kieba et al. 1989), collagenase (Robertson, Lantz et al. 1982), lipopolysaccharide (LPS) (Kiley and Holt 1980), fibroblast-inhibitory factor (Meghji, Wilson et al. 1992), bone resorption-inducing toxin (Slots and Genco 1984), polymorphonuclear leukocyte chemotaxis inhibitory factor (Van Dyke, Bartholomew et al. 1982), and lymphocyte suppressive factor (Shenker, Tsai et al. 1982; Shenker, Vitale et al. 1990; Kurita-Ochiai, Ochiai et al. 1992) and cytolethal distending toxin (Shenker, McKay et al. 1999; Shenker, Hoffmaster et al. 2000; Shenker, Hoffmaster et al. 2001).

Recent studies in our laboratory have shown that *A. actinomycetemcomitans* can induce a potent T cell activation response *in vitro*, where nearly half of all T cells are activated within 8 hours (Zadeh et al. 2001). However, the fate of these activated T cells was not known. The present study aimed to investigate whether the large number of T cells activated survive beyond activation. In view of the known cytolytic effects of *A. actinomycetemcomitans* on host cells, it will be of interest to determine whether the activated cells are eliminated, and if so, to define the mechanism.

A. actinomycetemcomitans has the ability to kill a variety of hematopoietic cells (Zadeh, Nalbant et al. 2001). Some strains of *A. actinomycetemcomitans* express leukotoxin, which is cytolytic toward both lymphoid and myeloid leukocytes. The mechanism by which *A. actinomycetemcomitans* leukotoxin kills target cells is currently under investigation. Two general mechanisms of eukaryotic cell death have been identified, i.e. necrosis and apoptosis. Recently it has been shown that leukotoxin-mediated killing of promyelocytic HL-60 cells involves the induction of apoptosis (Korostoff et al. 1998). Mangan, et al. have shown that purified leukotoxin can kill up to 70 % of human T cells with morphological features of apoptosis and necrosis (Mangan et al. 1991). Since T cells are an important component of the immune response, investigating microbial virulence factors that can affect their function are central to our understanding of the pathogenesis of periodontitis.

Apoptosis is a process, by which the cell participates by activating a cascade of molecular events, leading to its demise and disposal. Apoptosis is characterized by a variety of molecular and morphological changes. One of the earliest events in this cascade is plasma membrane changes, where the membrane phospholipid phosphatidylserine (PS) is translocated from the inner to the outer leaflet. Therefore, phosphatidylserine is exposed to the external cellular environment, while maintaining the plasma membrane integrity. This process can be monitored by using Annexin V, which is a calcium-dependent phospholipid-binding protein that binds preferentially to negatively-charged phospholipids such as phosphatidylserine (Koopman et al. 1994; Martin et al. 1995). The culmination of apoptosis is the loss of plasma membrane integrity. Propidium iodide is a DNA-intercalating dye that enters cells that have a breach in their plasma membrane integrity. Therefore, staining with Annexin V in conjunction with propidium iodide allows the identification of early apoptotic cells (Annexin V positive, propidium iodide negative). Apoptosis of hematopoietic cells normally occurs in physiological processes, e.g. during development or deletion of activated T cells that are no longer required in the immune response. Conversely, apoptosis may be part of a pathologic event, e.g. induced by pathogenic microorganisms (Thompson 1995).

In the present study, the capacity of *A. actinomycetemcomitans* to induce apoptosis among human peripheral T cells was investigated by examining early and late events of apoptosis. The results provide evidence for large-scale apoptosis of T cells during the course of the immune response to *A. actinomycetemcomitans*.

MATERIAL AND METHODS

Peripheral blood mononuclear cells

14 systemically and periodontally healthy subjects participated in this study. Subjects included 4 males and 10 females with mean age of 32 years. They were asked to sign an informed consent that was previously approved by the Institutional Review Board at the University of Southern California. Cultural analysis was used to select subjects for this study that did not harbor *A. actinomycetemcomitans* in their dental plaque. This entailed sampling of the dental plaque from 3 periodontal sulcus sites for each patient using paper points. The plaque samples were then inoculated on TSBV selective media (Slots 1982). Any cultivable colonies were examined for the presence of *A. actinomycetemcomitans* by colony morphology, Gram staining and positive catalase reaction.

Venous blood was drawn from healthy volunteers. Peripheral blood mononuclear cells (PBMC) were isolated by Ficoll-Hypaque density gradient centrifugation (Böyum 1968).

Bacteria

A. actinomycetemcomitans type strain (ATCC 33384, NCTC 9710, serotype c), which is generally recognized as a non-leukotoxin-producing or minimally leukotoxic strain (Spitznagel et al. 1991; Kolodrubetz et al. 1996), was purchased from ATCC (Rockville, MD). *A. actinomycetemcomitans* was grown as previously described [Sreenivasan, 1993 #144]. Briefly, bacteria were inoculated on MAN solid media plates containing Trypticase soy broth (Becton Dickinson and Co., Cockeysville, MD) supplemented with Trypticase soy agar (1.5% w/v; Becton Dickinson and Co., Cockeysville, MD), yeast extract (0.6% w/v; Becton Dickinson and Co., Cockeysville, MD) and 5% horse serum. Bacteria were cultured in a humidified atmosphere at 37°C and 10% CO₂ for 48 hours. The purity of bacteria was checked by cross streaking and subculture. Individual colonies grown were identified by Gram staining, H₂O₂ formation and PCR (Ashimoto et al. 1996). This ruled out contaminating colonies by demonstrating the absence of colonies testing negative by PCR for *A. actinomycetemcomitans*. To harvest the bacteria and their extracellular products, the surface of the plates were washed with 3 ml of PBS. After centrifugation (7000 rpm for 30 min), the supernatant was removed. This "plate wash" was concentrated (Slide-A-Lyzer ®

Concentrating Solution, Pierce Chemical Co., Rockford, IL) and dialyzed overnight against PBS to remove low molecular-weight components (SnakeSkin™ Dialysis Tubing; 10kDa Molecular Weight Cutoff, Pierce Chemical Co., Rockford, IL) (Stuart et al. 1992). Protein concentration was measured by Bradford method (Bio-Rad Laboratories, Richmond, CA). The bacterial plate wash was aliquoted and frozen at -70°C until use as stimulant for PBMC.

As a control, the surface of un-inoculated agar plates were washed in the same manner with PBS, demonstrating negligible presence of proteins. Hence, the preparation of bacterial extracellular material in this manner minimized the presence of bacterial media in the preparation, which is a problem when bacteria are grown in liquid media. The exact composition of the plate wash prepared in this manner is unknown. Detailed biochemical analyses are currently in progress to define the properties of some the molecules present in the plate wash.

Stimulation of mononuclear cells

PBMC were cultured in 12 x 75 mm culture tubes at a concentration of 2×10^6 cells/ml with or without *A. actinomycetemcomitans* plate wash in a humidified incubator at 37°C and 5% CO₂. The cells were incubated for various intervals between 0 to 96 hours. The medium consisted of RPMI-1640 supplemented with 10% FBS, 100 units/ml penicillin, 100 µg/ml streptomycin, and 25mM HEPES

buffer. *A. actinomycetemcomitans* plate wash was used at a concentration of 25 µg/ml, because dose-response studies in our laboratory showed that this was the optimal concentration for the induction of T cell activation (Zadeh, Nalbant et al. 2001) and preliminary studies confirmed the same with regard to Annexin V binding and propidium iodide staining (data not shown).

Immunofluorescence

Direct 3-color immunofluorescence assays of single cells in suspensions were performed as described (Zadeh, Nalbant et al. 2001). Briefly, 2×10^6 cells were incubated with a mixture of fluorochrome-conjugated monoclonal antibodies and probes (Annexin V or PI) for 15 minutes at room temperature (20-25 °C) in the dark. Labeled cells were washed twice with PBS containing 0.5% BSA and 0.1% sodium azide and analyzed by flow cytometry. Anti-CD69 and anti-CD3 were purchased from Becton Dickinson (San Jose, CA) conjugated with phycoerythrin (PE) and Peridinin Chlorophyll Protein (PerCP), respectively. Annexin V, conjugated with fluorescein isothiocyanate (FITC) was purchased from Pharmingen (San Diego, CA) and Propidium iodide was obtained from Becton Dickinson (San Jose, CA). Isotype-matched immunoglobulins conjugated with each of the fluorochromes were used as negative controls. Immunofluorescent labeling of peripheral blood mononuclear cells with monoclonal antibodies conjugated with each of the fluorochromes and known to react with these cells served as positive controls.

Detection of T cell activation

To identify activated T cells, anti-CD69 monoclonal antibody was used. CD69 is a very early human activation antigen that is present on activated T- and B-lymphocytes, as well as natural killer (NK) cells (Maino et al. 1995). CD69 is not expressed by resting peripheral blood lymphocytes.

Detection of apoptotic T cells

Annexin V conjugated with fluorescein isothiocyanate was used to determine the percentage of cells undergoing apoptosis. Cells cultured with or without *A. actinomycetemcomitans* plate wash were harvested, washed twice with cold PBS and then re-suspended in binding buffer at a concentration of 2×10^6 cells/ml. The binding buffer consisted of 10 mM HEPES/NaOH (pH 7.4), 140 mM NaCl, and 2.5 mM CaCl_2 . Annexin V-FITC and propidium iodide were added to the cells resuspend in binding buffer. The cells were gently vortexed and incubated for 15 minutes at room temperature (20-25 °C) in the dark. 400 μl of binding buffer was added to each tube and the cells were promptly analyzed by flow cytometry (within 1 hr). Cells with the phenotype of Annexin V positive propidium iodide negative were defined as early apoptotic cells. These are the cells that have presumably externalized their membrane phosphatidylserine and have an intact cell membrane. In contrast, cells that were positively labeled with propidium iodide have a breach in their membrane integrity and are dead. Once the membrane integrity is breached, Annexin V can potentially enter cells and

label the phosphatidylserine on the inner leaflet of the cell membrane; therefore, it would not be possible to predict whether cells, which are Annexin V positive propidium iodide positive, have died by apoptosis or necrosis.

Flow Cytometry

Immunofluorescently-labeled cells were processed on a FACSCalibur (Becton-Dickinson Immunocytometry Systems, San Jose, CA) flow cytometer equipped with an argon ion laser (488nm), and the data was analyzed using CellQuest software (Becton Dickinson). The data acquired by flow cytometry was limited to the cell subsets of interest by selection of electronic gates. Lymphocyte gates were selected on the basis of their forward and side scatter profiles and T cells were identified with anti-CD3 monoclonal antibody.

STATISTICAL ANALYSIS

Mean and standard deviation (S.D.) of data were calculated and are presented in Figures. The experimental treatment (*in vitro* culture with *A. actinomycetemcomitans* plate wash) was compared with control treatment (*in vitro* culture without stimulant) using repeated measures analysis of variance (ANOVA). Main effects included time of culture and treatment with *A. actinomycetemcomitans*. Since a large number of tests (8 for each set) were performed, Bonferoni adjustment for the p-values were made. Following this adjustment, a significance level of 0.0015 was used throughout the analyses.

RESULTS

Detection of phosphatidylserine (PS) exposure in T cells stimulated with *A. actinomycetemcomitans*

To investigate plasma membrane changes associated with apoptosis, freshly isolated PBMC from healthy donors were cultured with *A. actinomycetemcomitans* plate wash for various intervals from 2 to 96 hours. Cultured cells were labeled with Annexin V and anti-CD3, followed by flow cytometric analysis. Results demonstrated that the first observable increase in Annexin V labeling above background was at 48 hours (Figure 2.1). From that point, there was a progressive increase in the proportion of Annexin V⁺ T lymphocytes from 10% at 24 hours to 50% at 96 hours.

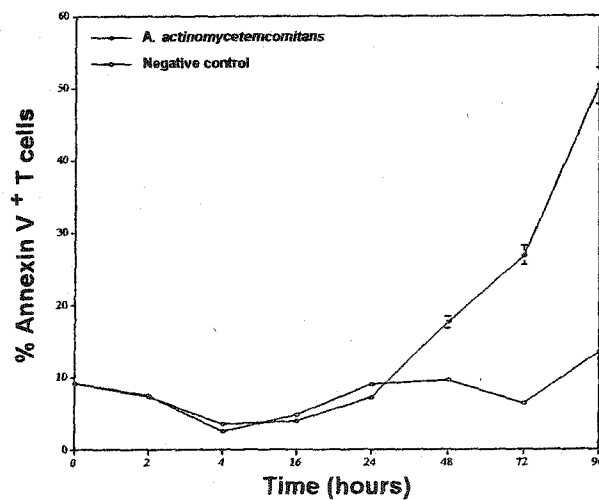


FIGURE 2.1. Detection of phosphatidylserine (PS) exposure on the surface of T cells in response to *A. actinomycetemcomitans*. Freshly isolated PBMC were incubated with or without *A. actinomycetemcomitans* plate wash for various intervals from 2 to 96 hours. Cultured cells were labeled with Annexin V and anti-CD3 monoclonal antibody, followed by flow cytometric analysis. A representative experiment that was repeated six times is presented, where the PBMC from a donor were cultured and analyzed in triplicate. Mean and standard deviation of the proportion of Annexin V positive T cells are shown. The differences between *A. actinomycetemcomitans* and negative control were significant at 48, 72 and 96 hours ($p = 0.0001$ using repeated ANOVA).

Identification of apoptotic T cells

To specifically identify the appearance of the phenotype associated with apoptotic cells, PBMC cultured in the presence or absence of *A. actinomycetemcomitans* plate wash were labeled with Annexin V and propidium iodide followed by flow cytometric analysis. Double labeling of cells with Annexin V and propidium iodide demonstrated that shortly after stimulation with *A. actinomycetemcomitans*, there was initially a marked increase in the proportion of Annexin V positive propidium iodide negative lymphocytes (Figure 2.2A). This was followed by a rise in the proportion of Annexin V positive propidium iodide positive lymphocytes, which first appeared at 48 hours, progressively increased thereafter and reached 21% by 96 hours.

To gain additional information about the phenotype of T cells that have externalized their phosphatidylserine, cells labeled with Annexin V were plotted against forward scatter, which is a measure of cell size (Figure 2.2B). This analysis illustrated that the Annexin V positive T cells that appeared after *A. actinomycetemcomitans* stimulation, were initially medium in size. Therefore, the phenotype of early apoptotic cells is associated with a normal lymphocyte size. The population of medium-sized Annexin V positive T cells increased from 10% at time zero to approximately 33% at 72 hours. However, these cells decreased back to baseline at 96 hours. Subsequently, a population of small-sized Annexin V positive T cells appeared. This population progressively rose with time and by

96 hours, it comprised approximately 46% of total T cells. The small cells were all Annexin V⁺ T cells. By 96 hours, there were five fold more small Annexin V⁺ T cells than the medium-sized ones. Analysis of propidium iodide labeling demonstrated that small Annexin V⁺ T cells were also propidium iodide positive (Figure 2.2C).

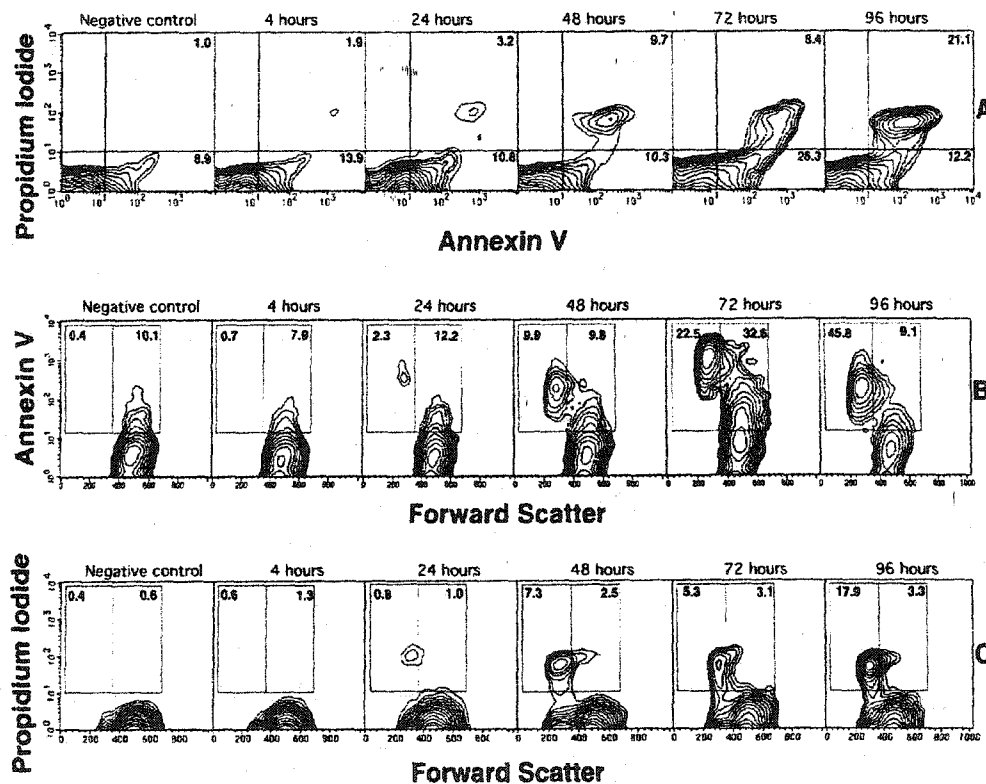


FIGURE 2.2. Gated contour dot plots of peripheral blood mononuclear cells cultured with or without *A. actinomycetemcomitans* at various time points. (A) Propidium iodide staining versus Annexin V labeling. Horizontal lines show propidium iodide positive lymphocytes, so that cells above the line are all propidium iodide positive. Vertical lines show Annexin V positive lymphocytes, so that cells on the right are Annexin V positive. The proportion of cells in each quadrant is annotated. Upper right quadrants represent propidium iodide positive Annexin V positive (apoptotic/necrotic) cells. (B) Gated contour dot plots of Annexin V labeling versus forward light scatter. The windows demarcate two subsets of Annexin V positive T lymphocytes: the left window being small-sized, the right being medium-sized. (C) Gated contour dot plots of propidium iodide staining versus forward light scatter. The windows demarcate two subsets of propidium iodide positive lymphocytes: the left window being small-sized, the right being medium-sized. Proportions of total Annexin V positive (pre-apoptotic T cells) and propidium iodide positive cells within each window are indicated.

Therefore, this data demonstrates the appearance of medium-sized Annexin V⁺PI⁺ cells followed by a population of small-sized Annexin V⁺PI⁺ lymphocytes.

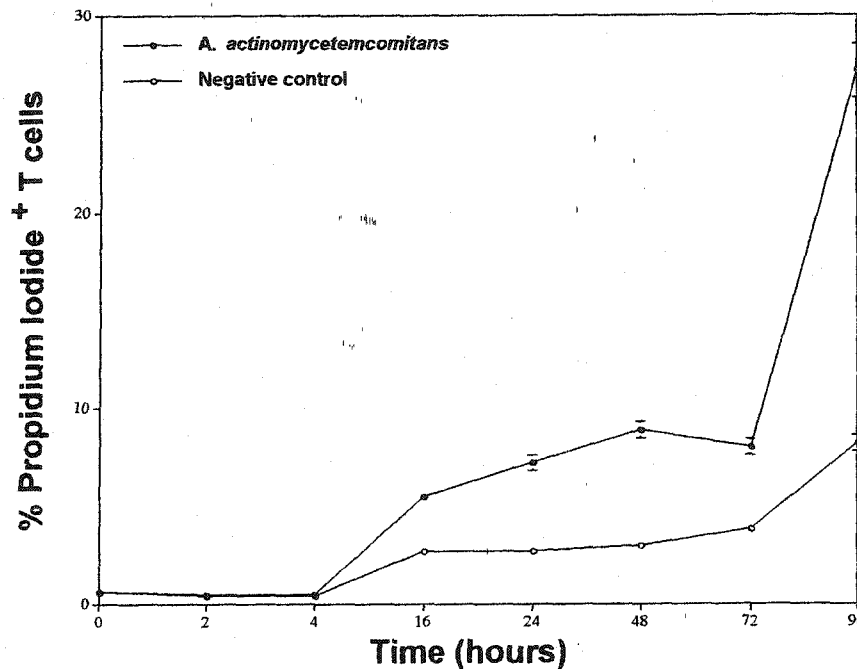


FIGURE 2.3. Proportion of propidium iodide positive lymphocytes among T cells. PBMC cultured with or without *A. actinomycetemcomitans* plate wash were dual-labeled with propidium iodide and anti-CD3 monoclonal antibody followed by flow cytometric analysis. One of five representative experiments. Mean and standard deviation of the proportion of propidium iodide positive lymphocytes among T cells are shown. Cultures with *A. actinomycetemcomitans* were significantly different from negative control cultures at 16, 48, 72 and 96 hours ($p = 0.0001$ using repeated ANOVA).

In order to prove that the lymphocytes labeled with propidium iodide were indeed T cells, the PBMC cultured with *A. actinomycetemcomitans* plate wash were dual labeled with propidium iodide and anti-CD3 monoclonal antibody. Results

demonstrated that the proportion of propidium iodide positive T lymphocytes remained low for the first 24 hours. Starting with the 48 hour time-point, the proportion of propidium iodide positive T lymphocytes significantly increased so that by 96 hours, approximately 30% of all T cells incubated with *A. actinomycetemcomitans*, but not those in control culture became propidium iodide positive (Figure 2.3). Therefore, there was a 48-hour lag in T cell death after stimulation with *A. actinomycetemcomitans*. Moreover, T cell death appeared to be significantly greater in magnitude in the presence of *A. actinomycetemcomitans*.

Annexin V binding among activated T cells

To determine the extent of apoptosis among the T cells that specifically responded to *A. actinomycetemcomitans*, PBMC were cultured with *A. actinomycetemcomitans* plate wash and labeled with anti-CD69 monoclonal antibody in concert with Annexin V-FITC. Results demonstrated that approximately one third of T cells that were activated (CD69⁺) at the onset of culture, labeled positively with Annexin V. This value declined to approximately 10% at 16 hours and thereafter progressively increased, so that at 72 hours 62% of CD69⁺ T lymphocytes were Annexin V positive. The kinetics of Annexin V labeling among activated T cells were similar in cultures with or without *A. actinomycetemcomitans* plate wash for most time points, except 2 and 24 hours (Figure 2.4A). This suggested that T cells either specifically activated by *A.*

actinomycetemcomitans or non-specifically activated in control cultures followed the same rate of apoptosis. In contrast, significantly fewer non-activated T cells (CD69⁺) in cultures with or without *A. actinomycetemcomitans* labeled positively with Annexin V (Figure 2.4B). Moreover, this proportion remained relatively low throughout the culture period (8 to 16% between 0 to 72 hours). This suggested that the majority (two-third) of activated T cells were committed to apoptosis.

***A. actinomycetemcomitans* -induced loss of T cell plasma membrane integrity**

To determine the time course of cell death following externalization of phosphatidylserine, simultaneous analysis of labeling with Annexin V and propidium iodide was performed on PBMC cultured in the presence or absence of *A. actinomycetemcomitans* plate wash. Results showed that the proportion of propidium iodide positive lymphocytes among Annexin V positive cells significantly increased after 48 hours and reached 21% at 96 hours (Figure 2. 2A), suggesting that there was a 48 hours lag before the onset of apoptosis among T lymphocytes responding to *A. actinomycetemcomitans*.

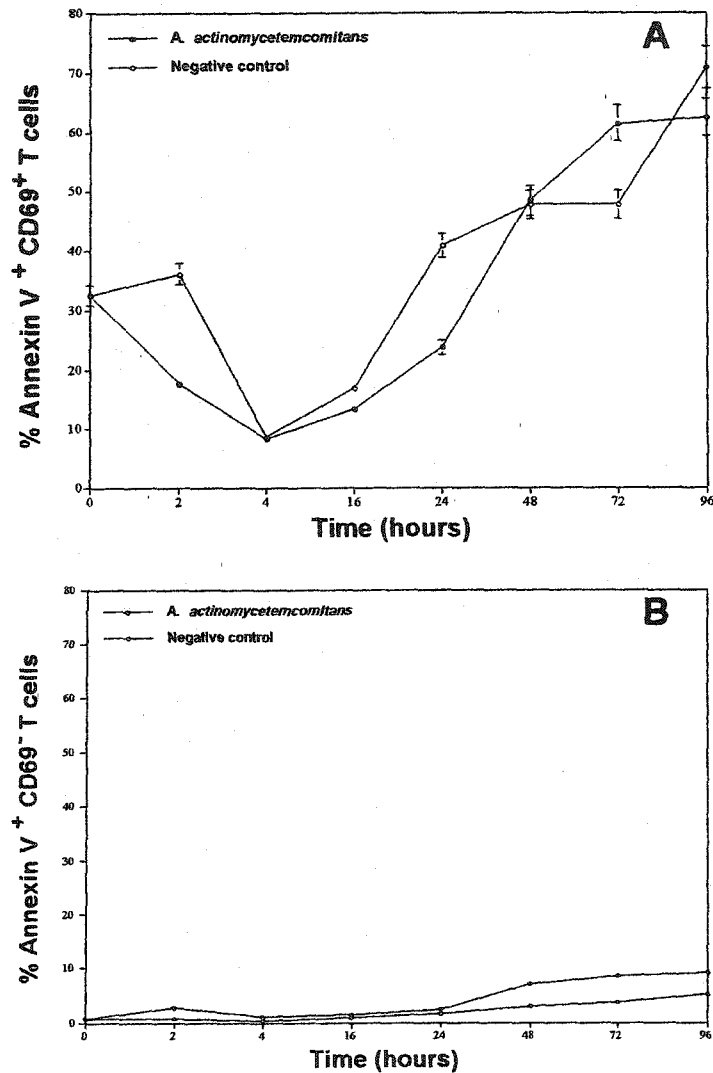


FIGURE 2.4. Proportion of activated (CD69⁺) and resting (CD69⁻) T cells that externalized phosphatidylserine (Annexin V⁺) in response to *A. actinomycetemcomitans*. PBMC were incubated in the presence or absence of *A. actinomycetemcomitans*. Cultured cells were labeled with anti-CD69 monoclonal antibody in concert with Annexin V and analyzed by flow cytometry. Mean and standard deviation of the proportion of Annexin V positive T cells among CD69⁺ (A) and CD69⁻ (B) T cells are shown. One of five representative experiments is shown, where PBMC from one donor were cultured and analyzed in triplicate. Among CD69⁺ T cells, *A. actinomycetemcomitans* -treated cells differed from negative controls at 2 and 24 hours ($P=0.0001$ and 0.0002 , respectively using repeated ANOVA). Among CD69⁻ T cells, *A. actinomycetemcomitans* -treated cells differed from negative controls at 2, 48, 72 and 96 hours ($P=0.0001$ using repeated ANOVA).

DISCUSSION

There is considerable evidence for killing of mammalian cells by *A. actinomycetemcomitans* (Taichman, 1980; Tsai, 1979; Mangan, 1991; Ohguchi, 1998; Korostoff, 1998; Kato, 1995; Muro, 1997). However, there is very little evidence for virulence factors of *A. actinomycetemcomitans* that are capable of killing T lymphocytes. Mangan, et al have reported killing of T cells by leukotoxin, which derived from cultures of *A. actinomycetemcomitans* JP2 (Mangan, Taichman et al. 1991). These investigators reported DNA fragmentation and morphological features consistent with apoptosis and necrosis. The present study investigated the potential of *A. actinomycetemcomitans* to induce apoptosis by studying plasma membrane changes in freshly isolated T cells that were cultured *in vitro* with *A. actinomycetemcomitans*.

Plasma membrane changes have been shown to occur very early in cells undergoing apoptosis (Andree et al. 1990; Creutz 1992; Fadok et al. 1993). These changes include cell surface exposure of phosphatidylserine that occurs prior to the loss of plasma membrane integrity. The plasma membrane exposure of phosphatidylserine was first described for erythrocytes (Connor et al. 1994) and platelets (Bervers et al. 1983; Zwaal et al. 1983; Zwaal et al. 1997). Later, these changes were also documented in nucleated cells undergoing apoptosis. Fadok and coworkers (Fadok et al. 1992; Fadok, Laszlo et al. 1993) have reported that human and murine leukocytes during apoptosis expose phosphatidylserine on the

outer leaflet of the plasma membrane. This cell surface-exposed phosphatidylserine apparently functions as a tag for specific recognition by phagocytes to clear dying cells. Verhoven, et al. have shown that apoptotic lymphocytes expose phosphatidylserine at their outer membrane early after onset of the execution phase of apoptosis (Verhoven et al. 1995). Phosphatidylserine exposure appears to last from the early execution phase of apoptosis until the final stage, at which time the cell is broken up into apoptotic bodies.

In the present study, utilizing Annexin V, it was shown that T cells cultured in the presence of plate wash of *A. actinomycetemcomitans* type strain exposed phosphatidylserine on the outer surface of their plasma membranes. Our data indicated that by 96 hours, approximately half of all T cells stimulated with *A. actinomycetemcomitans* had externalized their plasma membrane phosphatidylserine and thus underwent apoptosis. The proportion of apoptotic T cells appears to be even greater when the population of cells responding to *A. actinomycetemcomitans*, i.e. activated (CD69 positive) T cells was evaluated. These results showed that approximately two thirds of activated T cells bound Annexin V by 96 hours. It is interesting that the magnitude and kinetics of appearance of Annexin V positive T cells were similar in *A. actinomycetemcomitans*-stimulated and control cultures. However, it should be noted that the proportion of activated T cells in *A. actinomycetemcomitans*-stimulated cultures is roughly ten-fold greater, compared with control cultures

(Zadeh, Nalbant et al. 2001). Therefore, these results suggest that the majority of T cells activated *in vitro* are committed to apoptosis. This contention was further supported by the evidence that Annexin V binding among non-activated T cells (CD69 negative) remained low throughout the culture period, indicating that stimulation of T cells with *A. actinomycetemcomitans* leads to apoptosis primarily among activated T cells.

To provide further evidence that cells that have externalized their plasma membrane phosphatidylserine were committed to cell death, double labeling with Annexin V and propidium iodide was performed. These results showed that during the early time points, most of the Annexin V positive cells were propidium iodide negative. This was followed by the appearance of a population of cells that were Annexin V and propidium iodide positive. At later time points, most of the Annexin V positive cells incorporated propidium iodide. In fact, approximately 64% of Annexin V positive T cells became propidium iodide positive after 96 hours of culture. The results from labeling with propidium iodide and anti-CD3 revealed that propidium iodide positive T lymphocytes significantly increased by the 48 hour time-point and 30% of the T cells became propidium iodide positive at 96 hours. Another feature of the Annexin V positive, propidium iodide positive cells that appeared following stimulation with *A. actinomycetemcomitans* was their small size. Our data identified the phenotype of cells early (within 24 hours) after stimulation with *A. actinomycetemcomitans*, which was medium-sized

Annexin V⁺ PI⁻. After 48 hours, a population of small-sized Annexin V⁺ PI⁺ cells appeared. This phenotype is consistent with dead cells that have lost their membrane integrity and shrunk in size.

In general, stimulation of T cells through their antigen receptors can potentially lead to cellular activation, proliferation, differentiation or apoptosis. *In vitro* stimulation of human peripheral blood mononuclear cells with *A. actinomycetemcomitans* appears to induce large-scale expression of CD69, an early marker of activated T cells. Our previous data has shown that after *A. actinomycetemcomitans* stimulation for 24 hours, approximately half of all T lymphocytes become activated (Zadeh, Nalbant et al. 2001). The high level of T cell activation is clearly too large to be entirely accounted for by antigen-specific activation. It is possible that *A. actinomycetemcomitans* is capable of activating T cells by other mechanisms such as superantigenicity or polyclonal activation. It has been reported that superantigens can activate a large number of T cells that are later killed by apoptosis (McCormack et al. 1993; Wahl et al. 1993). Previous studies in our laboratory have provided evidence for superantigenic activation of T cells by *A. actinomycetemcomitans* (Zadeh et al. 1996). Therefore, more studies are required to determine the exact mode of activation of T cells in response to *A. actinomycetemcomitans*.

The fate of T cells responding to *A. actinomycetemcomitans* was monitored by following activated (CD69 positive) T cells. This analysis demonstrated that roughly two-thirds of activated T cells externalize their plasma membrane phosphatidylserine by 96 hours. Although this value was not significantly different in *A. actinomycetemcomitans*-stimulated or control cultures, there were about ten-fold as many activated T cells in *A. actinomycetemcomitans*-stimulated than control cultures. The rate of apoptosis, as determined by Annexin V binding in control cultures were almost the same as *A. actinomycetemcomitans* stimulated cultures, suggesting that T cells non-specifically activated in control cultures follow the same kinetics of cell death. On the other hand, the percentages of Annexin V binding among non-activated T cells (CD69 negative) did not change significantly at different time points, indicating that stimulation of T cells with *A. actinomycetemcomitans* leads to apoptosis primarily among activated T cells.

The data presented here raise a number of important questions to be investigated. Firstly, the specific bacterial molecules responsible for the apoptosis have not been identified. Secondly, the mechanism of interaction of *A. actinomycetemcomitans* molecules with the T cells has not been defined. In that respect, it is unclear whether *A. actinomycetemcomitans* components interact directly with T cells or act through intermediary cells such as antigen presenting cells or other T cell subsets that in turn induce apoptosis. Thirdly, the intracellular molecular pathways of apoptosis triggered in T cells has not been elucidated.

Fourthly, the consequences of apoptosis on the T cell repertoire and its potential implication on periodontitis has not been defined. Thus, the present study is preliminary in nature and lays the foundation for investigating a number of questions with potentially significant clinical implication.

Apoptosis of peripheral T cells usually requires the expression of functional Fas and Fas ligand by the T cell.¹⁰⁰ As qualitatively similar signals from the T cell antigen receptor (TCR) are responsible for both T cell expansion in inflammation and T cell elimination by apoptosis, regulating the balance between these opposing functions plays a crucial role in successful responses to pathogens (Russell 1995). The capacity of *A. actinomycetemcomitans* to initiate a process of apoptosis in lymphocytes implicates important molecular mechanisms by which *A. actinomycetemcomitans* could compromise the local cellular and humoral immune responses in periodontal lesions.

Another question is to identify the molecule(s) responsible for the cytolytic effects. Leukotoxin is the best characterized of the cytolytic factors produced by *A. actinomycetemcomitans*. Leukotoxin has been shown to kill a variety of mammalian cells, including: human polymorphonuclear leukocytes (PMNs), monocytes (Tsai et al. 1979; Taichman, Dean et al. 1980), T lymphocytes (Mangan, Taichman et al. 1991), B-cell hybridoma cell line HS-72 (Ohguchi et al. 1998), promyelocytic HL-60 cells (Korostoff, Wang et al. 1998), murine

macrophages cell line J774.1 (Kato et al. 1995; Muro et al. 1997) and mouse osteoblastic MC3T3-E1 cells (Morimoto et al. 1999). In view of the evidence for the induction of apoptosis by *A. actinomycetemcomitans* leukotoxin, the question arises about the involvement of leukotoxin in the apoptosis investigated in the present study. However, it is unlikely that the cytolytic effects observed here were due to leukotoxin, since the *A. actinomycetemcomitans* type strain used in this study is known to be a non-leukotoxin-producing or minimally leukotoxic strain (Spitznagel et al. 1995; Kolodrubetz, Spitznagel et al. 1996). Therefore, it is likely that a molecule other than leukotoxin is responsible for the apoptosis observed in this study.

Apoptosis has a physiological role in both lymphocyte development and function, serving to remove self-reactive T cells in the thymus, as well as activated peripheral T cells when they are no longer required in the immune response. On the other hand, disturbed levels of apoptosis have been demonstrated in certain disease conditions (Thompson 1995) such as Alzheimer's disease (Paradis et al. 1996; Vito et al. 1996; Wolozin et al. 1996), autoimmune diseases (Bellgrau et al. 1995; Benoist et al. 1997; Giordano et al. 1997), cancer (Fisher 1994; Hahne et al. 1996; Strand et al. 1996; Polyak et al. 1997; Rampino et al. 1997; Strasser et al. 1997) and AIDS (Groux et al. 1991; Groux et al. 1992; Gougeon et al. 1993; Karmochkine et al. 1998). The ability of *A. actinomycetemcomitans* to elicit apoptosis of host cells has also been shown. However, previous reports have not

firmly established the potential role of this property in the pathogenesis of periodontitis. Therefore, future studies need to evaluate the contribution of *A. actinomycetemcomitans* cytotoxic factors to the pathogenic potential of this microorganism in periodontitis. The demonstration of large-scale apoptosis of T cells in response to *A. actinomycetemcomitans* type strain merits further investigation to determine whether this represents a physiologic or pathologic phenomenon. It is believed that the pharmacological manipulation of apoptosis might offer new possibilities for prevention and treatment of some disease states.

The data presented here provide a potential mechanism for the immunomodulatory effect of *A. actinomycetemcomitans* on T cells. Understanding the role of apoptosis is likely to contribute to our understanding of the mechanisms involved in the pathogenesis of periodontal diseases.

CHAPTER III

Cellular and Molecular Mediators of *Actinobacillus actinomycetemcomitans*-Induced T cells Apoptosis

ABSTRACT

Actinobacillus actinomycetemcomitans expresses a number of toxins capable of inducing apoptotic cell death of T lymphocytes. However, the exact mechanism(s) has not been elucidated. The present study sought to investigate the involvement of the Fas (CD95)-mediated apoptotic pathway in *A. actinomycetemcomitans*-induced T cell apoptosis. To that end, peripheral blood mononuclear cells (PBMC) were cultured with or without *A. actinomycetemcomitans* cell free culture supernatant (CFCS) for 0 to 96 hours. The cells were then labeled with specific monoclonal antibodies followed by flow cytometry. Results demonstrated upregulation of Fas and activation of caspase-3 in T cells in response to *A. actinomycetemcomitans* CFCS. Monocytes were the only cells analyzed to constitutively express FasL, which was further upregulated in response to *A. actinomycetemcomitans* CFCS, while T cells expressed FasL only after this stimulation. Depletion of monocytes prior to stimulation with *A. actinomycetemcomitans* CFCS led to a marked decline in apoptosis. Blocking Fas-FasL interactions with anti-Fas monoclonal antibody or Fas:Fc fusion protein lead to a significant decline, but not abolition of T cell apoptosis. Nearly all T

cells expressed Bcl-2 at the outset of culture and Bcl-2 expression declined in T cells stimulated with *A. actinomycetemcomitans* CFCS. Collectively, these data provide evidence for the induction of T cell apoptosis by *A. actinomycetemcomitans* via Fas-mediated pathway, involving Caspase-3 and bcl-2. Moreover, this apoptotic response was dependent on the presence of monocytes.

INTRODUCTION

Microorganisms frequently use subversion of the host response as a mechanism of pathogenicity (Chen et al. 1994). The presence of a mechanism for the host to participate in its own destruction, i.e. apoptosis, has made an obvious target for the virulence factors of a number of microorganisms (Weinrauch et al. 1999). Pathogen-induced modulation of the host cell-death pathway may serve to eliminate key immune cells or evade host defenses that can act to limit the infection. *Actinobacillus actinomycetemcomitans* is an important human pathogen that has been shown to kill host cells by apoptosis (Mangan, Taichman et al. 1991; Kato, Muro et al. 1995; Korostoff, Wang et al. 1998; Ohguchi, Ishisaki et al. 1998; Morimoto, Morimoto et al. 1999; Weinrauch and Zychlinsky 1999; Yamamoto et al. 1999; Gadhavi et al. 2000; Kato et al. 2000; Korostoff et al. 2000; Nalbant et al. 2000; Shenker, Hoffmaster et al. 2001).

Recent studies in our laboratory, as well as by other investigator have reported that *A. actinomycetemcomitans* induces a potent apoptosis response among T cells (Mangan, Taichman et al. 1991; Nalbant and Zadeh 2000; Shenker, Hoffmaster et al. 2001). However, the cellular and molecular mechanisms of T cell apoptosis have not been defined.

Apoptosis may be induced through several pathways. One pathway initiates when Fas ligand (FasL) binds to Fas receptor (CD95) on target cells (Griffith et al. 1995). Another pathway involves binding of Tumor necrosis factor (TNF) to one of its cellular receptors (Screaton et al. 2000). Signals are transmitted intracellularly through the recruitment of signaling proteins, which initiate a cascade of events, involving activation of caspases. Caspases are a family of cysteine proteases with a critical role in apoptosis. The caspase cascade culminates in the activation of DNase, cleaving genomic DNA into 200 base pair fragments, a hallmark of apoptosis. A number of regulatory molecules are involved in the regulation of apoptosis. Members of the Bcl family of proteins are important regulators of apoptosis (Reed et al. 1991). Some of the members of this family, such as Bcl-2 and bcl-X_L inhibit apoptosis, while others such as Bax and Bad promote apoptosis. During apoptosis, mitochondria swell, releasing cytochrome c, which can contribute to the activation of caspases. Bcl-2 binds to mitochondrial surfaces, blocking its swelling and cytochrome c release, thereby inhibiting apoptosis (Yang et al. 1997). Normal homeostasis requires a delicate

balance between the induction of apoptosis and its inhibition, thereby maintaining a constant number of lymphocytes.

Understanding the cellular and molecular mediators utilized by *A. actinomycetemcomitans* in inducing T cell apoptosis will contribute to our understanding of the pathogenicity of this microorganism. To begin to elucidate the mechanism whereby *A. actinomycetemcomitans* induces apoptosis, the present study investigated the involvement of the Fas pathway. Results demonstrated that stimulation of T cells with *A. actinomycetemcomitans* leads to the up-regulation of Fas, Fas ligand and active caspase-3, accompanied by down regulation of Bcl-2, implicating the Fas pathway.

MATERIAL AND METHODS

Subjects

21 systemically and periodontally healthy subjects participated in this study. Subjects included 13 males and 8 females with mean age of 32 years. Subjects were requested to sign an informed consent that was previously approved by the Institutional Review Board at the University of Southern California. Cultural analysis was used to select subjects for this study that did not harbor *A. actinomycetemcomitans* in their dental plaque. This entailed sampling of the dental plaque from 3 periodontal sulcus sites for each patient using paper points. The plaque samples were then inoculated on TSBV selective media and cultivable

colonies were examined for the presence of *A. actinomycetemcomitans* (Slots 1982). The rationale for selecting *A. actinomycetemcomitans*-negative subjects was to exclude subjects, whose T cell repertoire had potentially been altered during the course of a chronic infection with *A. actinomycetemcomitans*.

Isolation of peripheral blood mononuclear cells

Venous blood was drawn from healthy volunteers. Peripheral blood mononuclear cells (PBMC) were isolated by Ficoll-Hypaque density gradient centrifugation (Böyum 1968).

Bacteria

A. actinomycetemcomitans type strain (ATCC 33384, NCTC 9710, serotype c) was purchased from ATCC (Rockville, MD). This strain is generally recognized to express very little or no leukotoxin (Spitznagel, Kraig et al. 1995; Kolodrubetz, Spitznagel et al. 1996). JP2 strain was obtained from Dr. Joseph Zambon (School of Dental Medicine, SUNY, Buffalo). *A. actinomycetemcomitans* was grown as previously described (Nalbant and Zadeh 2000). Bacteria and their extracellular products were harvested as described. Briefly, bacteria were grown on solid media plates containing Trypticase soy broth (Becton Dickinson and Co., Cockeysville, MD) supplemented with Trypticase soy agar (1.5% w/v; Becton Dickinson and Co., Cockeysville, MD), yeast extract (0.6% w/v; Difco Laboratories) and 10% horse serum. Bacteria were harvested by washing the

surfaces of plates with phosphate buffered Saline (PBS). The Cell-Free Culture Supernatant (CFCS) was separated from the cells by centrifugation (7000 rpm for 30 min). The material was dialyzed overnight against PBS (SnakeSkin™ Dialysis Tubing; 10kDa Molecular Weight Cutoff, Pierce Chemical Co., Rockford, IL). Protein concentration of the *A. actinomycetemcomitans* CFCS was measured by Bradford method (Bio-Rad Laboratories, Richmond, CA). *A. actinomycetemcomitans* CFCS was aliquoted and frozen at -70°C until use. As a control, the surface of un-inoculated media plates were washed in the same manner with PBS. This procedure yielded negligible proteins in the plate wash. This makes the contribution of the components of bacterial media improbable. Hence, the preparation of bacterial extracellular material in this manner minimized the presence of bacterial media in the preparation, which is a problem when bacteria are grown in liquid media.

Stimulants

The stimulatory agents included *A. actinomycetemcomitans* CFCS (25µg/ml, otherwise as indicated) or phorbol 12-myristate 13-acetate (PMA; Sigma-Aldrich, Milwaukee, WI) at 25 ng/ml, in conjunction with ionomycin (Sigma-Aldrich, Milwaukee, WI) at 1µg/ml, used as positive control.

Stimulation of mononuclear cells

PBMC were cultured in 96-well polypropylene microtubes (Corning Incorporated, Corning, NY), as described (Zadeh, Nalbant et al. 2001). Briefly, cells were incubated at a concentration of 2×10^6 cells/ml in the presence or absence of stimulants for various intervals between 0 to 96 hours in a humidified incubator at 37°C and 5% CO₂. The medium consisted of RPMI-1640 supplemented with 10% fetal[®] bovine serum (FBS), 100units/ml penicillin, 100 µg/ml streptomycin, and 25 mM HEPES buffer.

Monoclonal antibodies and flow cytometry reagents

All monoclonal antibodies except anti-FasL were directly conjugated with one of the following fluorochromes: Fluorescein isothiocyanate (FITC), Phycoerythrin (PE) or Peridinin Chlorophyll Protein (PerCP). The monoclonal antibodies with the following specificities were used in this study: CD3 (clone SK7; PerCP conjugated; Becton Dickinson, San Jose, CA), CD69 (clone L78; PE conjugated; Becton Dickinson, San Jose, CA), Fas (clone DX2; FITC conjugated; Pharmingen, San Diego, CA), CD14 (clone MφP9; FITC conjugated; Becton Dickinson, San Jose, CA), BrdU (clone ABFM18; FITC conjugated; Phoenix Flow Systems, San Diego, CA) and Bcl-2 (clone Bcl-2/100; FITC conjugated; Pharmingen, San Diego, CA). Anti-FasL (clone NOK-1; Pharmingen, San Diego, CA) was conjugated with Biotin and was developed with the secondary reagent, Streptavidin-PE (Becton Dickinson, San Jose, CA). Other flow cytometric

reagents included Annexin V-FITC (Pharmingen, San Diego, CA) and 7-amino-actinomycin D (7-AAD) (Pharmingen, San Diego, CA).

Annexin V labeling assay

The presence of cells with externalized phosphatidylserine (PS) was detected with Annexin V labeling by slight modification of previously published method (Nalbant and Zadeh 2000). The integrity of cellular plasma membrane was simultaneously evaluated by the ability of cells to exclude the viability probe, 7-AAD. Briefly, cultured cells were incubated with a cocktail of Annexin V-FITC, 7-AAD and anti-CD3-PE monoclonal antibody in binding buffer, containing 10 mM HEPES, 140 mM NaCl and 2.5 mM CaCl_2 , pH 7.4 for 20 minutes at 4°C. The cells were then washed twice with cold PBS and analyzed by flow cytometry within one hour. Annexin V-positive, 7-AAD-negative cells were defined as early apoptotic cells with an intact cell membrane that have externalized their membrane PS. In contrast, Annexin V-positive, 7-AAD-positive cells were defined as dead cells, by either apoptosis or necrosis.

TUNEL assay

DNA fragmentation was detected using transferase-mediated dUTP-biotin nick end labeling (TUNEL) assay (Phoenix Flow Systems, San Diego, CA) (Li et al. 1995). Briefly, cultured cells were harvested and washed with PBS containing 0.5% BSA and 0.1 % sodium azide. Cells were fixed with 70% ice-cold ethanol

for 30 minutes and frozen at -70°C for a minimum of 1 hour. Cells were then thawed at room temperature, washed and resuspended with DNA labeling solution, containing terminal deoxyribonucleotidyl transferase enzyme (TdT; 20 U/ μl), TdT reaction buffer containing (1M Sodium Cacodylate, 125 mM Tris, 1.25 $\mu\text{g}/\text{ml}$ BSA and 12.5 mM CaCl_2), bromodeoxyuridine triphosphate (BrdUTP; 54.7 $\mu\text{g}/\text{ml}$) and distilled water. Cells were incubated at 37°C for 60 minutes in a shaking water bath and then washed twice with rinse buffer, containing 10 mM Sodium phosphate monobasic, 150 mM NaCl, 0.1% TritonX-100, 0.05% sodium azide and 0.2% Gelatin. Immunofluorescent labeling of the cells was carried out with anti-BrdU monoclonal antibody and Propidium Iodide (PI) (5 $\mu\text{g}/\text{ml}$) in Rinse buffer containing RNase A (200 $\mu\text{g}/\text{ml}$) for 30 minutes. Cells were then analyzed by flow cytometry. Doublets and clumped cells were excluded by setting flow cytometric gates, using dual parameter display of DNA area versus DNA width. Apoptotic cells were identified as BrdU-positive gated cells.

Detection of Fas Ligand

For the detection of FasL on plasma membrane of various cell subsets, 10 μM of matrix metalloproteinase inhibitor (MMPI; KB8301 Pharmingen, San Diego, CA) was included in experimental or control cultures. KB8301 was added to increase the levels of membrane bound FasL by blocking FasL cleavage (Kayagaki et al.

1995; Oyaizu et al. 1997). Cultured cells were washed with PBS containing 0.1% sodium azide and 1.0% BSA and labeled with Biotin-conjugated anti-FasL monoclonal antibody for 20 minutes. These cells were washed and incubated with Streptavidin-PE in conjunction with fluorochrome-conjugated lineage-specific monoclonal antibodies, anti-CD3 and anti-CD14. Flow cytometric analysis of labeled cells was then performed.

Immunomagnetic isolation of monocytes

PBMC were labeled with anti-CD14 monoclonal antibody (Becton Dickinson, San Jose, CA). The cells were then washed and labeled with immunomagnetic beads conjugated with goat-anti-mouse IgG (Miltinyi Biotec, Auburn, CA). The immunomagnetically labeled cells were passed through a column filled with steel wires and held near a strong magnet (VarioMACS; Miltinyi Biotec, Auburn, CA). The non-adherent cells were washed off and the magnet was removed to elute the adherent cells. Immunofluorescent labeling was performed and demonstrated that the isolated adherent and non-adherent cells were >95% CD14⁺ and CD14⁻, receptively (data not shown).

Intracellular detection of Bcl-2 and active caspase-3

Intracellular expression of Bcl-2 and active caspase-3 were detected by immunofluorescent labeling and flow cytometry, as described (Boudet et al. 1996). PBMC were cultured with *A. actinomycetemcomitans* CFCS, positive

control stimuli or media alone as described above. Cultured cells were harvested, washed with cold PBS and fixed with Cytofix/Cytoperm solution (Pharmingen; San Diego, CA), which consists of PBS plus 4 % paraformaldehyde and 0.1% saponin for 30 minute on ice. Cells were then washed with wash buffer, containing PBS plus 0.1 % sodium azide and 1.0% FBS. Cells were re-suspended and permeabilized in permeabilization buffer containing PBS with 1% FBS, 0.1 % sodium azide and 0.1% saponin. Then, cells were stained for 20 minute at 4°C in the dark with monoclonal antibodies specific for Bcl-2, active caspase-3 and CD3. Following labeling, cells were analyzed by flow cytometry.

Caspase-3 inhibition assay

PBMC were pretreated with caspase-3 inhibitor, Ac-DEVD.FMK (Clontech Laboratories Inc., Palo Alto, Calif.) at 8 μ M or media alone for 2 hours prior to incubation with stimulants. Cultures were carried out for 48 hours. Annexin V labeling assay was then performed to determine the presence of apoptotic T cells in the presence and absence of caspase-3 inhibitor.

Blocking Fas-FasL interactions

Purified anti-human Fas monoclonal antibody and Fas:Fc chimeric fusion protein were purchased from Pharmingen (San Diego, CA). Fas:Fc is a chimeric protein that was expressed as a fusion of the extracellular domain of Fas with the Fc region of human Immunoglobulin G (Brunner et al. 1995). The Fas:Fc chimeric

protein functions as a potent antagonist of FasL biological activity (Rooney et al. 2000). PBMC were pretreated with anti-Fas monoclonal antibody (5 µg/ml), Fas:Fc chimeric fusion protein (10 µg/ml) or media alone for 30 minutes prior to incubation with stimulants. Cultures were carried out for 48 hours. Annexin V labeling and TUNEL assays were then performed to compare the induction of apoptotic T cells in the presence and absence of Fas:Fc chimeric fusion protein or anti-human Fas monoclonal antibody.

Flow Cytometry

Immunofluorescently-labeled cells were processed on a FACSCalibur (Becton-Dickinson Immunocytometry Systems, San Jose, CA) flow cytometer equipped with an argon ion laser (488nm) and the data were analyzed using CellQuest software (Becton Dickinson). The data acquired by flow cytometry were limited to the cell subsets of interest by selection of gates.

STATISTICAL ANALYSIS

Mean and standard error of the mean (S.E.M.) of data were calculated. To test the statistical significance of differences between experimental and control treatment of cell subsets, paired two-tailed Student's T-test were performed. *P* values of less than or equal to 0.05 were considered statistically significant.

RESULTS

Kinetics of Fas expression on T cells

To investigate the effects of *A. actinomycetemcomitans* on Fas expression, freshly isolated PBMC were cultured with *A. actinomycetemcomitans* CFCS for various intervals from 0 to 96 hours. Cultured cells were labeled with anti-Fas and anti-CD3 monoclonal antibodies, followed by flow cytometric analysis. Results demonstrated that approximately 40% of all freshly isolated T cells expressed Fas (Figure 3.1A, 3.1B). This level was maintained during the first 24 hours of culture with *A. actinomycetemcomitans* CFCS. Thereafter, Fas was rapidly upregulated in cell cultures containing *A. actinomycetemcomitans* CFCS, and during the 48 to 96 hours time points, approximately 80% of all T cells were Fas⁺. The response to the positive control stimulant, PMA and ionomycin, was comparable to *A. actinomycetemcomitans* CFCS. Fas expression in negative control cultures did not change significantly.

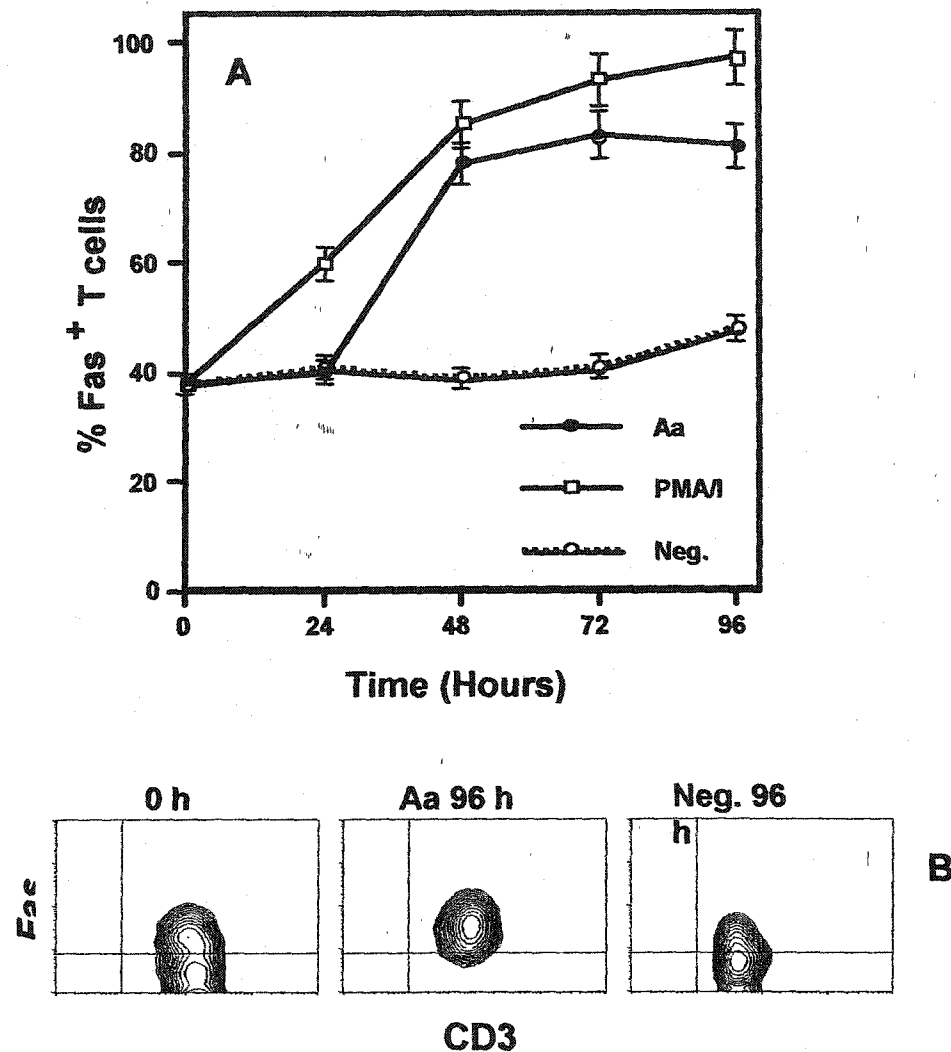


FIGURE 3.1. Expression of Fas on T cells in response to *A. actinomycetemcomitans*. PBMC were cultured for various time points in the presence of *A. actinomycetemcomitans* cell-free culture supernatant (Aa), PMA plus ionomycin (PMA/I) or media alone (Neg.). Immunofluorescent labeling of cells was carried out with anti-Fas and anti-CD3 monoclonal antibodies. Cells were then analyzed by Flow cytometry. Kinetics of expression of Fas on T cells in response to *A. actinomycetemcomitans* is shown. (1A) Mean and S.E.M. of the proportion of Fas⁺ T cells are shown. Representative flow cytometric profile of Fas expression on T cells is also shown (1B). Cells were gated for T lymphocytes, based on CD3 expression, in concert with forward and side scatter. The proportion of Fas⁺ T cells is indicated in the upper right quadrant of each panel. The data are representative of seven experiments.

Kinetics of FasL expression on T cells

The kinetics of FasL expression on T cells stimulated with *A. actinomycetemcomitans* CFCS was examined. Results revealed that very few freshly isolated T cells expressed FasL (Figure 3.2A). In cultures stimulated with *A. actinomycetemcomitans* CFCS, the proportion of FasL⁺ T cells increased slightly by 48 hours (12%). Thereafter, FasL expression increased more sharply, so that 22 to 30% of T cells were FasL⁺ between 72 to 96 hours. FasL expression in positive control cultures, containing PMA and ionomycin was somewhat higher, but followed similar kinetics. Very few T cells expressed FasL in negative control cultures.

Kinetics of FasL expression on monocytes

To determine whether monocytes contribute to T cell apoptosis, following stimulation with *A. actinomycetemcomitans* CFCS, FasL expression among monocytes was evaluated. Monocytes were the only cells examined that constitutively expressed significant levels of FasL, with approximately half being FasL⁺ at 0 hours (Figure 3. 2B). The expression of FasL progressively increased, so that by 96 hours, approximately 90% of monocytes were FasL⁺ in cultures stimulated with *A. actinomycetemcomitans* CFCS.

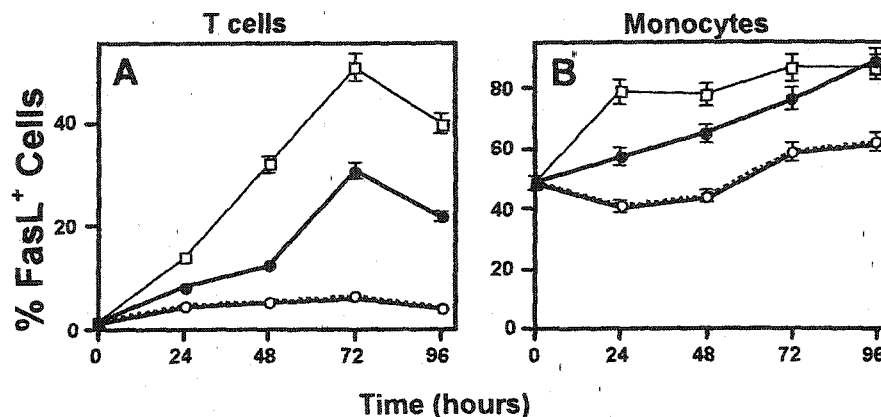


FIGURE 3.2. Kinetics of expression of FasL on T cells and monocytes in response to *A. actinomycetemcomitans*. Freshly isolated PBMC were incubated with *A. actinomycetemcomitans* cell-free culture supernatant (Aa), PMA plus ionomycin (PMA/I) or media alone (Neg.) in the presence of MMPI. FasL expression on T cells (A) and monocytes (B) were detected by immunofluorescent labeling and flow cytometry. The data represent mean and S.E.M. of 11 experiments.

Dependence of apoptosis on the presence of monocytes

To define the role of monocytes in *A. actinomycetemcomitans*-induced T cell apoptosis, CD14⁺ cells were depleted from PBMC. As a control for the influence of cell fractionation procedure, CD14⁺ cells were added back to CD14-depleted PBMC. Accordingly, unfractionated PBMC, CD14⁻ cells and CD14⁻ plus CD14⁺ cells were cultured with or without *A. actinomycetemcomitans* CFCS for 48 hours. T cell apoptosis was determined by Annexin V labeling assay. Results demonstrated a marked reduction of apoptosis among CD14-depleted-PBMC ($p < 0.001$). The addition of CD14⁺ cells back, appeared to restore the apoptosis response (Figure 3. 3).

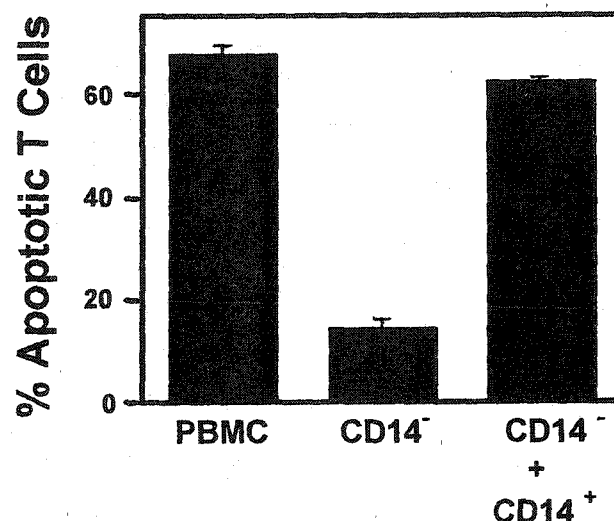


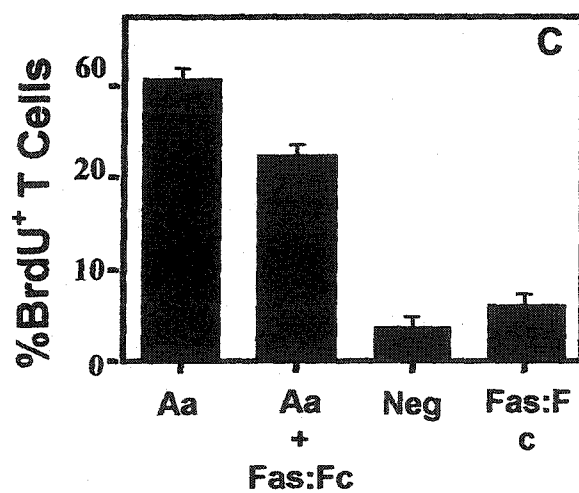
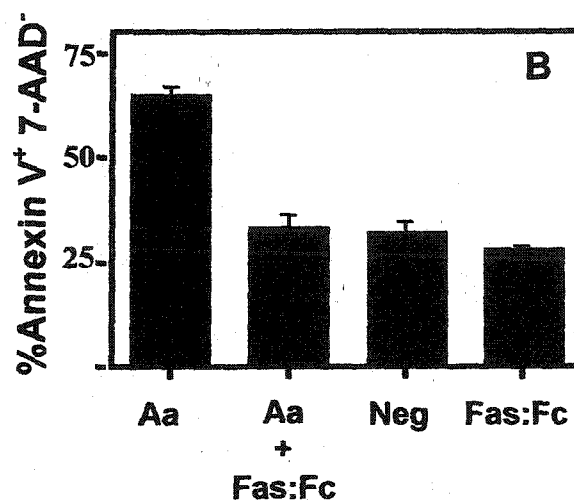
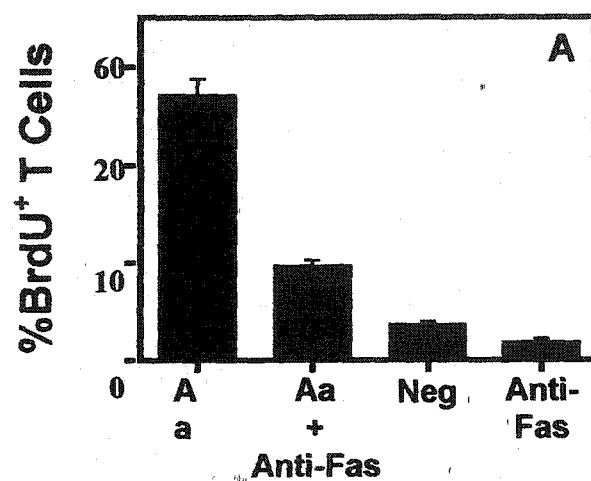
FIGURE 3.3. Dependence of the *A. actinomycetemcomitans*-induced T cell apoptosis on the presence of monocytes. Monocytes were isolated from PBMC by immunomagnetic sorting for CD14⁺ cells. Unfractionated PBMC, CD14-depleted PBMC or CD14-depleted PBMC plus CD14⁺ cells were incubated with *A. actinomycetemcomitans* cell-free culture supernatant for 48 hours. T cell apoptosis was detected by Annexin V labeling assays. Mean $\pm$ S.E.M. of triplicate samples are shown. The data are representative of three experiments.

The role of Fas and FasL in T cell apoptosis

To delineate the role of Fas-FasL interactions in *A. actinomycetemcomitans*-induced T cell apoptosis, anti-Fas monoclonal antibody was included in cell cultures. Results in Figure 3. 4A showed that blocking Fas-FasL interactions with anti-Fas monoclonal antibody lead to significantly reduced T cell apoptosis ($p < 0.05$). However this response was not completely abolished with anti-Fas monoclonal antibody, since residual apoptosis activity that was higher than background remained. To further investigate the role of Fas-FasL interactions, Fas:Fc chimeric protein was used as a competitive inhibitor of FasL. Results demonstrated that when PBMC were cultured with *A. actinomycetemcomitans*

CFCS in the presence of Fas:Fc, there was a significant reduction in the proportion of apoptotic T cells, as measured by Annexin V labeling (Figure 3. 4B; $p<0.05$) and TUNEL assay (Figure 3. 4C; $p<0.05$). However, Fas:Fc protein was also not able to completely inhibit *A. actinomycetemcomitans*-associated T cell apoptosis, since a higher number of cells with DNA fragmentation were observed in the presence of *A. actinomycetemcomitans* along with Fas:Fc than with Fas:Fc alone ($p<0.0001$).

FIGURE 3.4. The role of Fas-FasL interactions in T cell apoptosis mediated by *A.actinomycescomitans*. PBMC were pretreated for 30 minutes with Anti-Fas monoclonal antibody (A) at 5 µg/ml or Fas:Fc chimeric fusion protein (B, C) at 10 µg/ml or media alone. Cells were then incubated in the presence or absence of *A. actinomycescomitans* cell-free culture supernatant (Aa) for 48 hours. TUNEL (A, C) or Annexin V labeling assays (B) were performed to detect apoptotic T cells in the presence and absence of anti-Fas monoclonal antibody or Fas:Fc chimeric fusion protein. Mean and S.E.M. of data are shown. Selected comparisons of experimental and control treatments, using paired two-tailed Student's T-test, are shown. The data are representative of four experiments.



Expression of active caspase-3

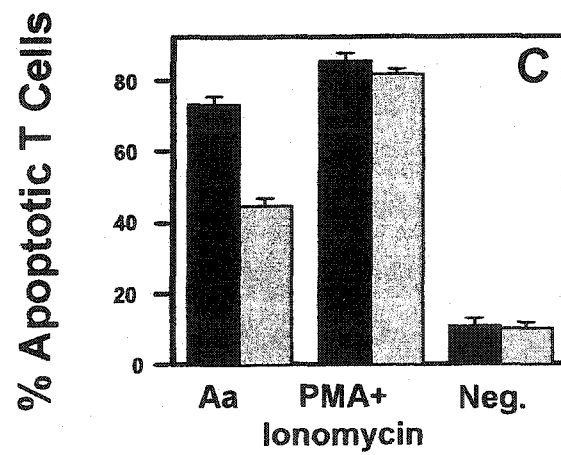
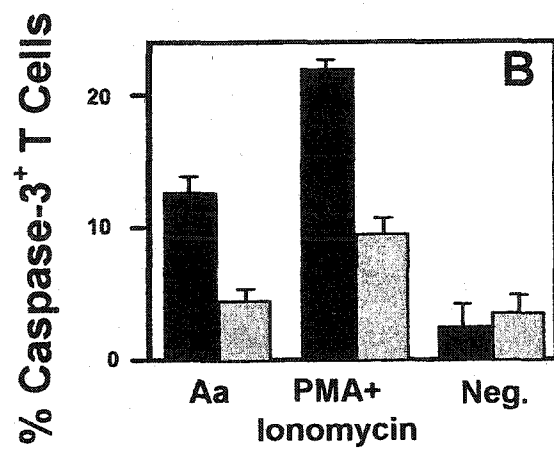
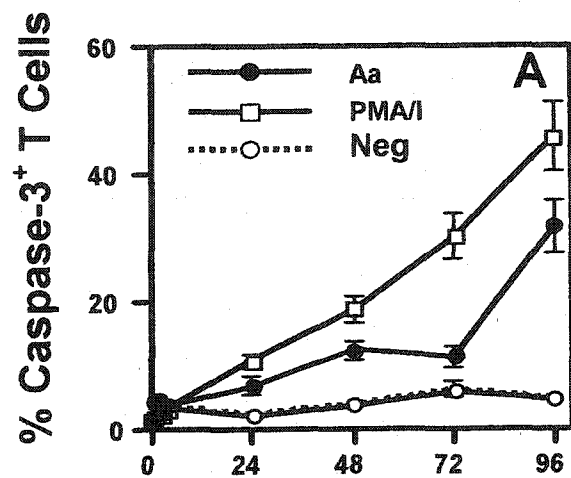
The involvement of caspases in the T cell apoptotic response to *A. actinomycetemcomitans* was studied by determination of active caspase-3 expression. Flow cytometric detection of active caspase-3 in PBMC cultured with *A. actinomycetemcomitans* CFCS demonstrated a time-dependent increase in the appearance of active caspase-3-positive T cells (Figure 3. 5A). The proportion of T cells expressing active caspase-3 increased from 1.8% at 0 hour (range 0.7% to 3.9%) to 31.4% at 96 hours (range 12.5% to 64.7%). In positive control cultures stimulated with PMA and Ionomycin approximately 46% of T cells expressed active caspase-3 at 96 hour. The level of active caspase-3 expression did not change in negative control cultures.

Effects of caspase-3 inhibitor on T cell apoptosis

To further investigate the role of caspase-3 in *A. actinomycetemcomitans*-induced T cells apoptosis, PBMC were pre-treated with the caspase-3 inhibitor, Ac-DEVD.FMK, for 2 hours prior to incubation with *A. actinomycetemcomitans* CFCS (Tsai et al. 1999). Results showed that pre-treatment of PBMC with Ac-DEVD.FMK indeed inhibited approximately 70% of caspase-3 activation in response to *A. actinomycetemcomitans* CFCS ($P=0.006$) (Figure 3. 5B). Pre-treatment with caspase-3 inhibitor was also associated with a modest, yet significant reduction of apoptosis by 17% ($P=0.05$), among T cells cultured with *A. actinomycetemcomitans* CFCS (Figure 3. 5C). In negative control cultures, the

proportion of apoptotic T cells did not change significantly when Ac-DEVD.FMK was included (Figure 5C).

FIGURE 3.5. Involvement of caspase-3 in *A. actinomycetemcomitans*-mediated T cells apoptosis. Kinetics of active caspase-3 expression in T cells stimulated with *A. actinomycetemcomitans* cell-free culture supernatant (Panel A). Active caspase-3 expression was detected by flow cytometry after intracellular labeling of cultured PBMC with monoclonal antibodies specific for CD3 and active caspase-3. The data represent mean and SEM of 9 experiments. The inhibitory effects of the caspase-3 inhibitor, Ac-DEVD.FMK, on active caspase-3 expression (Panel B), as well as apoptosis (Panel C) are shown. PBMC were pretreated with Ac-DEVD.FMK (8 μ M) for 2 hours prior to a 48-hour culture with stimulants. Flow cytometric expression of active caspase-3 is represented. T cell apoptosis was determined by Annexin V labeling assay. The data for caspase-3 inhibition assay are representative of three experiments. Mean and S.E.M. of data are shown.



Bcl-2 expression among T cells

To investigate possible regulatory mediators of T cell apoptosis, flow cytometric expression of Bcl-2 among T cells stimulated with *A. actinomycetemcomitans* CFCS was assessed. Results in Figure 6 showed that nearly all T cells expressed Bcl-2 at 0 hour. Bcl-2 expression declined in T cells stimulated with *A. actinomycetemcomitans*. Approximately 49% and 76% of T cells were Bcl-2⁺ at 24 and 48 hours, respectively. Thereafter, Bcl-2 levels gradually rose back, so that approximately 84% and 90% of T cells were Bcl-2⁺ at 72 and 96 hours, respectively.

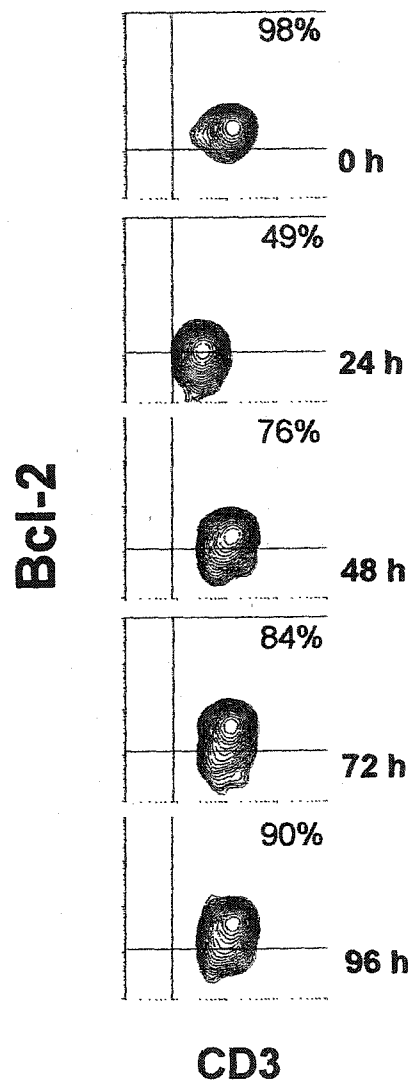


FIGURE 3.6. Representative flow cytometric profile of Bcl-2 expression of T cells. PBMC were incubated in the presence of *A. actinomycetemcomitans* cell-free culture supernatant for 0 to 96 hours. Intracellular expression of Bcl-2 was detected by flow cytometric analyses of immunofluorescently labeled cells. Cells were gated for T lymphocytes, based on CD3 expression, in concert with forward and side scatter. The proportion of Bcl-2⁺ T cells is indicated in the upper right quadrant of each panel. The data presented are representative of five experiments.

DISCUSSION

The present study investigated the involvement of the Fas pathway in the molecular and cellular mechanisms of *A. actinomycetemcomitans*-induced T cell apoptosis. The data regarding the molecular mechanism of apoptosis demonstrated that T cells exposed to a crude preparation of *A. actinomycetemcomitans* exhibit upregulation of Fas and FasL, down-regulation of Bcl-2, as well as activation of caspase-3. This is the first report of the involvement of the Fas/FasL pathway in *A. actinomycetemcomitans*-induced apoptosis of T lymphocytes. Based on these data, investigation of other apoptosis pathways is also merited, since blockage of Fas-FasL interactions lead to partial inhibition of apoptosis. Activation of the caspase cascade, which is an important intracellular event during apoptosis, has previously reported in response to *A. actinomycetemcomitans* CDT (Shenker, Hoffmaster et al. 2001) as well as Leukotoxin (Korostoff, Yamaguchi et al. 2000). The down-regulation of bcl-2 following incubation of T cells with *A. actinomycetemcomitans* preparation suggests a regulatory role for bcl-2 in *A. actinomycetemcomitans*-induced apoptosis. This contention is also supported by Shenker, et al, who found that overexpression of the bcl-2 gene in a B lymphoblastoid cell line, reduces *A. actinomycetemcomitans* CDT-induced apoptosis (Shenker, Hoffmaster et al. 2001).

The kinetics of apoptosis observed for T cells is significantly slower than that of other cell types killed by *A. actinomycetemcomitans* (Mangan, Taichman et al. 1991; Nalbant and Zadeh 2000; Shenker, Hoffmaster et al. 2001). *A. actinomycetemcomitans* rapidly kills neutrophils (Tsai, McArthur et al. 1979) and monocytes (Taichman, Dean et al. 1980) within minutes to a few hours. On the other hand, there is a 48-hour lag in the appearance of apoptotic T cells following stimulation with *A. actinomycetemcomitans* (Nalbant and Zadeh 2000). A potential reason for differences in the apoptosis rate between T cells and other cell types may be due to different apoptotic mechanisms involved. We have hypothesized that *A. actinomycetemcomitans* indirectly induces apoptosis of T cells, by first activating other effector cell types that in turn kill target T cells. This hypothesis is supported by the data in the present study showing FasL expression by monocytes, as well as T cells. During the first 48 hours of culture with *A. actinomycetemcomitans* CFCS, monocytes were the only cells examined that constitutively expressed FasL. During the second half of culture, T cells also exhibited elevated FasL expression. In addition, we have shown that B cells are induced to upregulate FasL in response to *A. actinomycetemcomitans* (data not shown). We have also observed that depletion monocytes from PBMC, significantly reduced apoptosis in response to *A. actinomycetemcomitans*. Hence, it is possible that the *A. actinomycetemcomitans*-induced T cell apoptosis is mediated by monocytes, B cells and T cells, each contributing at different time points. The relative contribution of various cell subsets as mediators of *A.*

actinomycetemcomitans-induced T cell apoptosis is an area that is being experimentally tested in our laboratory. Further studies are required to delineate the role of intermediary cells, such as monocytes, B cells and killer T cells in mediating the apoptosis response initiated by *A. actinomycetemcomitans*. It is possible that these intermediary cells are responsible for delivering the apoptosis-inducing signal. Alternatively monocytes may be involved in activation of T cells, making them susceptible to apoptosis-inducing signals.

In order for a cell to undergo death by apoptosis, extracellular signals have to be delivered to a susceptible cell. In the Fas-mediated apoptosis pathway, upregulation of Fas in conjunction with down-regulation of Bcl-2 makes a cell susceptible to apoptosis (Yoshino et al. 1994; Boudet, Lecoecur et al. 1996; Mitsiades et al. 1998; Spanaus et al. 1998; Takahashi et al. 1999). In the present study, there is evidence for a progressive upregulation of Fas, as well as down-regulation of Bcl-2 in response to *A. actinomycetemcomitans*. Moreover, the presence of FasL may function as the extracellular signal to initiate the apoptosis cascade. Our data also demonstrated upregulation of FasL by monocytes, B cells and T cells. Depletion of monocytes, as well as intervention with Fas-FasL interactions by Fas:Fc chimeric protein or anti-Fas monoclonal antibody also lead to reduced apoptosis. Collectively, these data suggest the involvement of the Fas-mediated apoptosis pathway in T cells stimulated with *A. actinomycetemcomitans*. In view of the data that Fas:Fc chimeric protein or anti-Fas monoclonal antibody

did not completely inhibit *A. actinomycetemcomitans*-induced apoptosis, investigation of the involvement of other apoptotic pathways, such as the TNF pathway is merited. The elucidation of other potential pathways of apoptosis will be important in order to obtain a more comprehensive picture of the mechanism of pathogenicity of *A. actinomycetemcomitans*.

Activation or prevention of cell death could be a critical factor in the outcome of an infection. Apoptosis has been observed as a response to infection by a wide range of animal and plant pathogens and is mediated by an array of pathogen-encoded virulence determinants (Weinrauch and Zychlinsky 1999). However, the clinical significance of apoptosis, as a virulence factor of *A. actinomycetemcomitans*, is not known. In view of the data that approximately 70% of all T cells exhibited apoptotic cell death, this response represents a major outcome of T cell activation response to this microorganism (Nalbant and Zadeh 2000). Therefore, it is likely that response of this magnitude would have significant effects on the immune response. We have hypothesized that during chronic infections with *A. actinomycetemcomitans*, which in many cases lasts for decades, there are repeated cycles of T cell activation and apoptosis. This may potentially lead to a dramatic skewing of the T cell repertoire. In this regard, we and others have reported that the T cell receptor repertoire in periodontitis lesion sites is markedly skewed (Yamazaki et al. 1996; Karimzadeh et al. 1999).

Whether the shift in T cell repertoire has any functional implications on the ability of the host to deal with microbial challenges is yet to be determined.

The exact bacterial molecules responsible for the Fas-mediated apoptosis have also not been defined. The bacterial strain used in this study was a low leukotoxin producer. However, we have tried other strains, including the JP2 strain, which is a high leukotoxin producer and obtained similar results with regard to Fas and FasL expression (data not shown). Identification of the molecules involved in T cell apoptosis and defining the cellular and molecular pathways of their actions will promote our understanding of the pathogenesis of *A. actinomycetemcomitans* infections and aid the selection of specific molecular targets for therapeutic intervention.

CHAPTER IV

Evidence for *Actinobacillus actinomycetemcomitans*

Induced T Cell Apoptosis Independent of Cytolethal Distending Toxin and Leukotoxin

ABSTRACT

The pathogenic bacterium *Actinobacillus actinomycetemcomitans* is known to express a leukotoxin (ltx) and cytolethal distending toxin (CDT) that can induce apoptosis. CDT also induces a G₂ cell cycle block. The extent of contribution of each toxin, as well as other potential toxin(s) to the cytolytic and cytostatic activities of this microorganism have not been defined and were the aims of this study. To that end, a competent *A. actinomycetemcomitans* clinical isolate (D7S-smooth) was used to construct a series of isogenic deletion mutants ($\Delta cdtA$, $\Delta cdtB$, $\Delta cdtC$, $\Delta cdtABC$, Δltx , $\Delta cdtABC/ltx$). Human peripheral blood mononuclear cells (PBMC) were incubated with cell-free and cell-associated bacterial preparations. The ability of wild-type parent and isogenic mutant strains to induce T cell apoptosis and cell cycle arrest was compared. The expression of *ltx* and each of the *cdt* gene loci partially contributed to the cytotoxicity of *A. actinomycetemcomitans*, since each of the isogenic mutants exhibited reduced

apoptosis for T cells. Double mutants with deletion of *ltx*, as well as all 3 *cdt* gene loci retained significant ability to induce T cell apoptosis. Neutralization of CDT proteins with specific monoclonal antibodies in a minimally leukotoxic *A. actinomycetemcomitans* strain showed significant T cell apoptosis. On the other hand, the ability to induce a cell cycle block was abolished in each of the *cdt* isogenic mutants. Collectively, these data provide evidence for the expression of other cytolytic molecule(s) of *A. actinomycetemcomitans* distinct from CDT and leukotoxin, while suggesting that the cytostatic function is dependent on the expression of all 3 *cdt* gene loci.

INTRODUCTION

Actinobacillus actinomycetemcomitans is an important pathogenic bacterium, implicated in a number of diseases, including periodontitis (Fives_Taylor et al. 1999), as well as in a number of non-oral infections including cardiovascular, intracranial, thoracic and skin infections (van Winkelhoff et al. 1999). There is evidence that the immune response to *A. actinomycetemcomitans* is inadequate to deal with the virulence challenges of this pathogen (Shenker et al. 1982; Shenker, Tsai et al. 1982; Shenker et al. 1997). Therefore, this bacterium likely uses some mechanisms to subvert the host response.

A. actinomycetemcomitans has been shown to kill a number of freshly isolated cells, as well as cell lines, including T cells (Mangan, Taichman et al. 1991; Nalbant and Zadeh 2000; Shenker, Hoffmaster et al. 2001), monocytes (Mangan et al. 1993), epithelial cells (Kato, Nakashima et al. 2000), B cell hybridoma cell line HS-72 (Ohguchi, Ishisaki et al. 1998), murine macrophage cell line J774.1 (Kato, Muro et al. 1995), promyelocytic HL-60 cells (Korostoff, Wang et al. 1998), MG63 human osteosarcoma-derived cells (Gadhavi, Wilson et al. 2000), mouse osteoblastic MC3T3-E1 cells (Yamamoto, Mogi et al. 1999) and human osteoblastic MG63 cells (Morimoto, Morimoto et al. 1999). Apoptosis has been proposed as a mechanism of cell death with respect to the effect on some of these cells (Mangan, Mergenhagen et al. 1993; Kato, Muro et al. 1995; Korostoff, Wang et al. 1998, Gadhavi, 2000 #1136; Ohguchi, Ishisaki et al. 1998; Morimoto, Morimoto et al. 1999; Yamamoto, Mogi et al. 1999; Kato, Nakashima et al. 2000; Nalbant and Zadeh 2000; Shenker, Hoffmaster et al. 2001).

A. actinomycetemcomitans expresses two known toxins with cytolytic properties against human cells, namely leukotoxin (ltx) and cytolethal distending toxin (CDT). Leukotoxin is a 116-kDa member of the repeats-in toxins (RTX) family of pore-forming proteins, whose members include hemolysins and leukotoxins (Lally,

Golub et al. 1989; Lally, Kieba et al. 1989). Leukotoxin is variably expressed by different strains of *A. actinomycetemcomitans* and enables them to kill both lymphoid and myeloid leukocytes (Taichman, Dean et al. 1980; Tsai, Shenker et al. 1984; Lally, Golub et al. 1989; Lally, Kieba et al. 1989; Mangan, Taichman et al. 1991; Ohta et al. 1993; Lally et al. 1994; Kolodrubetz, Spitznagel et al. 1996). Leukotoxin has been shown to kill human T cells (Mangan, Taichman et al. 1991), promyelocytic HL-60 cells (Korostoff, Wang et al. 1998) and B cells (Korostoff, Yamaguchi et al. 2000) by apoptosis.

CDT is a soluble protein encoded by a cluster of three genes designated *cdtABC* (Pickett et al. 1999). The molecular mass of polypeptide products of these three genes is approximately 25-35 kDa. CDT is expressed by a number of gram-negative organisms such as *A. actinomycetemcomitans* (Sugai et al. 1998; Shenker, McKay et al. 1999; Shenker, Hoffmaster et al. 2000), *Haemophilus ducreyi* (Cope et al. 1997), *Campylobacter jejuni* (Pickett et al. 1996; Whitehouse et al. 1998), *Escherichia coli* (Pickett et al. 1994; Scott et al. 1994; Peres et al. 1997), *Shigella* (Okuda et al. 1995), and *Helicobacter spp.* CDT causes irreversible G2 arrest and subsequent cell death (Shenker, McKay et al. 1999; Shenker, Hoffmaster et al. 2000; Sato et al. 2002). *A. actinomycetemcomitans*, as well as, *H. ducreyi* CDT have been shown to induce apoptosis of T cells

(Gelfanova et al. 1999; Shenker, Hoffmaster et al. 2001). However, CDT can only induce the G2 arrest and apoptosis among T cells that had been previously activated. In the *in vitro* model T cells were first activated with phytohemagglutinin (PHA) or other polyclonal T cell activators prior to exposure to CDT (Shenker, Hoffmaster et al. 2001). Inasmuch as this does not represent the physiologic conditions under which CDT acts, it was sought to determine the role of CDT in intact *A. actinomycetemcomitans* cells. Moreover, the relative contribution of CDT and leukotoxin, as well as other potential toxin(s) to the cytolytic and cytostatic properties of *A. actinomycetemcomitans* have not been elucidated. To that end, single and double isogenic *A. actinomycetemcomitans* mutants with deletion of *cdt* and/or *ltx* gene loci were constructed. Results demonstrated that *cdt* and *ltx* genes and their associated proteins, partially contributed to the cytotoxicity of *A. actinomycetemcomitans*, but could not account for all of its activity. Our data provide evidence for the expression of other molecule(s) with cytolytic function, distinct from CDT and leukotoxin.

MATERIAL AND METHODS

Subjects

25 systemically and periodontally healthy subjects participated in this study. Subjects included 15 males and 10 females with mean age of 35 years. Subjects

were requested to sign an informed consent that was previously approved by the Institutional Review Board at the University of Southern California. Cultural analysis was used to select subjects for this study that did not harbor *A. actinomycetemcomitans* in their dental plaque. This entailed sampling of the dental plaque from 3 periodontal sulcus sites for each patient using paper points. The plaque samples were then inoculated on TSBV selective media and cultivable colonies were examined for the presence of *A. actinomycetemcomitans* (Slots 1982). The rationale for selecting *A. actinomycetemcomitans* culture-negative subjects was to exclude subjects, whose T cell repertoire had potentially been altered during the course of a chronic infection with *A. actinomycetemcomitans*.

Isolation of peripheral blood mononuclear cells

Venous blood was drawn from healthy volunteers. Peripheral blood mononuclear cells (PBMC) were isolated by Ficoll-Hypaque density gradient centrifugation (Böyum 1968).

Bacteria

A spontaneously occurring non-fimbriated mutant, designated as strain D7S-smooth, which was derived from a clinical *A. actinomycetemcomitans* isolate (strain D7S) was used in this study. Strain D7S was originally recovered from a patient with aggressive periodontitis. *A. actinomycetemcomitans* was grown as

previously described (Nalbant and Zadeh 2000). Briefly, *A. actinomycetemcomitans* was inoculated on solid media plates containing Trypticase soy broth (Becton Dickinson and Co., Cockeysville, MD) supplemented with Trypticase soy agar (1.5% w/v; Becton Dickinson and Co., Cockeysville, MD), yeast extract (0.6% w/v; Difco Laboratories) and 10% horse serum for 48 hours at 37° C in atmosphere supplemented with 5% CO₂.

Bacteria and their extracellular products were harvested as previously described (Nalbant and Zadeh 2000). *A. actinomycetemcomitans* cells were cultured as described above. Cultured cells and their extracellular products were removed from media plates by washing the surfaces of plates with 3 ml of phosphate buffered Saline (PBS), followed by centrifugation (7000g for 30 min). The supernatant, which contains the extracellular products of bacteria was used for the preparation of cell free culture supernatant (CFCS). To prepare bacterial cellular extract (CE), cells were washed and frozen at -70°C and thawed. Bacterial cells were then resuspended in a nonionic detergent (CellLytic B II; Sigma, St Louis, MO) along with deoxyribonuclease (DNase) I (5µg/ml; Sigma, St Louis, MO) and incubated for 15 minutes with shaking at room temperature. Cellular debris and unlysed bacteria were removed by centrifugation (10,000g for 30 min.). The CFCS and CE preparations were dialyzed overnight against PBS (SnakeSkin™ Dialysis Tubing; 10kDa Molecular Weight cut-off, Pierce Chemical Co., Rockford, IL).

The resultant fractions were sterilized by filtration through a 0.2 μm filter (Nalgene, Rochester, NY). The protein concentration of bacterial preparations was measured by Bradford method (Bio-Rad Laboratories, Richmond, CA) and samples were divided into aliquots and frozen at -70°C until use.

As a control for the CFCS preparation, the surface of un-inoculated agar plates was washed in the same manner with PBS. This demonstrated negligible presence of proteins. Hence, the preparation of bacterial extracellular material in this manner minimized the presence of bacterial media in the preparation, which is a problem when bacteria are grown in liquid media.

Stimulants

The stimulatory agents included *A. actinomycetemcomitans* CFCS (0.1 to 100 $\mu\text{g/ml}$), *A. actinomycetemcomitans* CE (0.1 to 100 $\mu\text{g/ml}$) or phorbol 12-myristate 13-acetate (PMA, 25 ng/ml) in conjunction with ionomycin (1 $\mu\text{g/ml}$). Purified leukotoxin was a kind gift of Dr. Edward Lally at the School of Dental Medicine, University of Pennsylvania (Lally, Golub et al. 1989; Lally, Kieba et al. 1989).

Construction and characterization of specific gene deletion mutants of *A. actinomycetemcomitans*

A wild type clinical isolate of *A. actinomycetemcomitans*, designated D7S-smooth strain, was used for the construction of specific single- and double-knockout mutants. A novel gene replacement strategy was employed. The details of the gene replacement strategy is described elsewhere (Y. Wang and C. Chen, submitted for publication). Briefly, two DNA fragments flanking the target gene locus were generated by PCR. The amplicons were specifically ligated to an antibiotic-resistance cassette. The ligation mixture was directly used to transform *A. actinomycetemcomitans* strain D7S-smooth and selected for the marker. The *cdtABC* gene cluster was replaced by the Spe cassette (LeBlanc et al. 1991). *A. actinomycetemcomitans* leukotoxin (*ltxA*) deletion makes this bacterium incapable of synthesizing leukotoxin. (Lally, Golub et al. 1994; Tatum et al. 1998). The *ltxA* gene was replaced by the *tet(O)* gene (Wang et al. 1991). The $\Delta cdtABC/ltxA$ double-knockout mutant was generated by transforming the D7S-smooth ($\Delta cdtABC$) with the DNA from the $\Delta ltxA::tet(O)$ mutant.

The sequences of the primers were designed based on the information of *A. actinomycetemcomitans* strain HK1651 at the University of Oklahoma (www.uoklahoma.edu). The primers for amplification of the upstream region of the *cdt* gene cluster were *cdtA-E* (5'-GTGGATATCGAGATTTCTGA) and *cdtA-*

S1 (5' TGAGTCGACGAACAAGCCACTAAACC). Primers for the downstream region of the *cdt* gene cluster were *cdtC*-S (5'-GAGTGGTCGACCCTTTTCG), *cdtC*-P (5'-CTCACTGCAGCAAAAGACAG). Primers for the amplification of upstream region of *ltxA* were LAp1 (5'-GGTATGTGGCTTGGTTATGG 3') and LAp2 (5'-GAGGATCCAAACTGTGCAGCTTGCTGT 3'). Primers for the amplification of downstream region of *ltxA* were LApst4 (5'-CGGGATCCGCTTCGCCATTGCAGA 3') and LApst4 (5'-CGGGAGGAATAAACCAGGTA 3').

All mutants were confirmed by PCR to verify (1) the deletion of the target gene using primers within the target gene, (2) the insertion of the antibiotic cassette using primers flanking the target site, and (3) the direction of the inserted antibiotic cassette using a primer upstream of the target site and a forward or reverse primer within the cassette. Mutants with the antibiotic cassette inserted in the same direction as the target gene were used for the following experiments to reduce or eliminate the potential polar effect from the cassette insertion.

Stimulation of mononuclear cells

PBMC were cultured in 96-well polypropylene microtubes (Corning Incorporated, Corning, NY) at a concentration of 2×10^6 cells/ml. Cells were incubated in the presence or absence of stimulants for various intervals in a humidified incubator at

37°C and 5% CO₂. The medium consisted of RPMI-1640 supplemented with 10% fetal bovine serum (FBS), 100 units/ml penicillin, 100 µg/ml streptomycin, and 25 mM HEPES buffer.

Annexin V labeling

The presence of cells with externalized phosphatidylserine (PS) was detected with Annexin V labeling by slight modification of previously published methods (Nalbant and Zadeh 2000). The integrity of cellular plasma membrane was simultaneously evaluated by the ability of cells to exclude the viability probe, 7-amino-actinomycin D (7-AAD). Briefly, cultured cells were incubated with a cocktail of Annexin V-FITC, 7-AAD and anti-CD3 monoclonal antibody in binding buffer, containing 10 mM HEPES, pH 7.4, 140 mM NaCl and 2.5 mM CaCl₂ for 20 minutes at 4°C. The cells were then washed twice with cold PBS and analyzed by flow cytometry within one hour. Annexin V-positive 7-AAD-negative cells were defined as early apoptotic cells with an intact cell membrane, that have externalized their membrane PS. In contrast, Annexin V-positive, 7-AAD-positive cells were defined as dead cells, by either apoptosis or necrosis.

TUNEL assay

DNA fragmentation was detected using transferase-mediated dUTP-biotin nick end labeling (TUNEL) assay (Phoenix Flow Systems, San Diego, CA) (Darzynkiewicz et al. 1992; Li and Darzynkiewicz 1995). Briefly, cultured cells were harvested and washed with PBS containing 0.5% bovine serum albumin (BSA) and 0.1 % sodium azide and fixed with 70% ice-cold ethanol for 30 minutes. Fixed cells were frozen at -70°C for a minimum of 1 hour. Cells were then thawed at room temperature, washed and resuspended with DNA labeling solution, containing terminal deoxynucleotidyl transferase enzyme (TdT; 20 U/ μl), TdT reaction buffer containing (1M Sodium Cacodylate, 125 mM Tris, 1.25 $\mu\text{g}/\text{ml}$ BSA and 12.5 mM CaCl_2), bromodeoxyuridine triphosphate (BrdUTP; 54.7 $\mu\text{g}/\text{ml}$) and distilled water. Cells were incubated at 37°C for 60 minutes in a shaking water bath and then washed twice with rinse buffer, containing (10 mM Sodium phosphate monobasic, 150 mM NaCl, 0.1% TritonX-100, 0.05% sodium azide and 0.2% Gelatin). Immunofluorescent labeling of the cells was carried out with anti-BrdU monoclonal antibody and Propidium Iodide (PI) (5 $\mu\text{g}/\text{ml}$) in Rinse buffer containing RNase A (200 $\mu\text{g}/\text{ml}$) for 30 minutes. Cells were then analyzed by flow cytometry. Doublets and clumped cells were excluded by setting flow cytometric gates, using dual parameter display of DNA area versus DNA width. Apoptotic cells were identified as BrdU-positive gated cells.

Cell cycle analysis

The presence of cells in various phases of cell cycle was demonstrated by analysis of DNA ploidy (Li and Darzynkiewicz 1995). Cultured cells were harvested and washed with PBS containing 0.5% bovine serum albumin (BSA) and 0.1 % sodium azide and fixed with 70% ice-cold ethanol for 30 minutes. Fixed cells were frozen at -70°C for a minimum of 1 hour. Cells were then thawed at room temperature, washed and permeablized with a rinse buffer, containing 10 mM Sodium phosphate monobasic, 150 mM NaCl, 0.1% TritonX-100, 0.05% sodium azide and 0.2% Gelatin (Phoenix Flow Systems, San Diego, CA). Immunofluorescent labeling of the cells was carried out with Propidium Iodide (PI) ($5\mu\text{g/ml}$) in Rinse buffer containing RNase A ($200\mu\text{g/ml}$; Phoenix Flow Systems, San Diego, CA) for 30 minutes at room temperature in the dark. Cells were then analyzed by flow cytometry. Doublets and clumped cells were excluded by setting flow cytometric gates, using dual parameter display of DNA area versus DNA width. The degree of PI incorporation into PBMC following various cultures was used to measure DNA ploidy. Diploid (G_0/G_1), quadraploid (G_2/M) and cells with intermediate ploidy (S) were identified based on the intensity of PI fluorescence using DNA area flow cytometric display.

Neutralization of CDT activity with specific monoclonal antibodies

Anti-CDT monoclonal antibodies were a kind gift of Dr. Eric Hansen of the University of Texas Southern Medical Center, Texas. The source of the monoclonal antibodies was hybridoma culture supernatant fluids from murine hybridoma cell lines secreting monoclonal antibody clone 1G8 (directed against *H. ducreyi* CdtA), clone 20B2 (directed against *H. ducreyi* CdtB) and clone 8C9 (directed against *H. ducreyi* CdtC), as previously described (Stevens et al. 1999). These monoclonal antibodies have been demonstrated to cross-react with *A. actinomycetemcomitans* CDT's.

Stimulation of mononuclear cells was carried out as described above, except for pre-incubation of stimulants with neutralizing monoclonal antibodies. CFCS of *A. actinomycetemcomitans* D7S-smooth (25µg/ml) were incubated with 20µl of hybridoma culture fluid supernatants containing monoclonal antibodies specific for *H. ducreyi* CdtA, CdtB, and CdtC for 60 minutes at room temperature. PBMC were added to this mixture and the remainder of the culture period was carried out as described above.

Flow Cytometry

Immunofluorescently-labeled cells were processed on a FACSCalibur (Becton-Dickinson Immunocytometry Systems, San Jose, CA) flow cytometer equipped

with an argon ion laser (488 nm), and the data were analyzed using CellQuest software (Becton Dickinson). The data acquired by flow cytometry were limited to the cell subsets of interest by selection of electronic gates. Lymphocyte gates were selected on the basis of their forward and side scatter profiles. T cells were identified with anti-CD3 monoclonal antibody.

Statistical analysis

Mean and standard error of the mean (S.E.M.) of data were calculated. To test the statistical significance of differences between experimental and control treatment of cell subsets, two-tailed Student's t-test was used.

RESULTS

Role of *cdt* gene loci in contributing to T cell apoptosis

To assess the relative contribution of *A. actinomycetemcomitans* CDT to T cell apoptosis, a specific knockout mutant of *cdt* was generated, where all 3 *cdt* gene loci were deleted ($\Delta cdtABC$). The cytotoxicity of CFCS preparation of wild type *A. actinomycetemcomitans* D7S-smooth and isogenic $\Delta cdtABC$ strains were determined and compared. Results in Figure 1 revealed dose-dependent T cell

apoptosis with maximal cytotoxic effects in the presence of 1 to 10 µg/ml of wild type or isogenic mutant preparations. The isogenic $\Delta cdtABC$ strain exhibited markedly reduced cytotoxicity ($p < 0.0001$), as demonstrated by a 30% reduction in the proportion of apoptotic T cells in the presence of $\Delta cdtABC$ CFCS, compared with the wild type parent strain. Nevertheless, the $\Delta cdtABC$ retained significant cytotoxicity, compared with negative control ($p < 0.01$).

To map the *cdt* loci responsible for T cell apoptosis response to *A. actinomycetemcomitans* CDT, individual *cdt* mutants with deletion in each of the 3 *cdt* loci were generated. The cytotoxicity of CFCS preparations made from wild type parent and isogenic mutant strains for T cells was determined and compared. Results in Figure 2 revealed that a large number of T cells (nearly 80%) exhibited apoptosis after 72 hours of culture with CFCS of wild type strain.

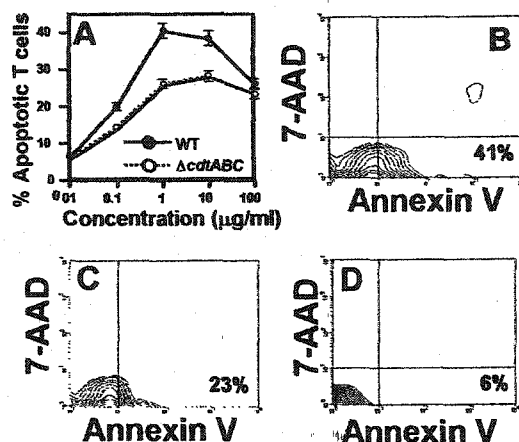
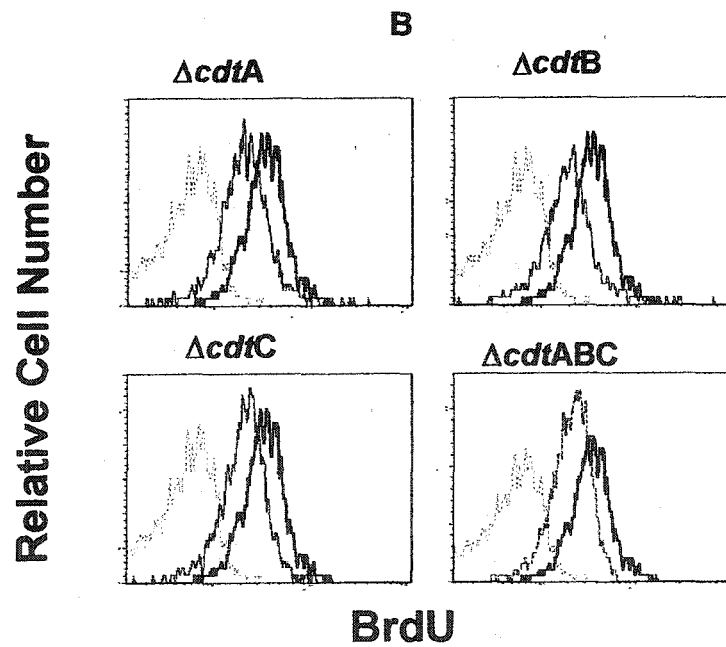
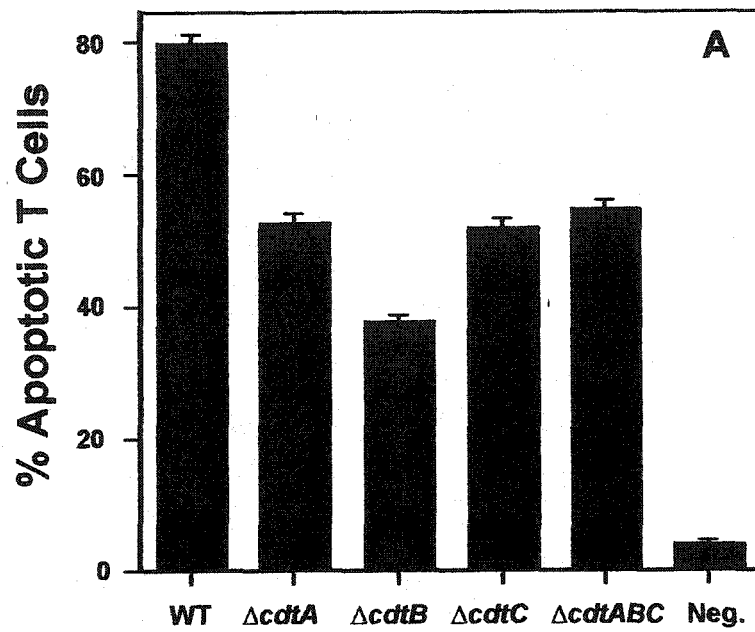


FIGURE. 4.1. Apoptosis in T cells exposed to varying concentrations of CFCS of wild type (WT) and specific *cdt* knockout mutant of *A. actinomycetemcomitans* ($\Delta cdtABC$). Freshly isolated PBMC were incubated for 48 hours with *A. actinomycetemcomitans* wild type or $\Delta cdtABC$. Cultured cells were labeled with Annexin V, anti-CD3 monoclonal antibody and 7-AAD, followed by flow cytometric analysis. The data is representative of five independent experiments, where the PBMC from a donor were cultured and analyzed in triplicate. Mean and S.E.M. of the proportion of Annexin V-positive 7-AAD-negative T cells are shown. Representative flow cytometric profile of apoptosis in T cells exposed to CFCS (10µg/ml) of wild type (B), specific *cdt* knockout mutant (C) and media alone (D).

Deletion of any of the 3 *cdt* gene loci was associated with significantly reduced cytotoxicity for T cells, compared with wild type parent; $\Delta cdtA$ ($p < 0.0005$), $\Delta cdtB$ ($p < 0.0001$), $\Delta cdtC$ ($p < 0.0005$) and $\Delta cdtABC$ ($p < 0.0005$). The *cdtB* isogenic mutant strain exhibited the most significant loss of cytotoxicity. Nevertheless, CFCS of *cdt* mutants possessed significant cytotoxicity, compared to negative control cultures $\Delta cdtA$ ($p < 0.01$), $\Delta cdtB$ ($p < 0.0005$), $\Delta cdtC$ ($p < 0.0005$) and $\Delta cdtABC$ ($p < 0.01$). In view of the fact that CDT proteins are secreted, CFCS preparations were used here as the source of stimulants. However, similar results were obtained with CE preparations (data not shown).

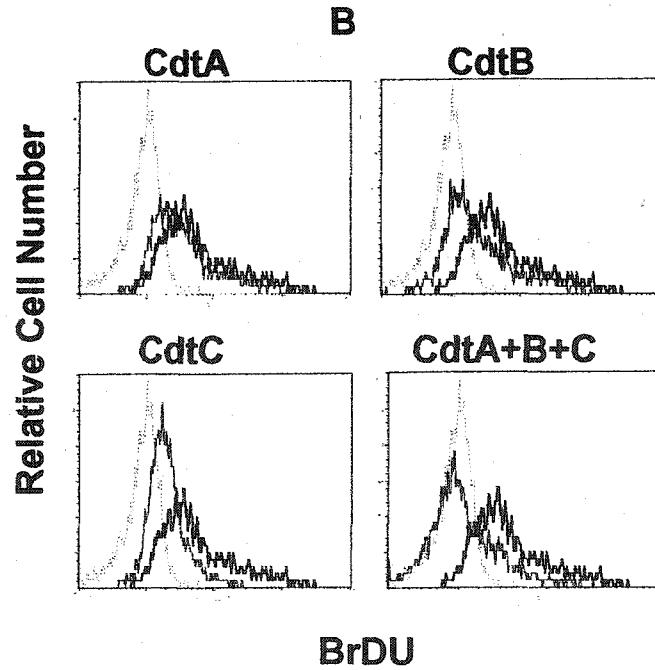
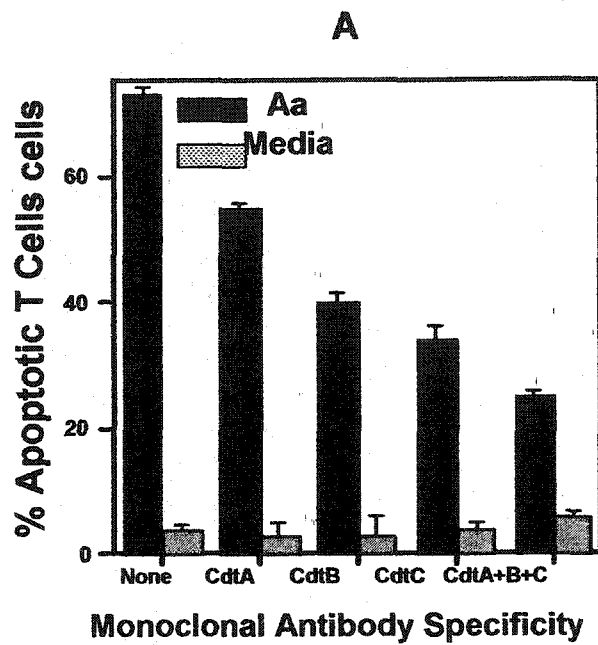
FIGURE 4. 2. Apoptosis of T cells stimulated with CFCS of *A. actinomycetemcomitans* wild type (WT), as well as specific knockout mutants of *cdt*. Freshly isolated PBMC were incubated for 72 hours with CFCS (10⁶g/ml) of *A. actinomycetemcomitans* wild type parent or isogenic mutants with deletion in each of the *cdt* gene loci, *ΔcdtA*, *ΔcdtB*, *ΔcdtC* and *ΔcdtABC*. Cultured cells were fixed, permeabilized and labeled with BrdU and PI, followed by flow cytometric analysis. (A) A representative experiment that was repeated five times is presented, where the PBMC from a donor were cultured and analyzed in triplicate. Mean and S.E.M. of the proportion of cells with fragmented DNA are shown. (B) Representative flow cytometric histograms of apoptosis in T cells cultured in the presence of CFCS of wild type (bold lines), individual *cdt* knockout mutants (solid lines) or media alone (dotted lines) are shown.



Role of CDT proteins in contributing to T cell apoptosis

To further investigate the contribution of CDT proteins to *A. actinomycetemcomitans*-mediated cytotoxicity, CDT-specific monoclonal antibodies were included in cell cultures stimulated with *A. actinomycetemcomitans* CFCS. Results in Figure 3 revealed significantly reduced T cell apoptosis in the presence of anti-CdtA ($p<0.0001$), anti-CdtB ($p<0.0005$), anti-CdtC ($p<0.001$) or a mixture of all 3 monoclonal antibodies ($p<0.0005$). However, the anti-CDT monoclonal antibodies did not abolish the apoptosis response. Residual cytotoxicity that was significantly higher than background levels of negative control cultures was retained in the presence of anti-CdtA ($p<0.0005$), anti- CdtB ($p<0.005$), anti- CdtC ($p<0.05$) or a mixture of all 3 monoclonal antibodies ($p<0.01$).

FIGURE 4. 3. Apoptosis in T cells stimulated with *A. actinomycetemcomitans* in the presence and absence of anti-CDT monoclonal antibodies. PBMC were incubated with or without *A. actinomycetemcomitans* CFCS. Cell cultures were carried out for 72 hours in the presence or absence of a mixture of monoclonal antibodies specific for cdtA, cdtB and cdtC. Apoptotic cells were detected by TUNEL assay. In panel A, mean and S.E.M. of the proportion of BrdU positive T cells are shown. The data presented is representative of 5 experiments. In panel B, representative single parameter histograms show apoptosis in T cells exposed to *A. actinomycetemcomitans* CFCS alone (dark solid lines), *A. actinomycetemcomitans* CFCS and a mixture of monoclonal antibodies specific for cdtA or cdtB or cdtC or cdtA+B+C (thin solid lines) or the CDT-specific monoclonal antibodies alone (gray dotted lines).



Role of *ltx* gene locus in contributing to T cell apoptosis

To determine the relative contribution of *A. actinomycetemcomitans* leukotoxin to T cell apoptosis, a specific knockout mutant of leukotoxin (Δltx) was generated. The T cell apoptosis response to CFCS of wild type and Δltx strains were measured and compared. Results in Fig. 4 showed dose-dependent T cell apoptosis in both cultures. The CFCS of Δltx strain exhibited significantly reduced ability to induce apoptosis ($p < 0.0005$), as demonstrated by a 15 to 23% reduction cytotoxicity in the presence of Δltx CFCS (10 to 100 $\mu\text{g/ml}$), compared with the wild type. The Δltx CFCS possessed significant cytotoxicity for T cell, compared with negative control ($p < 0.00005$).

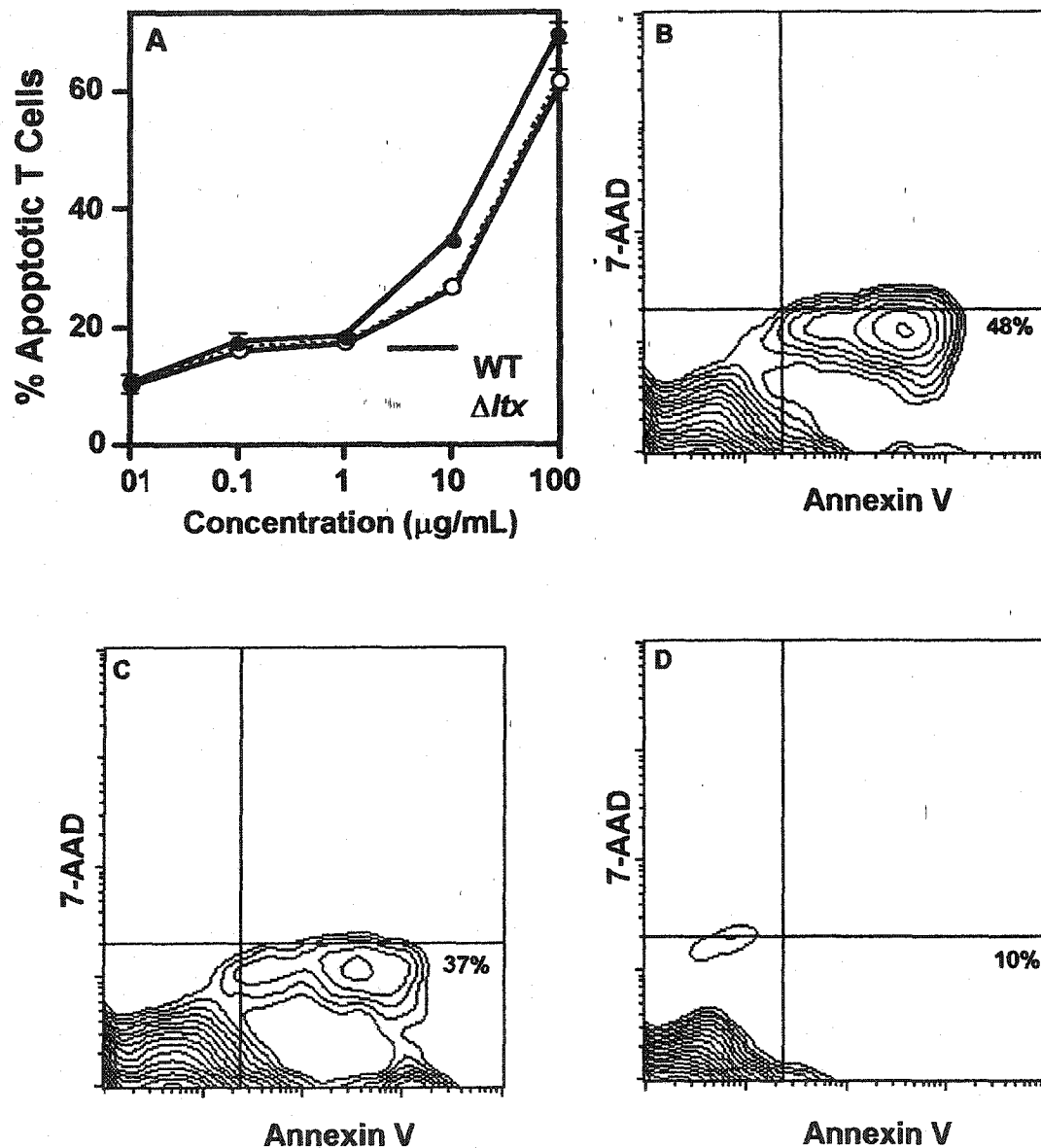


FIGURE 4. 4. Apoptosis in T cells exposed to varying concentrations of CFCS of wild type (WT) and specific *ltxA* knockout mutant of *A. actinomycetemcomitans* (A). Freshly isolated PBMC were incubated for 72 hours with *A. actinomycetemcomitans* wild type or Δ *ltxA*. Cultured cells were labeled with Annexin V, anti-CD3 monoclonal antibody and 7-AAD, followed by flow cytometric analysis. The data is representative of five independent experiments, where the PBMC from a donor were cultured and analyzed in triplicate. Mean and S.E.M. of the proportion of Annexin V-positive 7-AAD-negative T cells are shown. Representative flow cytometric profile of apoptosis in T cells exposed to wild type (B), specific *ltxA* knockout mutant (C) of *A. actinomycetemcomitans* and media alone (D) are shown.

Role of leukotoxin protein in contributing to T cell apoptosis

To further investigate the magnitude of T cell apoptosis response to *A. actinomycetemcomitans* leukotoxin, PBMC were cultured in the presence of varying concentrations of purified recombinant leukotoxin (Fig. 5). Results demonstrated dose-dependent increase in T cell apoptosis in the presence of leukotoxin. Maximal level of apoptosis was observed in the presence of 10 ng/ml of leukotoxin, where 27% of the T cells underwent apoptosis.

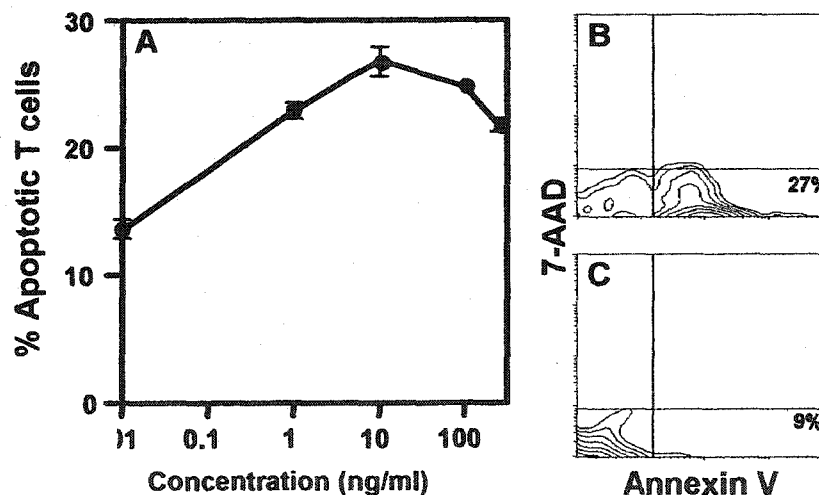


FIGURE 4. 5. Concentration-dependence of apoptosis in T cells exposed to *A. actinomycetemcomitans* pure-leukotoxin (A). Freshly isolated PBMC were incubated with the indicated concentrations of *A. actinomycetemcomitans* leukotoxin for 48 hours. Cultured cells were labeled with Annexin V, anti-CD3 monoclonal antibody and 7-AAD, followed by flow cytometric analysis. The data presented is representative of 3 experiments. Mean and S.E.M. of the proportion of Annexin V-positive 7-AAD-negative T cells are shown. Representative flow cytometric profile of apoptosis in T cells exposed to *A. actinomycetemcomitans* leukotoxin (B) or media alone (C) are shown.

T cell apoptosis in leukotoxin and CDT double mutants

To investigate whether leukotoxin and CDT are the only cytotoxins expressed by *A. actinomycetemcomitans*, a double knockout mutant of leukotoxin and CDT ($\Delta ltx/cdtABC$) was constructed. The cytotoxicity of CE and CFCS preparations made from wild type and $\Delta ltx-cdtABC$ strains were measured and compared. Results showed significant level of T cell apoptosis in cultures stimulated with CE of wild type, as well as double-mutant, $\Delta ltx/cdtABC$ strain (Fig. 6).

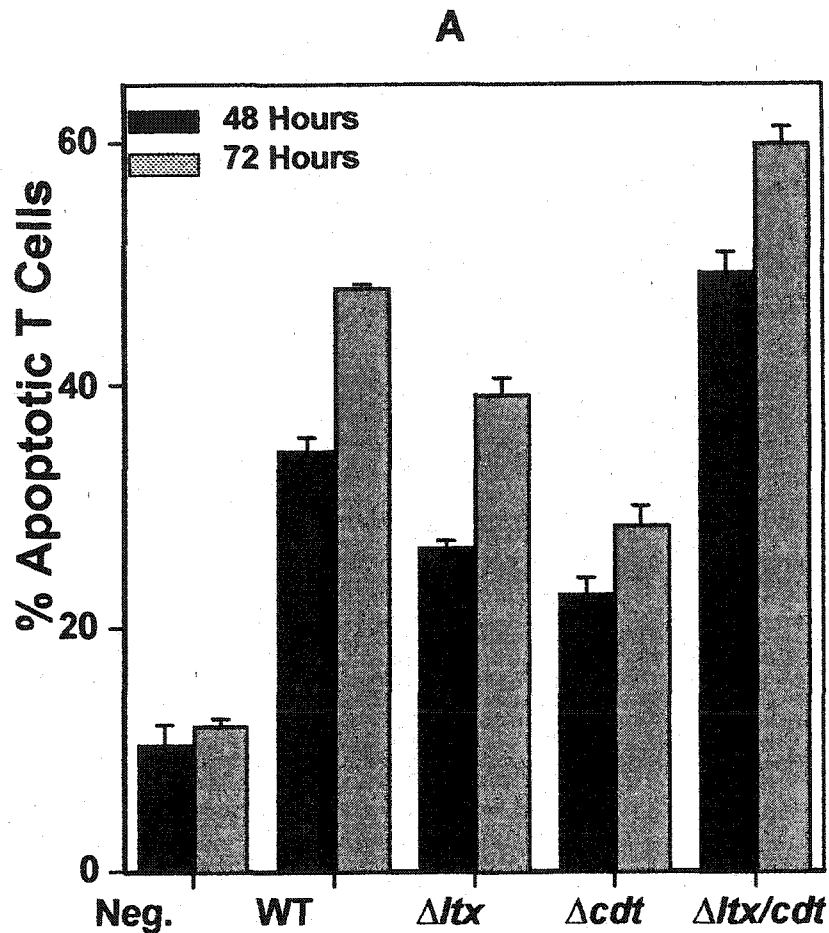


FIGURE 4. 6A. Cell-associated cytotoxicity of *A. actinomycetemcomitans* wild type (WT) and CDT/leukotoxin mutant strains. T cell apoptosis response to CE of wild type (WT) parent, as well as isogenic knockout mutants of *cdt*, *ltx* or *cdt/ltx* double mutants. Freshly isolated PBMC were incubated for 48 or 72 hours with CE of *A. actinomycetemcomitans* wild type parent, or isogenic mutants with deletion in *cdt*, *ltx* or both *cdt* and *ltx* loci, Δcdt , Δltx or $\Delta cdt/ltx$. Cultured cells were labeled with Annexin V, anti-CD3 monoclonal antibody and 7-AAD, followed by flow cytometric analysis. A representative experiment that was repeated three times is presented, where the PBMC from a donor were cultured and analyzed in triplicate. Mean and S.E.M. of the proportion of Annexin V-positive 7-AAD-negative T cells are shown (panel A).

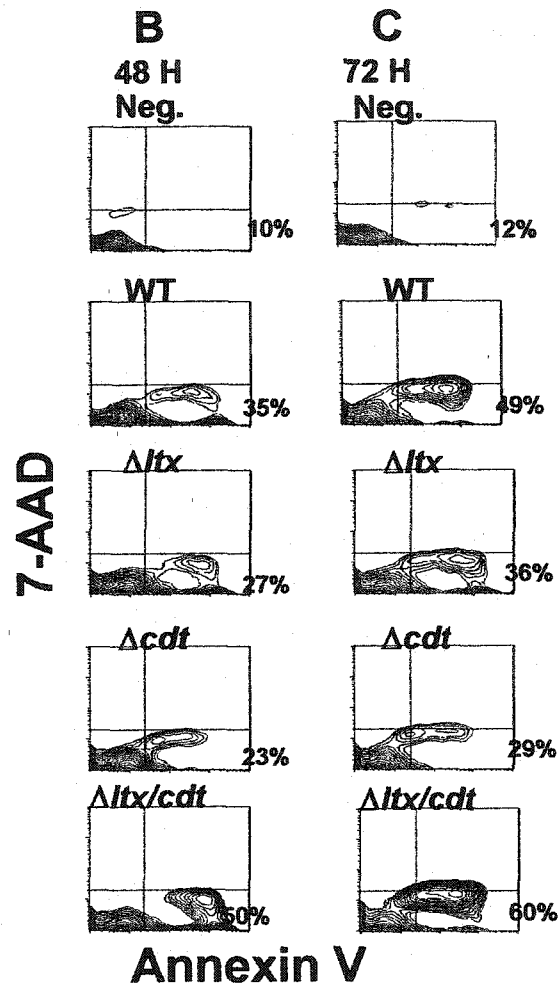


FIGURE 4. 6B and C. Cell-associated cytotoxicity of *A. actinomycetemcomitans* wild type (WT) and CDT/leukotoxin mutant strains. T cell apoptosis response to CE of wild type (WT) parent, as well as isogenic knockout mutants of *cdt*, *ltx* or *cdt/ltx* double mutants. Freshly isolated PBMC were incubated for 48 or 72 hours with CE of *A. actinomycetemcomitans* wild type parent, or isogenic mutants with deletion in *cdt*, *ltx* or both *cdt* and *ltx* loci, Δcdt , Δltx or $\Delta cdt/ltx$. Cultured cells were labeled with Annexin V, anti-CD3 monoclonal antibody and 7-AAD, followed by flow cytometric analysis. A representative experiment that was repeated three times is presented, where the PBMC from a donor were cultured and analyzed in triplicate. Representative flow cytometric profile of apoptosis in T cells exposed to media, wild type, specific *ltxA* knockout mutant, specific *cdt* knockout mutant, specific *ltxA/cdtABC* knockout mutant of *A. actinomycetemcomitans* at 48 hours (panel B) versus at 72 hours (panel C) are shown.

Surprisingly, the CE of the double-mutant, $\Delta ltx/cdtABC$ strain, exhibited significantly higher cytotoxicity than the Wild Type ($p < 0.0005$). This was demonstrated by a 43% increase in the proportion of apoptotic T cells in the presence of CE made from $\Delta ltx-cdtABC$, compared with the wild type strain. In contrast CFCS preparation made from double-mutant $\Delta ltx/cdtABC$ strain was apparently devoid of cytotoxicity (Fig. 7).

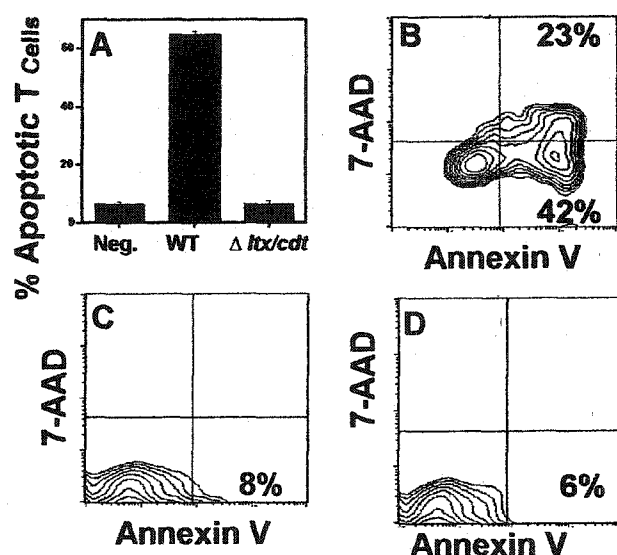


FIG. 7. Cytotoxicity of extracellular products of *A. actinomycetemcomitans* wild type (WT) and CDT/Leukotoxin mutant strains. Cytotoxicity of CFCS preparation of WT and isogenic double knockout mutant of *cdt/ltx*. Freshly isolated PBMC were incubated for 72 hours with CFCS of *A. actinomycetemcomitans* wild type parent or isogenic mutant with deletion of both *cdt* and *ltx* loci. Cultured cells were labeled with Annexin V, anti-CD3 monoclonal antibody and 7-AAD, followed by flow cytometric analysis. Mean and S.E.M. of the proportion of Annexin V-positive 7-AAD-negative T cells are shown (A). Representative flow cytometric profile of apoptosis in T cells exposed to CFCS of *A. actinomycetemcomitans* wild type (B), *ltxA/cdtABC* knockout mutant (panel C) and media alone (D) are shown.

Role of CDT in contributing to cell cycle arrest

The relative contribution of *A. actinomycetemcomitans* CDT to the cytostatic effects of this organism was investigated. To that end, progression through cell cycle among lymphocytes exposed to CFCS of wild type and isogenic CDT mutants were analyzed and compared. Results demonstrated that approximately 75% of lymphocytes exposed to CFCS of wild type bacteria were arrested in the G₂/M phase after 72 hours of culture ($p < 0.00001$) compare to lymphocytes exposed to media alone (Fig. 8). Significant loss of cytostatic function was noted in CFCS of isogenic mutants of *cdtA* ($p < 0.00001$), *cdtB* ($p < 0.00001$), *cdtC* ($p < 0.00001$) and *cdtABC* ($p < 0.000001$). The cytostatic ability of *cdt* mutants were abolished, since the proportion of lymphocytes in G₂/M phase in cultures stimulated with CFCS of *cdt* mutants were not significantly different than in negative control cultures.

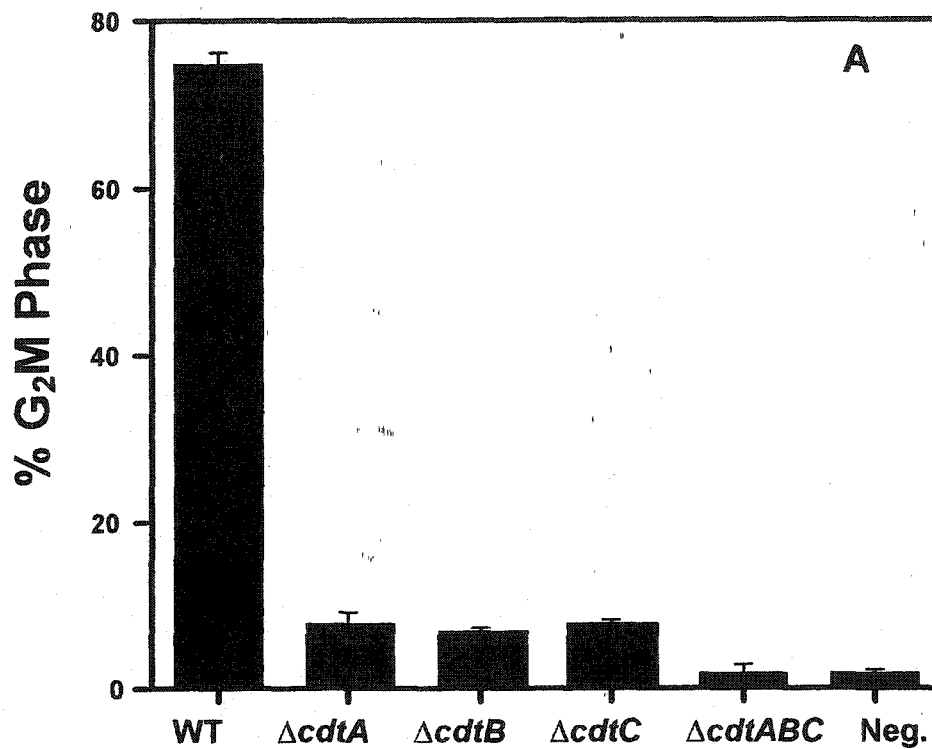


FIGURE 4. 8A. Ploidy of T cells stimulated with CFCS of *A. actinomycetemcomitans* wild type (WT), as well as specific knockout mutants of *cdt*. Freshly isolated PBMC were incubated for 72 hours with *A. actinomycetemcomitans* wild type parent or isogenic mutants with deletion in each of the *cdt* loci, *cdtA*Δ, *cdtB*Δ, *cdtC*Δ and *cdtABC*Δ. Cultured cells were fixed, permeabilized and labeled with PI, followed by flow cytometric analysis. (A) A representative experiment that was repeated five times is presented, where the PBMC from a donor were cultured and analyzed in triplicate. Mean and S.E.M. of the proportion of cells with different degrees of ploidy are shown. (B) Representative flow cytometric profile of apoptosis in T cells exposed to CFCS of wild type and specific *cdt* knockout mutants of *A. actinomycetemcomitans*.

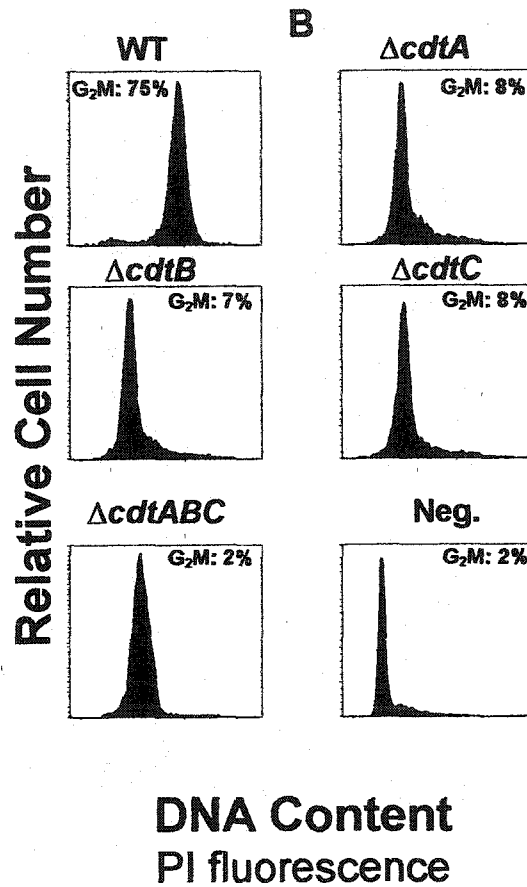


FIGURE 4. 8B. Representative flow cytometric profile of apoptosis in T cells exposed to CFCS of wild type and specific *cdt* knockout mutants of *A. actinomycetemcomitans*. Freshly isolated PBMC were incubated for 72 hours with *A. actinomycetemcomitans* wild type parent or isogenic mutants with deletion in each of the *cdt* loci, *cdtAA*, *cdtBA*, *cdtCA* and *cdtABCA*. Cultured cells were fixed, permeabilized and labeled with PI, followed by flow cytometric analysis. Panel (B); Representative flow cytometric profile of apoptosis in T cells exposed to CFCS of wild type and specific *cdt* knockout mutants of *A. actinomycetemcomitans* are shown.

DISCUSSION

Recent studies from our laboratory have demonstrated that following stimulation of PBMC with cell-associated and cell-free preparations of *A. actinomycetemcomitans*, T cells exhibit large-scale apoptosis in a time- and dose-dependent manner; up to 70% at 96 hours (Nalbant and Zadeh 2000). It had previously been demonstrated that specific toxins of *A. actinomycetemcomitans*, namely leukotoxin (Mangan, Taichman et al. 1991) and CDT (Shenker, Hoffmaster et al. 2001), under certain circumstances, are capable of inducing T cell apoptosis. However, it is not known whether these molecules are the exclusive cytotoxins that *A. actinomycetemcomitans* expresses. The extent to which the cytotoxicity of *A. actinomycetemcomitans* is dependent on the presence of CDT and leukotoxin is also not known. To that end, we developed the tools necessary to investigate the contribution of these cytotoxins, as well as probe the existence of other potential cytotoxins. Thus, successful construction of single and double knock-out mutants of *A. actinomycetemcomitans* leukotoxin and CDT provide valuable tools for this and future investigations.

We evaluated the contribution of leukotoxin and CDT to T cell apoptosis by deletion of the *ltx* and/or the *cdtABC* genes. The individual Leukotoxin or CDT mutant strains, each exhibited reduced capacity to induce T cell apoptosis. The response to CDT mutants was reduced by approximately 30%. This data is

consistent with the report by Shenker, et al, who found that recombinant CDT induces apoptosis of approximately 24% to 48% of lymphocytes at 72 hours (Shenker, Hoffmaster et al. 2001). Mangan, et al have previously demonstrated loss of plasma membrane integrity and the presence of chromosomal fragments on agarose gel, following exposure of T cells to *A. actinomycetemcomitans* leukotoxin (Mangan, Taichman et al. 1991). The findings of the present study complement the previous report. Our present data demonstrated that, compared with the wild type parent, preparations of leukotoxin-mutant had a relatively small (18%) but significant decrease in T cell apoptosis. The reason for the relatively low decrease in T cell apoptosis following leukotoxin deletion is probably due to the fact that the wild type parent strain of *A. actinomycetemcomitans* (D7S-smooth) used, expresses low levels of leukotoxin (data not shown). Purified recombinant leukotoxin was also found to induce apoptosis of approximately 27% of T cells in the present study. Collectively, these data demonstrate that CDT and leukotoxin, each partially contribute to the observed T cell apoptosis activity.

Our data also suggest that deletion of any of the *cdt* gene loci was associated with roughly 30-35% decrease in the ability of their cell free culture supernatant of *cdt* mutants to induce apoptosis. These data is consistent with previous report that the expression of CdtA, CdtB, and CdtC is necessary for *H. ducreyi*-derived culture supernatant fluid to exhibit cytotoxicity for HeLa cells (Lewis et al. 2001). On the

other hand, whole cell extracts from *H. ducreyi cdtA* mutant was found to retain partial cytotoxicity. Therefore, it has been proposed that CdtA may be primarily associated with the *H. ducreyi* cell envelope, whereas both CdtB and CdtC to be present primarily in the soluble fraction from sonicated cells. These data is consistent with our antibody inhibition experiment showing that neutralization of CdtA protein had the lowest effect on T cell apoptosis. Other investigators have suggested that CdtB and CdtC^{hu} are possibly the biologically active components of CDT (Gelfanova et al. 1999; Shenker, Hoffmaster et al. 2000). However, without more data about the nature of the CDT holotoxin, the exact role of each component in apoptosis cannot be ascertained.

Deletion of both CDT and leukotoxin led to the loss of cytotoxicity in extracellular preparation of *A. actinomycetemcomitans*, while the cell associated preparation retained significant cytotoxicity. These findings provides direct evidence for the expression of other cytolytic toxin(s) distinct from CDT and leukotoxin in the cell-associated preparations. The putative cytolytic molecule is likely not released extracellularly, since CFCS preparations made from double mutant strain lacked cytotoxicity. The existence of additional cytolytic molecules is also corroborated by data showing significant ability to induce apoptosis in response to preparations of naturally occurring low leukotoxin-producing *A. actinomycetemcomitans* strains (D7S-smooth and 33384-data not shown), where CDT was neutralized with

specific monoclonal antibodies. It should be noted that the monoclonal antibodies used were raised against *H. ducreyi* CDT. Though, these monoclonal antibodies have been shown to cross-react with *A. actinomycetemcomitans* CDT (Stevens, Latimer et al. 1999). Moreover, the CdtA, CdtB and CdtC proteins from the two species have 91.9%, 96.8 and 93.5% homology, respectively (Pickett and Whitehouse 1999). Nevertheless, it cannot be rule out that the persistence of cytotoxic activity is a result of incomplete neutralization, due to minor species-specific differences.

The ability of double-deletional mutants of leukotoxin and CDT to induce higher level of apoptosis than the wild type parent was surprising. The mechanism of such increase is not known. It may be speculated that the lack of expression of leukotoxin and CDT may contribute to an upregulation of other cytotoxins. The identification of novel cytolytic molecules expressed by *A. actinomycetemcomitans* is a current focus of our investigation. It is important to note that the bacterial preparations used here had been dialyzed to remove molecules of less than 10kD in molecular weight. This rules out the involvement butyric acid, which is an extracellular metabolite of *A. actinomycetemcomitans* that has been reported to induce apoptosis of human and murine T cells (Kurita-Ochiai et al. 2000). Recent data from chromatographic purification and characterization of cytolytic proteins of *A. actinomycetemcomitans* have identified 60kD GroEL-like protein as a

potential candidate (manuscript in preparation). Our results have also shown that addition of anti-GroEL neutralizing antibody to cultures of PBMC stimulated with double deletional mutant strain ($\Delta ltx/cdtABC$), abolishes the residual cytotoxicity (data not shown).

With regard to the cytostatic function of *A. actinomycetemcomitans*, it appears that deletion of any of the 3 *cdt* loci is sufficient to abolish the ability of *A. actinomycetemcomitans* to induce a cell cycle arrest. Therefore, these data suggest that the cytostatic function of *A. actinomycetemcomitans* is dependent on the expression of all 3 *cdt* gene loci. This may imply that CDT is the only cytostatic molecule that *A. actinomycetemcomitans* expresses. However, these data do not rule out the possibility of the existence of other cytostatic molecules that depend on the expression of *cdt* genes.

The mechanisms by which CDT and leukotoxin induce T cell apoptosis and whether there are any interactions is not known. Shenker, et al have recently reported that CDT can induce T cell apoptosis, only in conjunction with polyclonal stimulants such as PHA or anti-CD3 plus anti-CD28 monoclonal antibodies (Shenker, Hoffmaster et al. 2001). In order to establish the physiologic relevance of this phenomenon, it was shown that crude preparations of *A. actinomycetemcomitans* were able to induce apoptosis, in the absence of any other

stimulants. It is possible that some of the components present in crude preparations of *A. actinomycetemcomitans*, such as lipopolysaccharide (LPS) or putative superantigens (Zadeh, Nalbant et al. 2001) provide the same function as the polyclonal stimulants in enhancing apoptosis.

Apoptosis is an active process by which the target cell activates a cascade of events leading to its demise. It is well established that apoptosis plays a major role in maintaining immunological homeostasis. However, a number of microorganisms utilize apoptosis as a virulence factor (Chen and Zychlinsky 1994). A number of gram negative microorganisms such as *Haemophilus ducreyi*, *Escherichia coli*, *Shigella* and *Campylobacter* are known to induce apoptosis of T cells (Cortes-Bratti et al. 1999; Gelfanova, Hansen et al. 1999; Stevens, Latimer et al. 1999). The clinical significance of apoptosis induction in the pathogenicity of *A. actinomycetemcomitans* is currently not known. However, the expression of multiple apoptosis-inducing toxins, as well as the occurrence of large-scale cytotoxicity are likely to have a major impact on the immune response to this organism and contribute to its pathogenicity. The availability of the deletional mutants of *A. actinomycetemcomitans* provide valuable tools for investigation of the role of CDT and leukotoxin in the pathogenicity of *A. actinomycetemcomitans*.

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