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*J Immunol* 1999; 162:4773-4780;
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Actinobacillus actinomyctemcomitans Immunosuppressive Protein Is a Member of the Family of Cytolethal Distending Toxins Capable of Causing a G₂ Arrest in Human T Cells

Bruce J. Shenker,* Terry McKay,* Sugandha Datar,* Mark Miller,* Rohini Chowhan,* and Donald Demuth†

We have previously shown that Actinobacillus actinomyctemcomitans produces an immunosuppressive factor (ISF) capable of impairing human lymphocyte function by perturbing cell cycle progression. We now report that ISF is the product of the cdTB gene, one of three genes encoding the family of cytolethal distending toxins (Cdts). The ISF polypeptide exhibits ≥95% identity with Hemophilus ducreyi CdTB protein and ≥60% homology with Escherichia coli or Campylobacter jejuni CdTB. Pretreatment of PHA-activated lymphocytes with 5–25 ng ISF results in G₂ arrest of CD4+ and CD8+ T cells. Similarly, treatment of HeLa cells results in G₂ arrest and cell elongation and distension. However, lymphocytes are at least 5 times more sensitive to ISF than HeLa cells and do not undergo the elongation and distension that characterizes interactions of Cdts with cell lines. ISF-treated lymphocytes express normal cyclin A and B1 levels, but contain reduced levels of cell cycle-dependent kinase-1 (Cdk1). Additionally, the majority of Cdk1 is in the hyperphosphorylated, inactive, form. In contrast, PHA-induced G₂ cells contain elevated levels of the hypophosphorylated, active Cdk1. Failure of ISF-treated cells to dephosphorylate Cdk1 is not associated with decreased availability of Cdc25. These studies suggest that the CdTB protein alone is capable of inducing G₂ arrest in lymphocytes and cell cycle arrest, elongation, and distension of HeLa cells. Our studies also suggest that lymphocytes may be primary targets for A. actinomyctemcomitans CdTB (ISF) and possibly for other Cdt family members as well. Thus, Cdts may function to impair host immunity and contribute to the pathogenesis of disease associated with Cdt-producing organisms. The Journal of Immunology, 1999, 162: 4773–4780.

Microbial virulence may sometimes be the consequence of their ability to resist, escape, or subvert host defense mechanisms. The ability of micro-organisms to evade or suppress the immune response of the host not only affects the course of initial infection by facilitating spread, multiplication, and persistence, but may also lead to enhanced susceptibility to infection by secondary pathogens (reviewed in Refs. 1 and 2). Such modulation of the immune response may be a critical event in the outcome of numerous diseases, including measles, rubella, candidiasis, tuberculosis, trypanosomiasis, and syphilis among others. Perhaps the most prominent example of this relationship between host and pathogen is HIV infection, which ultimately results in destruction and elimination of a subpopulation of T lymphocytes. Actinobacillus actinomyctemcomitans, a nonmotile, Gram-negative coccobacillus, is associated with several human diseases. These include endocarditis, meningitis, osteomyelitis, s.c. abscesses, and periodontal disease (3–8). Although the pathogenic mechanism(s) by which A. actinomyctemcomitans acts to cause disease is not known, it does produce several potential virulence factors capable of facilitating colonization, destroying host tissue, inhibiting tissue repair, and interfering with host defenses (reviewed in Ref. 7). With respect to the latter, several studies suggest that impaired host defense mechanisms may contribute to infectious diseases associated with A. actinomyctemcomitans (reviewed in Refs. 7 and 9). In this regard we have previously shown that A. actinomyctemcomitans produces a heat-labile immunosuppressive factor (ISF) that is capable of inhibiting both human T and B cell function (10–13). While the exact mechanism by which this immunoregulatory agent acts is not yet known, our previous studies provide strong support for the ability of ISF to induce aberrations in the normal progression of the lymphocyte cell cycle. This is characterized by both an accumulation of cells in the G₂ and M phase of the cell cycle as well as the generation of a subset of T cells with an abnormal phenotype. The latter cells are not only CD3 positive, but are CD4− and CD8− as well; furthermore, these cells are predominantly in the S and G₂ phases of the cell cycle.

The cytolethal distending toxins (Cdts) are a newly described family of heat-labile protein cytotoxins produced by several different bacterial species. These include diarrheal disease-causing enteropathogens such as some Escherichia coli isolates, Campylobacter jejuni and Shigella dysenteriae (14–17). More recently, related toxins have been identified in Hemophilus ducreyi, a human pathogen responsible for the formation of chancreoid ulcers and buboes, and A. actinomyctemcomitans strain Y4 (18, 19). The Cdts cause progressive cellular distension and finally death in some cell lines; it should be noted that the gross cellular changes...
associated with Cdt activity are clearly different from those caused by other known toxins that induce rapid morphological alterations culminating in cell death (18, 20, 21). There is now clear evidence that Cdt is encoded by three genes, designated \( cdtB \), \( cdtC \), and \( cdtA \), which are arranged in an apparent operon. These three genes specify polypeptides with predicted or apparent molecular masses of approximately 25–35 kDa. We now report that the \( A. \) actinomycetemcomitans ISF is a member of the family of Cdt. N-terminal amino acid analysis of purified \( A. \) actinomycetemcomitans ISF indicates 98% identity with the CdtB toxin of \( H. \) ducreyi (18).

Subsequently, the entire gene encoding ISF was isolated and was shown to be 95% identical with the CdtB protein of \( H. \) ducreyi. Moreover, we have determined that the purified ISF is capable of inducing a G2 arrest in the cell cycle of both lymphocytes and human cell lines. However, lymphocytes are 5-fold more sensitive to the toxin than are HeLa cells and do not exhibit the morphologic effects that are commonly observed with cell lines. The G2 arrest appears to be associated with a failure to dephosphorylate cell cycle-dependent kinase-1 (Cdk1). These studies suggest that the primary host target of \( A. \) actinomycetemcomitans ISF and possibly all Cdt toxins may be lymphocytes.

Materials and Methods

Culture of \( A. \) actinomycetemcomitans and preparation of ISF

ISF was prepared from \( A. \) actinomycetemcomitans strain 652 using a modification of the procedure we previously described (12). Briefly, the bacteria were grown for 48 h at 37°C in PYG medium containing 0.4% sodium bicarbonate. Harvested organisms were washed with PBS and extracted in 50 mM Tris buffer, pH 8.0, containing 10 mM NaCl, 5 mM EDTA, 0.1 mM PMSF, l-lysine (10 \( \mu \)M), and DNase (0.5 \( \mu \)g/ml). Following one cycle of freeze-thawing, resulting bacterial cells were removed by centrifugation at 10,000 \( \times \) g, and the supernatant was ultrafiltrated for 60 min at 100,000 \( \times \) g. The ISF was then purified to homogeneity as described in Results.

Protein and gene sequencing \( A. \) actinomycetemcomitans ISF (\( cdtB \))

The purified ISF was immobilized onto PVDF membrane (Bio-Rad, Hercules, CA) and subjected to N-terminal amino acid analysis using Edman degradation. Sequencing was performed on an ABI Procise sequencer (Applied Biosystems, Foster City, CA) using the manufacturer’s software by the Wistar Protein and Molecular Biology Core facility (Wistar Institute; Philadelphia, PA). The derived N-terminal peptide sequence was compared with known protein sequences using the NCBI Blast program.

To clone the gene encoding ISF, PCRs were conducted using \( A. \) actinomycetemcomitans strain 652 genomic DNA as the template and degenerate oligonucleotide primers based upon the ISF N-terminal amino acid sequence determined above and the deduced amino acid sequence of CdtC of \( H. \) ducreyi (residues 23–28) (18). The resulting fragment was cloned in pGEM-T (Promega) and transformed into \( E. \) coli DH5\( \alpha \). Sequencing was conducted on an ABI 377 by the DNA Sequencing Core Facility at the University of Pennsylvania. To complete the sequencing of the ISF gene (\( cdtB \)), additional primers were synthesized based upon the \( A. \) actinomycetemcomitans sequence already determined. Subsequently, the complete \( cdt \) locus was isolated from an \( A. \) actinomycetemcomitans library in AEML3 by screening with the \( cdtB \) gene as a probe. One clone with a 15-kbp insert containing the entire \( cdt \) operon was chosen for further study. The sequences of the \( A. \) actinomycetemcomitans \( cdtB \) and \( cdtC \) genes were determined from DNA fragments flanking the \( cdtB \) gene that were derived from this clone. The \( A. \) actinomycetemcomitans strain 652 \( cdtB \) gene sequence has been submitted to the GenBank database; the accession number is AF102554.

Cell isolation and culture

Human PBMC (HPBMC) were prepared as described previously (10). Briefly, HPBMC were isolated from 100–200 ml of heparinized venous blood obtained from healthy donors. The blood was diluted with an equal volume of RPMI 1640 and the HPBMC were fractionated on Ficoll-Hypaque (Pharmacia, Piscataway, NJ). The HPBMC were washed twice with RPMI 1640, and counts of viable cell were performed by assessing trypan blue dye exclusion.

For selected experiments purified populations of T cells were obtained by E rosette formation as described previously (12). Briefly, sheep erythrocytes were washed and treated with 0.14 M 2-aminoethylisothiouronium bromide at pH 9 for 15 min. After four washes, the erythrocytes were incubated with HPBMC as a cell pellet for 60 min. The cells were then gently resuspended; nonrosetted cells were separated from rosetted cells on Ficoll-Hypaque as described above. The rosetted cells found in the pellet were lysed to remove erythrocytes and contained >98% T cells when stained with anti-CD3 mAb (Becton Dickinson Immunocytometry Systems, San Jose, CA) and analyzed by flow cytometry. HPBMC or purified T cells were incubated in 1-ml cultures in 24-well plates containing 1.5 \( \times \) 10\(^6\) cells/ml, PHA (1 \( \mu \)g/ml; Murex, Dartfield, U.K.), and ISF (5–25 ng/ml) in RPMI 1640, antibiotics, and 2% heat-inactivated human AB serum. The cells were incubated for 72 h (or as indicated), harvested, and analyzed by flow cytometry or extracts prepared for Western blot analysis as described below.

Column fractions were monitored for ISF activity by measuring their relative ability to inhibit \(^{[3 \text{H}]\text{thymidine incorporation of HPBMC as previously described (10). Briefly, 2 \times 10^5 \text{cells were plated into each well of flat-bottom microculture plates. Each culture received 0.1 \text{ml of medium or 0.1 ml of varying amounts of individual column fractions diluted in medium. The cells were incubated for 60 min at 37°C, at which time the cells received an optimal mitogenic dose of Con A (1 \mu g; Calbiochem, La Jolla, CA). The cells were incubated for 96 h, labeled with \(^{[3 \text{H}]\text{thymidine, and harvested as previously reported (12). One unit of ISF activity was calculated as that volume of material required to reduce \(^{[3 \text{H}]\text{thymidine incorporation to 50% maximum (Con A alone).}}\)

Assessment of \( A. \) actinomycetemcomitans ISF on \( \text{HeLa cells}\)

HeLa cells were obtained from the American Type Tissue Culture Collection (Manassas, VA) and maintained in RPMI 1640 containing antibiotics and 10% FBS. For cell cycle analysis, 1-ml cultures were established containing 2.5 \( \times \) 10\(^6\) cells in 24-well plates. Cultures received varying amounts of ISF or medium (control) and incubated for 72–96 h, at which time the cells were trypsinized and subjected to cell cycle analysis by flow cytometry (see below). Any nonadherent cells found in the culture were removed and included in the cell cycle analysis. For morphologic evaluation, 1 \times 10\(^5\) cells were added to microscope chamber slides (Lab-Tek, Naperville, IL) in 0.4 ml of medium in the presence or the absence of ISF. Following 96-h incubation, the cells were stained with hematoxylin and eosin and analyzed.

Flow cytometric analysis of cell cycle

Cell cycle analysis was performed on lymphocytes and HeLa cells as previously reported (12). Briefly, T cells (1.5 \times 10^6) were first washed (PBS containing 0.1% NaCl) and then stained with anti-CD4- or anti-CD8-conjugated FITC (Becton Dickinson Immunocytometry Systems) 72 h after exposure to medium, PHA, or PHA and ISF. The cells were washed and fixed for 60 min with cold 0.25% paraformaldehyde. After washing, the cells were permeabilized with 0.2% Tween 20 in PBS for 15 min at 37°C. DNA was then stained by incubating cells with propidium iodide (10 \( \mu \)g/ml containing 1 mg/ml RNase) for 30 min. Samples were analyzed on a Becton Dickinson FACStar flow cytometer. FITC and propidium iodide fluorescence were excited by an argon laser operating at 488 nm, and fluorescence was measured with a 530/30-nm bandpass filter (FITC) or a 630/22-nm bandpass filter (propidium iodide). A minimum of 30,000 events were collected on each sample. Immunofluorescence data were collected on a log scale, and propidium iodide emissions were collected using linear amplification.

For the analysis of total RNA, protein, and cyclins, the cells were harvested and washed in PBS and then resuspended in cold 80% ethanol. To measure cell cycle along with total RNA and protein, the cells were harvested and washed with Hoechst 33342 (2 \( \mu \)g/ml; Molecular Probes, Eugene, OR) and pyronine Y (4 \( \mu \)g/ml; Sigma, St. Louis, MO) or with FITC (0.1 \( \mu \)g/ml; Molecular Probes), respectively (22, 23). The cells were analyzed by flow cytometry; Hoechst fluorescence was excited with one laser operating at 488 nm, and fluorescence was detected through a 424/44-nm bandpass filter. Both pyronine Y and FITC were excited with a second laser operating at 488 nm; emission was detected with a 575/26-nm bandpass filter (pyronine Y) or a 535/30-nm bandpass filter (FITC). The cyclins were analyzed after fixation for 2 h at -20°C when the cells were washed, resuspended in 0.25% Triton X-100/PBS, and incubated for 5 min at 2–5°C. The cells were washed and then stained with FITC-conjugated Abs to cyclin A or B1 (PharMingen, San Diego, CA) for 30 min; control cells were stained with appropriate isotype controls. After washing, the cells were resuspended in propidium iodide solution (10 \( \mu \)g/ml) and analyzed as described above.
Western blot analysis for the analysis of Cdc2 and Cdc25

T cells were incubated with PHA in the presence or the absence of ISF for 72 h. The cells were harvested, and replicate wells were pooled and solubilized in 50 mM Tris containing 0.5% Nonidet P-40, 150 mM NaCl, 1 mM sodium vanadate, 50 mM sodium fluoride, 1 mM benzamidine, and 25 μg/ml aprotinin, leupeptin, and trypsin inhibitor (Sigma). Cell debris was removed by centrifugation (10,000 × g). Proteins of interest were detected by Western blot analysis using mAbs to the Cdc-2 or using actin (PharMingen) or goat polyclonal antisera to Cdc25B and Cdc25C (PharMingen) as previously described (24). Briefly, total solubilized cellular protein (10 μg) was separated by 12% SDS-PAGE and then transferred to nitrocellulose. The membrane was blocked with BLOTTO and then incubated with one of the primary Abs listed above for 18 h at 4°C. Membranes were washed, incubated with rabbit anti-mouse Ig (or anti-goat) serum conjugated to the primary Abs listed above for 18 h at 4°C. Membranes were washed, incubated with rabbit anti-mouse Ig (or anti-goat) serum conjugated to horseradish peroxidase (Fisher Scientific, Pittsburgh, PA); the blots were developed using chemiluminescence (New England Nuclear, Boston, MA). The relative quantity of each band was determined by scanning densitometry.

Results

ISF was purified from *A. actinomycetemcomitans* strain 652 using a modification of the procedure we previously described (12). Bacterial extracts were first fractionated by ammonium sulfate precipitation; all activity precipitated between 30–55%. Following dialysis in 50 mM Tris containing 10 mM NaCl, the sample was applied to an ion exchange column (Mono Q 10, Pharmacia Biotech, Uppsala, Sweden) pre-equilibrated in 50 mM Tris, pH 7.0, containing 10 mM NaCl. The column was washed and eluted with a 250–550 mM NaCl gradient (see Fig. 1A). Fractions were collected and monitored for both A 280 and ISF activities; for these purposes we defined 1 LD50 unit of ISF as that amount (volume) of material required to reduce Con A-induced [3H]Tdr incorporation to 50% of the maximum. As shown in Fig. 1A, ISF activity was eluted with 300–400 mM NaCl; the active fractions were pooled, dialyzed, and applied to a chromatofocusing column equilibrated at pH 7 (Mono P, Pharmacia). After extensive washing, the column was eluted with a linear pH 7.0–6.0 gradient (Polybuffer, Pharmacia); >90% of the ISF activity was obtained in a single fraction corresponding to pH 6.6 (Fig. 1B). The ISF activity was concentrated and further fractionated by gel filtration chromatography (Superdex 75, Pharmacia). Column fractions were monitored for ISF activity as described above (Fig. 1C); ISF activity eluted from the column in a volume that corresponds to a m.w. of 30–40 kDa. These fractions were further assessed for purity by SDS-PAGE analysis; as shown in Fig. 1D, the ISF obtained by this purification scheme yields a single band corresponding to a *M* of approximately 35 kDa.

Purified ISF was immobilized onto a PVDF membrane and subjected to N-terminal amino acid analysis. As shown in Fig. 2, the first 15 amino acids of the N-terminus of the protein were identified. Comparison of this sequence to known protein sequences revealed 95% identity with the deduced amino acid sequence of the CdtB protein of *H. ducreyi*. Therefore, we used a series of degenerative primers based upon both the *A. actinomycetemcomitans* and *H. ducreyi* protein sequences to determine the entire gene sequence of the *A. actinomycetemcomitans* ISF (*cdtB*) gene (Fig. 2). The gene consists of 848 bp with a RBS site just upstream to the start codon (residue 34). Analysis of the deduced amino acid sequence revealed that the *A. actinomycetemcomitans* *cdtB* gene encodes for a protein consisting of 283 amino acids with a molecular mass of 32 kDa. Moreover, the gene encodes an additional 22 amino acids that comprise a signal sequence. Comparison of the complete *A. actinomycetemcomitans* *cdtB* gene sequence with other *cdt* genes indicates 94% identity with the *cdtB* of *H. ducreyi* and only 66 and 59% identities with the *cdtB* genes of *C. jejuni* and *E. coli*, respectively. Likewise, as shown in Table I, analysis of the deduced amino acid sequence of the CdtB proteins indicates that the *A. actinomycetemcomitans* protein is almost identical (94%) with the *H. ducreyi* CdtB protein; identities with *C. jejuni* or *E. coli* are 60 and 57%, respectively. Further sequencing upstream and downstream from the *A. actinomycetemcomitans* *cdtB* gene identified genes corresponding to *cdtA* and *cdtC*, respectively (not shown), suggesting that the structure of the *A. actinomycetemcomitans* *cdtB* locus is similar to those of other *cdt* gene clusters. Subsequent to these analyses Sugai et al. (19) reported the cloning of a *cdt* toxin gene cluster from *A. actinomycetemcomitans* strain Y4. Comparison of the *cdtB* genes from the two *A. actinomycetemcomitans* strains was identical with the exception of a single nucleotide; this translated to a single amino acid difference in the deduced sequence of the polypeptides.

Our previous studies indicated that the *A. actinomycetemcomitans* ISF impairs normal immune function by causing aberrations in the cell cycle of activated human T cells (10, 12). Of particular relevance, the family of Cdts also appeared to function by causing G2 arrest in the cycle of toxin-treated cell lines. Therefore, we first evaluated and compared the ability of purified ISF to interfere with
the normal cell cycle progression of PHA-activated human CD3\(^+\) CD4\(^+\) and CD3\(^+\) CD8\(^+\) T cells as well as HeLa cells. Based upon DNA analysis with the fluorochrome propidium iodide, nonactivated CD4\(^+\) and CD8\(^+\) T cells formed a single well-defined population of cells in the G0/G1 phase of the cell cycle (Fig. 3, A and F). By contrast, the addition of PHA to T lymphocyte cultures resulted in cell activation characterized by the appearance of cells in both the S (14–20%) and G2 M (4–5%) phases of the cell cycle (Fig. 3, B and G). Treatment of lymphocytes with 5–25 ng of ISF resulted in a 4- to 6-fold increase in the population of G2M cells (Fig. 3, C–E and H–J). In the presence of 5 ng of ISF, the percentage of G2M cells increased to 34 and 17% for CD4\(^+\) and CD8\(^+\) T cells, respectively. Increasing the ISF concentration to 25 ng did not further increase the numbers of CD4\(^+\) cells in the G2/M phase of the cell cycle, whereas the percentage of CD8\(^+\) T cells in this phase of the cell cycle increased to 25%. No effect was observed when the ISF concentration was reduced to <1 ng/ml (data not shown). Furthermore, incubation of the ISF-treated T cells for 96 h failed to significantly increase the percentage of G2M cells over that observed at 72 h.

HeLa cells were also susceptible to the effects of ISF, but were significantly less sensitive than lymphocytes. As shown in Fig. 4, treatment of HeLa cells with 25 ng of ISF for 96 h resulted in a 2-fold increase in the percentage of G2 cells over that observed in untreated cultures. In contrast to human T cells, no effect of ISF was noted at either 10 or 5 ng of ISF. Similarly, treatment of HeLa cells with 25 ng of ISF resulted in the typical morphologic changes previously described for other toxins of this family (Fig. 5, A and B). These cells were severalfold larger than the untreated cells and exhibited the cytoplasmic elongation and distension previously noted (25). These morphological alterations were not a feature of human T cells arrested in the G2 phase of the cell cycle as a result of exposure to ISF (Fig. 5, C and D).

To further characterize the ISF-treated lymphocytes we employed the fluorochromes propidium iodide, pyronin Y, and fluorescein to simultaneously analyze cell cycle, and RNA and protein contents, respectively. Resting lymphocytes (Figs. 6, A and D) contained amounts of RNA and protein below those detectable with the fluorochromes employed. Both the G1 and G2M populations of the PHA-activated cells contained elevated levels of RNA; the mean channel fluorescence (MCF) values for these cells were 283 and 608, respectively. Treatment with ISF resulted in a small, but reproducible, decrease in the content of RNA in both G1 (MCF = 240) and G2M (MCF = 587) cells. Similarly, PHA-activated cells contained high levels of protein in both the G1 (MCF = 272) and G2M (MCF = 616) populations; the total protein content was also slightly reduced in ISF-treated cells. The MCF values in these cells were 192 (G1 cells) and 560 (G2M cells).

Our experiments clearly indicate that in the presence of A. actinomycetemcomitans ISF, mitogen-induced T cell activation leads to an arrest in the G2/M phase of the cell cycle. We next conducted a series of experiments to determine whether ISF blocks specific events associated with the progression through this phase of the cell cycle. Critical to the G2 phase of the cell cycle is the activation of Cdk1; activation of Cdk1 requires cyclins A and B as well as dephosphorylation of the kinase itself. Therefore, we employed multiparameter flow cytometry and Western blot analysis to assess the expression of these key G2 regulatory elements. Lymphocytes were activated with PHA in the absence or the presence of 5 ng of ISF and then were stained with propidium iodide and FITC-conjugated Abs to cyclin A or B. The relative levels of cyclin A and B are presented in Fig. 7 in relationship to the DNA content of the cells. Cyclin A was found essentially only in G2/M cells; 66% of the G2/M cells of both the PHA-treated and ISF-treated cultures contained elevated levels of RNA; the mean channel fluorescence (MCF) values for these cells were 283 and 608, respectively. Treatment with ISF resulted in a small, but reproducible, decrease in the content of RNA in both G1 (MCF = 240) and G2M (MCF = 587) cells. Similarly, PHA-activated cells contained high levels of protein in both the G1 (MCF = 272) and G2M (MCF = 616) populations; the total protein content was also slightly reduced in ISF-treated cells. The MCF values in these cells were 192 (G1 cells) and 560 (G2M cells).

Table I. Comparison of CdtB protein identitya

<table>
<thead>
<tr>
<th>A. actinomycetemcomitans</th>
<th>H. ducreyi</th>
<th>C. jejuni</th>
<th>E. coli</th>
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<tbody>
<tr>
<td>CdtB (ISF)</td>
<td>100</td>
<td>94</td>
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<tr>
<td>CdtB (ISF)</td>
<td>100</td>
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Note: Percent identity of CdtB proteins.
PHA; data not shown), nor were they detected in any of the G₁ cells from PHA activated cultures with or without ISF.

In contrast to the cyclins, we observed a striking difference in the expression of Cdk1. This kinase can be detected in two distinct forms; as an inactive kinase it is hyperphosphorylated and appears as a larger entity than the smaller, dephosphorylated, active kinase. Inactive T cells (Fig. 8A, lane 1) do not express detectable levels of either form of Cdk1. By comparison, PHA-activated T cells contain significantly elevated levels of Cdk1 (Fig. 8A, lane 2); the majority of Cdk1 in these cells is present as the active, dephosphorylated kinase. Activation of T cells in the presence of 5–25 ng of ISF results in reduced expression of Cdk1 (Fig. 8A, lanes 3–5); moreover, the majority of Cdk1 in these cells is in the inactive, hyperphosphorylated form. Finally, we evaluated cells for the presence of Cdc25, the phosphatase responsible for dephosphorylating Cdk1. As shown in Fig. 8B, active T cells contain minimally detectable levels of Cdc25; these levels increase in PHA-activated T cells. Surprisingly, cells also treated with ISF contain up to more than twice the amount of Cdc25 as that observed in cells activated by PHA alone.

**Discussion**

In this study we demonstrate that purified *A. actinomycetemcomitans* ISF is a product of the *cdtB* gene and belongs to the Cdt family. Furthermore, *A. actinomycetemcomitans* ISF (CdtB) interferes with both CD4⁺ and CD8⁺ T cell activation by inducing G₂/M arrest in the cell cycle. These results extend our earlier observations that indicated ISF caused perturbations in the cell cycle of activated lymphocytes (12); this included accumulation of cells in the G₂/M phase of the cell cycle as well as the appearance of CD3-positive T cells that coexpress CD4 and CD8. The dual positive cells were also found predominantly in the S and G₂/M phases of the cell cycle. Furthermore, we previously observed that [³H]thymidine incorporation in T cells treated with ISF was normal through 48 h (10); inhibition was not detected until 72 h and was even greater at 96 h. These results can now be explained by our current observations and provide further support for the idea that ISF-treated cells are able to properly transit the S phase of the cell cycle but then become irreversibly arrested in the G₂/M phase.
The family of CdtS represent a novel toxic activity whose members have been identified in several Gram-negative bacterial species. These include some strains of *E. coli*, *S. dysenteriae*, *Campylobacter* sp., and more recently *H. ducreyi* and *A. actinomycetemcomitans* (14, 15, 17–19, 25). Regardless of the species, the genes encoding Cdt form a three-gene operon, *cdtA*, *cdtB*, and *cdtC*, encoding three proteins with similar molecular masses (20–35 kDa). The Cdt were originally defined based upon their effects on mammalian cell lines, HeLa, Hep-2, Vero, and Chinese hamster ovary cells. Exposure to Cdt causes these cells to arrest in the G2 phase of the cell cycle; these cells also exhibit morphologic alterations, including elongation and distension (20, 25). To date there is no documentation or evidence pointing to a possible host target cell for these putative virulence factors. Furthermore, several questions remain regarding the relationship among the three Cdt gene products: CdtA, CdtB, and CdtC. For instance, are the three proteins required to act together as a holotoxin for full expression of biologic activity or is each of the Cdt toxins fully capable of functioning individually to induce cell cycle arrest?

**FIGURE 5.** Effect of *A. actinomycetemcomitans* ISF on the morphology of HeLa cells and human T cells. HeLa cells were incubated in the absence (A) or the presence (B) of 25 ng of ISF for 72 h. T cells were incubated for 72 h in the presence of PHA (C) or PHA plus 5 ng of ISF (D). Results are representative of three experiments. HeLa cells (A and B) are shown at ×25 magnification, and lymphocytes (C and D) are shown at ×100 magnification.

**FIGURE 6.** Effect of *A. actinomycetemcomitans* on PHA-induced RNA and protein content in relationship to cell cycle. T cells were incubated in the presence of medium (A and D), PHA (B and E), or PHA and 10 ng of ISF (C and F). After 72 h the cells were stained with Hoechst 33342 (cell cycle) and pyronin Y (RNA; A–C) or with Hoechst and FITC (protein; D–F). The cells were then analyzed by multiparameter flow cytometry. Data are plotted as the DNA content (Hoechst fluorescence) vs the RNA content (pyronin Y fluorescence) or protein content (fluorescein fluorescence). The results are representative of three experiments; at least 30,000 cells were analyzed.

**FIGURE 7.** Effect of *A. actinomycetemcomitans* ISF on cyclin A and B expression in relationship to cell cycle. T cells were incubated in the presence of PHA alone (A, B, D, and E) or PHA and 10 ng of ISF (C and F). The cells were then permeabilized and stained with FITC-conjugated Abs to cyclin A (B and C) or cyclin B (E and F). A and D represent controls (conjugate) on which analytical gates were set to identify cells positive for cyclin fluorescence. Data are presented as the cyclin content (FITC fluorescence intensity) vs the DNA content (propidium iodide fluorescence). Results are representative of three experiments, each performed in duplicate; at least 30,000 cells were analyzed per sample.
arrest? Alternatively, only one of the peptides may be the active toxin. The other two components may be required for proper expression and/or secretion of the active toxin by the bacteria.

The studies described in this paper address several issues regarding the possible role that Cdt toxins might play during microbial infection. For instance, it is clear from our studies that at least one of the Cdt polypeptides, CdtB, is capable of expressing all of the biologic properties previously associated with this family of toxins. Thus, cell cycle arrest, cell elongation, and cell distention are induced in HeLa cells treated with 25 ng of highly purified A. actinomycetemcomitans ISF (CdtB), which is free of CdtA and CdtC. This was confirmed by the presence of a single protein band on SDS-PAGE and the generation of a single unambiguous N-terminal sequence by Edmund degradation of the purified ISF sample. However, our results do not exclude the possibility that CdtA and CdtC are biologically active as well or are required to activate CdtB. Indeed, Sugai et al. (19) report that none of the cdt genes expressed individually in E. coli possessed biologic activity, but expression of all three genes generated active toxin. In light of our results showing that purified CdtB is active, this would suggest that post-translational modification of CdtB may occur. Also, it is noteworthy that we previously reported that partially pure preparations of A. actinomycetemcomitans extracts were capable of inhibiting fibroblast proliferation (26). This activity was separate from the ISF and perhaps represents activity associated with either CdtA or CdtC.

Our studies also define the lymphocyte as a target for the family of Cdt toxins. Human T cells are at least 5-fold more sensitive to the action (i.e., cell cycle arrest) of CdtB then are HeLa cells. The basis for the tropism of CdtB for human T cells is not clear and is currently under investigation. In addition, the morphologic changes, for example cell elongation and distension, often seen in Cdt-treated cell lines do not occur with lymphocytes treated with A. actinomycetemcomitans ISF (CdtB) and arrested in the G2 phase of the cell cycle. Thus, it is possible that the terminology for this family of potential virulence factors may be misleading, especially with regard to their effects on possible host target cells that may be relevant to the pathogenesis of disease-caused Cdt-producing bac-

teria. In this regard, it may be more appropriate to define Cdt toxins in relation to their effects on lymphocytes (i.e., as immunosuppressive factors) than in the context of cell lines. Thus, impairment of lymphocytes to progress normally through the cell cycle would certainly be expected to lead to impaired immunity and benefit the microbe, leading to increased susceptibility of the host to infection and resulting disease. Indeed, several studies provide support for the idea that A. actinomycetemcomitans infection is associated with immune abnormalities (reviewed in Refs. 7 and 9). It has also been suggested that the failure of the host to generate a rapid and robust immune response may be the determining factor as to whether A. actinomycetemcomitans produces a generalized and wide-spread infection as opposed to localized disease.

There is, in fact, growing evidence that perturbation of the ability of lymphocytes to properly transit through the cell cycle represents a strategy used by several human pathogens to interfere with host immunity. For instance, the activity of A. actinomycetemcomitans ISF (CdtB), in particular, and the family of CdtS, in general, closely resemble the activity of the Vpr protein of human immunodeficiency virus type (27, 28). In this instance, a G2 cell cycle arrest may benefit the virus by leading to increased rates of HIV-1 replication. We have also shown that other pathogens, such as Fusobacterium nucleatum, use a similar strategy to inhibit lymphocyte function by interfering with the cell cycle; this immuno-suppressive factor inhibits mitogen-activated T cells by causing an arrest in the mid-G2 phase of the cell cycle (24). The G2 arrest is associated with the failure of these cells to express proliferating cell nuclear Ag. It is also noteworthy that this class of microbial-derived cell cycle inhibitors may eventually prove to be useful as immunosuppressive drugs, as other naturally occurring agents, such as cyclosporine A, have revolutionized organ transplantation through their ability to prevent graft rejection.

Cell cycle regulation is generally accepted to be controlled at discrete points, called checkpoints, by complexes of cyclins and associated with the failure of these cells to express proliferating cell cycle regulation. Cell cycle regulation is generally accepted to be controlled at discrete points, called checkpoints, by complexes of cyclins and associated with the failure of these cells to express proliferating cell nuclear Ag. It is also noteworthy that this class of microbial-derived cell cycle inhibitors may eventually prove to be useful as immunosuppressive drugs, as other naturally occurring agents, such as cyclosporine A, have revolutionized organ transplantation through their ability to prevent graft rejection.

As for CdtA, the ISF-treated T cells not only express less kinase activity (i.e., cell cycle arrest) of CdtB then are HeLa cells. The basis for the tropism of CdtB for human T cells is not clear and is currently under investigation. In addition, the morphologic changes, for example cell elongation and distension, often seen in Cdt-treated cell lines do not occur with lymphocytes treated with A. actinomycetemcomitans ISF (CdtB) and arrested in the G2 phase of the cell cycle. Thus, it is possible that the terminology for this family of potential virulence factors may be misleading, especially with regard to their effects on possible host target cells that may be relevant to the pathogenesis of disease-caused Cdt-producing bac-

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FIGURE 8. Western blot analysis of Cdk1 and Cdc25 in ISF-treated cells. T cells were incubated for 72 h in medium alone (lane 1), PHA alone (lane 2), or PHA plus 25 ng of ISF (lane 3), 10 ng of ISF (lane 4), or 5 ng of ISF (lane 5). Cellular extracts were then prepared and analyzed by Western blot for both the hyperphosphorylated (Cdk1-p) and hypophosphorylated (Cdk1) forms of Cdk1 (A). The ratio of Cdk1 (active) to Cdk1-p (inactive) is shown. The same cell extracts were also analyzed for the presence of the phosphatase Cdc25 (B); the percentage of Cdc25 relative to that in PHA controls is presented. Blots were also stained for actin as a control reference.
To further define the mechanism for the failure to activate Cdk1 in A. actinomycetemcomitans ISF-treated lymphocytes, we explored the possibility that the defect was related to the availability of Cdc25, the phosphatase responsible for dephosphorylating the Cdk1. However, our studies indicate that Cdc25 is not only present in these cells, but the ISF-treated cells contain higher levels of Cdc25 than do the control PHA-activated cells. Studies are currently being conducted to further define the molecular lesions in these G2 arrested T cells. In this regard, one possibility is that the ISF-treated cells contain damaged DNA, which is known to result in cell cycle arrest at either the G1 or G2 checkpoint (29, 30). Consistent with this possibility is our preliminary observation that ISF-treated lymphocytes arrested in G2 eventually undergo apoptosis. However, it is not clear at this time how A. actinomycetemcomitans ISF (CdtB) might directly or indirectly induce DNA damage, which, in turn, results in an arrest of the cell cycle at the G2 checkpoint.

In summary, the avoidance or modulation of the immune response by invading pathogens may be a critical event in determining the outcome of numerous infectious processes. Our results suggest that the CdtB must now be considered members of the class of immunoregulatory agents capable of impairing immunologic responsiveness by interfering with lymphocyte activation. Such immunosuppressive factors could lead to a state of hyporesponsiveness that favors colonization by the initiating organism or by other opportunistic organisms. Although the immunologic mechanism involved in the pathogenesis of A. actinomycetemcomitans infections has not been clearly defined, there is growing evidence that suppressed host defense mechanisms may contribute to the disease process. This disturbance would, in turn, adversely affect the development of normal immunologic defense mechanisms. We propose that such immunologic perturbations could contribute to the pathogenesis of diseases associated not only with A. actinomycetemcomitans, but with other Cdt-producing organisms as well, by impairing host protection.

Acknowledgments
We thank Rose Espiritu for technical expertise as well as the SDM Flow Cytometry Facility and the Wistar Institute Protein and Molecular Core Facilities for their support of these studies. We also thank Dr. Edward T. Lally for helpful discussions.

References