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Immunosuppressive Properties of *Actinobacillus actinomycetemcomitans* Leukotoxin

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Actinobacillus actinomycetemcomitans **produces a leukotoxin that kills human polymorphonuclear cells (PMNs) and monocytes but not lymphocytes. In this study, we examined *A. actinomycetemcomitans* leukotoxin for its ability to alter human peripheral blood lymphocyte (HPBL) responsiveness. After a 90-min exposure to the leukotoxin, all monocytes were killed and HPBL responsiveness to mitogens and antigens was significantly inhibited. The ability of the leukotoxin to inhibit HPBL responses was not surprising, since monocytes and macrophages are required for many lymphocyte functions. However, we were unable to totally restore HPBL responsiveness when adherent autologous monocytes were added back to cultures of leukotoxin-treated lymphocytes. These studies demonstrate that *A. actinomycetemcomitans* leukotoxin may also exert nonlethal effects directly on lymphocytes. Furthermore, impaired lymphocyte function did not appear to be the result of indirect effects of products released by dying monocytes. Although it is not clear how *A. actinomycetemcomitans* acts to cause disease, several investigators have proposed that impaired host defenses may play a pivotal role. Several studies have demonstrated defects in PMN, monocyte, and lymphocyte function in patients with periodontal disease. These findings, along with the data presented in this paper, support the hypothesis that patients who harbor *A. actinomycetemcomitans* could suffer from local or systemic immune suppression. The effects of this suppression may be to enhance the pathogenicity of *A. actinomycetemcomitans* itself or that of some other opportunistic organism.**

Actinobacillus actinomycetemcomitans, a gram-negative, capnophilic bacterium, is a suspected etiologic agent in certain forms of periodontal disease and has been isolated in relatively high concentrations from the pathological pockets of juvenile periodontitis patients (for a review, see reference 46). The organism also has been identified in the blood samples of patients with bacterial endocarditis, meningitis, and abscesses of the jaw and face (4, 9, 10, 19, 40). Although the mechanism by which *A. actinomycetemcomitans* might contribute to the development of periodontal disease is not clear, it is known that this organism produces several biologically active mediators, including an immunosuppressive factor capable of activating human T suppressor cells (28, 29), an endotoxin capable of promoting bone resorption (13), a polyclonal B-cell activator (44), a factor(s) capable of inhibiting fibroblast, endothelial, and epithelial cell proliferation (12, 26, 33), and a leukotoxin capable of killing human polymorphonuclear cells (PMNs) and monocytes (1, 2, 16, 32). The leukotoxin may be particularly important because of the critical role that monocytes and macrophages play in host defense in general and in the immune response in particular.

Although the nature and contribution of the immune system to the pathogenesis of periodontal disease are poorly understood, there is a vast literature supporting the view that the immune system plays a beneficial role in protecting and limiting bacterial infection in the gingival region (3, 7, 20, 21). Furthermore, there is evidence that immune dysfunction, which may be characterized as a deficiency or defect in either cellular or humoral immunity, may contribute to susceptibility to, and progression of, periodontal disease (15, 23, 31, 34, 35). It is in this context that we initiated the present study. We have examined *A. actinomycetemcomitans* leukotoxin (LTX) for its ability to alter human lymphocyte function and found that it inhibits lymphoid responsiveness to both mitogens and antigens. Although part of this inhibitory effect can be attributed to the depletion of monocytes, our study indicates that the LTX also has direct, nonlethal effects on lymphocytes as well.

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MATERIALS AND METHODS

Isolation and preparation of HPBLs. Human peripheral blood lymphocytes (HPBLs) were prepared as described previously (25). Briefly, HPBLs were prepared from 100 to 200 ml of heparinized venous blood of healthy donors. The blood was first centrifuged at 300 × *g* for 15 min at 5°C; the plasma was removed, and the cells were brought back to the original volume with Hanks balanced salt solution (HBSS). Erythrocytes were eliminated by sedimentation through 1.25% dextran (Dextran T500; Pharmacia Fine Chemicals, Piscataway, N.J.). HPBLs were then isolated by buoyant density centrifugation on Ficoll-Hypaque (LSM; Litton Bionetics, Kensington, Md.) by the method of Boyum (6). The HPBLs were washed twice with HBSS and diluted to 4×10^7 viable cells per ml of HBSS. Viable cell counts were performed by assessing trypan blue exclusion.

Exposure of HPBLs to LTX. HPBLs (2×10^7) were exposed to 100 ng of purified LTX in a total volume of 1 ml; LTX was purified as previously described (38; E. T. Lally, D. Simpson, N. S. Taichman, and J. M. DiRienzo, J. Dent. Res. **66**:169, 1987), free of other *A. actinomycetemcomitans*-derived biologically active factors previously described (26, 29; kindly provided by N. S. Taichman, University of Pennsylvania, Philadelphia). HPBLs were incubated at 37°C for 90 min with either LTX, medium, or LTX pretreated with monoclonal antibody, previously shown to neutralize the cytotoxic activity of LTX (8). After the incubation period, the number of viable cells was determined by trypan blue

exclusion. The cells were washed three times in HBSS and suspended to 2×10^6 viable cells per ml of RPMI 1640 (GIBCO Laboratories, Grand Island, N.Y.) containing 10 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) buffer (Research Organics, Cleveland, Ohio), 2% heat-inactivated fetal bovine serum (FBS), 2 mM L-glutamine, 100 U of penicillin per ml, and 100 μg of streptomycin per ml. Cell smears were prepared with the aid of a cytocentrifuge (Shandon, Sewickley, Pa.) and examined for the presence of monocytes by both morphologic appearance and nonspecific esterase staining (41); at least 200 cells per slide were examined.

Assay of DNA synthesis. A 0.1-ml quantity of HPBL suspension containing 2×10^5 HPBLs was placed into each well of flat-bottom microculture plates (Becton Dickinson, Lincoln Park, N.J.). Each culture received 0.1 ml of medium or 0.1 ml of an optimal mitogenic dose of concanavalin A (ConA [5 μg/ml]; Calbiochem, San Diego, Calif.), phytohemagglutinin (PHA [0.5 μg/ml]; Wellcome Reagents Ltd., Beckenham, England), pokeweed mitogen (PWM [10 μg/ml]; Calbiochem), or formalinized *Staphylococcus aureus* (1 μg/ml; Calbiochem). For antigen stimulation, cultures received 0.1 ml of either tetanus toxoid (10 ng/ml; Wyeth Laboratories, Marietta, Pa.) or streptokinase-streptodornase (5 U of streptokinase and 1 U of streptodornase per ml; Lederle Laboratories, Pearl River, N.Y.). The cells were incubated for 96 (mitogen-induced DNA synthesis) or 144 h (antigen-induced DNA synthesis) at 37°C in humidified air containing 5% CO_2.

DNA synthesis was assayed by the incorporation of [^{3}H]thymidine ([^{3}H]TdR) as previously described (25). Briefly, cells were incubated as described above with the addition of 0.25 μCi of [^{3}H]TdR (specific activity, 42 Ci/mmol; Amersham Corp., Arlington Heights, Ill.) for the last 4 h of culture. The cultures were harvested onto glass fiber filter paper with an automatic cell harvester (Cambridge Technology, Cambridge, Mass.). Radiolabeled precursor incorporation was determined by counting in a Beckman LS3801 liquid scintillation spectrometer. Incorporation of radiolabeled precursor into cells was expressed as a percentage of the incorporation observed in control cultures (exposed to medium).

Assessment of immunoglobulin production. HPBLs were prepared as described and suspended to 2×10^6 cells per ml in RPMI 1640 containing antibiotics, 2 mM glutamine, and 10% heat-inactivated FBS (GIBCO). Cultures of 1 ml (in triplicate) were established containing 10^6 cells and 0.5 μg of PWM. The cells were incubated for 8 days at 37°C. Culture supernatants were obtained by centrifugation at $300 \times g$, filtered through a 0.22-μm-pore-size Millipore filter, and maintained at −20°C until assayed for immunoglobulin as described below.

Culture supernatants were assayed for total secreted immunoglobulin M (IgM) and IgG by enzyme-linked immunosorbent assay (14). Flat-bottomed (Falcon 3912; Becton Dickinson) microculture plates were precoated with 200 μl of a solution (5 μg of protein per ml) of either affinity-purified goat anti-human IgG [specific for F(ab')$_2$] (Cooper Biomedical, Malvern, Pa.) or goat anti-human IgM (specific for μ chain; Cooper Biomedical) in carbonate buffer (pH 9.6, room temperature, 4 h). The plates were washed with phosphate-buffered saline, and unbound sites were blocked by incubating the wells with 200 μl of BLOTTO (11) (room temperature, 60 min) and stored at −20°C until further use. For assay, the plates were thawed and washed with phosphate-buffered saline, and 100 μl of an appropriate dilution (diluted in 1 M diethanolamine buffer containing 0.5% bovine serum albumin) of culture supernatant was added to duplicate wells. After a 4-h incubation, the wells were washed and the amount of IgG or IgM bound to the wells was determined by the addition of 200 μl of goat anti-human immunoglobulin (IgG, IgM, and IgA; Boehringer Mannheim Biochemicals, Indianapolis, Ind.) conjugated to alkaline phosphatase. The plates were incubated overnight at 5°C, washed, and then developed by the addition of 200 μl of a solution containing *p*-nitrophenylphosphate (1 mg/ml; Sigma Chemical Co., St. Louis, Mo.) in 1 M diethanolamine buffer (pH 9.8). After 30 min, 50 μl of 2 M NaOH was added to each well to stop the enzymatic reaction. Supernatant (150 μl) from each well was transferred to a clean polystyrene microculture plate (Nunc, Roskilde, Denmark), and the amount of color development (optical density) was detected on a Dynatech microplate reader (Dynatech Laboratories, Alexandria, Va.). The optical density values were related to immunoglobulin concentration on a standard curve (generated by linear regression analysis) simultaneously prepared with pure IgG or IgM (Cooper Biomedical and Calbiochem, respectively). Culture supernatants were diluted so that the immunoglobulin levels in supernatants fell within the range of the standard curve (5 to 125 ng/ml).

Monocyte purification. Monocyte-enriched cell populations were prepared as previously described (27). Briefly, HPBLs were diluted to 2×10^6 cells per ml in RPMI 1640 containing 10% FBS. The cell suspension (12 to 15 ml) was incubated in 75-cm^2 flasks (Corning Plastics, Corning, N.Y.) that had been preincubated at 37°C with 10 ml of RPMI 1640 containing 10% FBS for at least 30 min. After 1 h of incubation at 37°C, the nonadherent cells were removed and the flasks were washed twice with warm medium. Cells still remaining attached to the plastic surface were then removed by adding 10 ml of cold phosphate-buffered saline containing 10% FBS and gently scraping the surface with a rubber policeman. The adherent cells were washed two times and suspended to 2×10^5 cells in culture medium; these cells were found to routinely contain >90% monocytes when assessed by both morphologic appearance and nonspecific esterase staining.

RESULTS

The ability of *A. actinomycetemcomitans* to kill human monocytes and PMNs has been described by Taichman and colleagues (1, 2, 16, 32); cytotoxicity can be demonstrated by release of lactate dehydrogenase or ^{51}Cr, as well as by a decrease in the ability of cells to exclude trypan blue. Killing is time, dose, and temperature dependent. We first wanted to determine if all monocytes present in our HPBL preparations were killed by the LTX and verify that lymphocyte viability remained unaltered. We first determined the percentage of monocytes present in the HPBL population by both morphologic appearance and nonspecific esterase staining; we routinely observed the presence of 15 to 20% monocytes (Table 1). After the cells were exposed to LTX, we examined the cells for viability by trypan blue exclusion. Cells exposed to LTX contained 12.5 to 15% dead cells as assessed by trypan blue exclusion; this represents 80 to 85% of the total monocytes present. We suspect that the actual percentage of dead cells was slightly higher, however; because of the long exposure period (90 min), some dead cells most likely distintegrated before microscopic evaluation (Norton Taichman, personal communication). Furthermore, no monocytes could be identified by morphology or

TABLE 1. Killing of monocytes by LTX

Expt	% Monocytes[a]		% Nonviable cells[b]		
	Morphology	Nonspecific esterase staining	Control	Treated with LTX	Treated with monoclonal antibody to LTX
1	17.7	18.2	1.0	15.0	3.0
2	19.4	17.0	0	14.0	12.6
3	15.6		0	12.6	1.6

[a] Percentage of monocytes present in control populations before exposure to LTX. Percentages were calculated after examining at least 200 cells per slide and three slides per experiment.
[b] Percentage of cells taking up trypan blue after indicated treatment as described in Materials and Methods.

nonspecific esterase staining after treatment with LTX. No change in cell viability was observed in cultures exposed to medium (control), to LTX previously treated with a neutralizing monoclonal antibody, or to LTX previously heated (56°C). As reported by Taichman and co-workers (32, 37), lymphocyte viability was not altered after a 90-min exposure to the toxin.

Effect of LTX on lymphocyte function. The dependency on monocytes and macrophages for full expression of human lymphocyte responsiveness is well documented (36, 39). Therefore, to further verify LTX depletion of monocytes, we examined the effects of toxin treatment on several aspects of lymphocyte responsiveness. The proliferative response to both B- and T-cell mitogens was reduced in cells exposed to LTX compared with control cells; we observed inhibition of 75, 30, 65, and 50% for ConA-, PHA-, PWM-, and formalinized *S. aureus*-activated cells, respectively (Fig. 1). We also observed similar effects on RNA and protein synthesis (data not shown). We have previously shown that in addition to LTX, *A. actinomycetemcomitans* produces an immunosuppressive factor that acts via activation of T suppressor cells. It was, therefore, important to demonstrate that the observed inhibition of HPBL proliferation was in fact due to LTX, rather than the immunosuppressive factor. This was unlikely, however, since we used pure LTX. Nevertheless, we also used a monoclonal antibody to LTX which has been shown to neutralize toxic activity but not the *A. actinomycetemcomitans*-immunosuppressive factor. This antibody protected monocytes from the lethal effects of LTX and lymphocytes from the inhibitory effects of the toxin (Fig. 1; Table 1). We also examined lymphocytes for responses to antigens streptokinase-streptodornase and tetanus toxoid after exposure to LTX. Proliferative responses to both antigens were significantly suppressed after treatment with the LTX (Fig. 1). Finally, we examined lymphocytes for their ability to synthesize and secrete immunoglobulin. Exposure to LTX resulted in 95% inhibition of IgG and 80% inhibition of IgM production (Fig. 2).

Effect of readdition of monocytes on LTX-mediated lymphocyte suppression. Thus far, we have demonstrated that LTX is capable of inhibiting a wide range of lymphocyte responses. These findings were not surprising since we also demonstrated that the LTX killed virtually all monocytes present in our cell population. In vitro and in vivo lymphocyte responses to both mitogens and antigens are complex processes involving the interaction of several cells, including subsets of lymphocytes (T helper, T suppressor, B, etc.) and monocytes (or macrophages) (18, 36, 39). To verify that the immunosuppressive effects of the LTX were in fact due to monocyte depletion, we performed experiments in which purified monocytes were added back to LTX-treated HPBL cultures and the cells were then tested for mitogen respon-

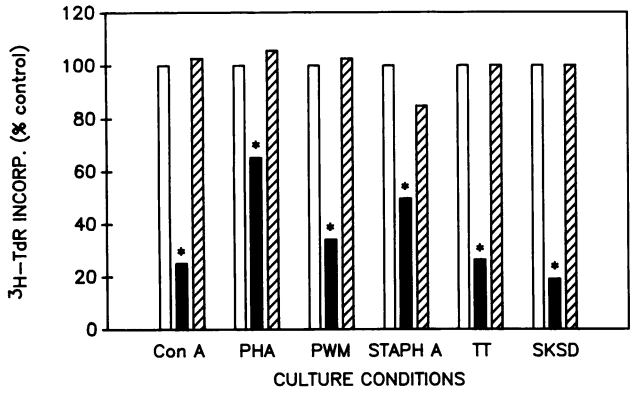

FIG. 1. Effect of LTX on lymphocyte responsiveness to mitogens and antigens. HPBLs were incubated with medium (□), LTX (■), or monoclonal antibody to LTX (▨) as indicated in Materials and Methods. After being washed extensively, the cells were cultured and incubated with the indicated mitogen or antigen. The results represent the mean of three experiments, each performed in quadruplicate, and are plotted as a percentage of the [^{3}H]TdR incorporation observed in control cultures. Standard errors were within 5% of the mean. *, Significantly different from control (medium) cultures ($P < 0.01$). Abbreviations: Staph A, formalinized *S. aureus*; TT, tetanus toxoid; SKSD, streptokinase-streptodornase.

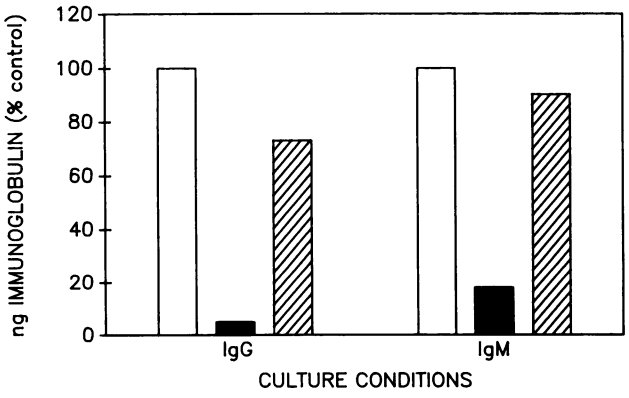

FIG. 2. Effect of LTX on immunoglobulin production. HPBLs were incubated with medium (□), LTX (■), or monoclonal antibody to LTX (▨) and then cultured with PWM. Culture supernatants were collected and assayed for IgG and IgM levels by enzyme-linked immunosorbent assay as described in Materials and Methods. The data are plotted as a percentage of the amount of immunoglobulin secreted from control cultures and represent the mean of quadruplicate cultures of a typical experiment. Standard errors were within 10% of the mean.

siveness (Fig. 3). As already documented, HPBLs treated with LTX exhibited significant impairment in their ability to proliferate in response to ConA, PHA, and PWM. However, when monocytes were added back at concentrations of either 10 or 20% of the total culture, we observed only slight recovery of ConA and PWM responsiveness. Similar effects were observed with PWM-induced immunoglobulin production as well (data not shown). On the other hand, PHA responsiveness was totally restored. These results suggest that the depletion of monocytes alone cannot account for all the suppression observed after exposure to LTX. Another possible explanation is that lymphocytes are indirectly damaged by LTX through the killing of monocytes. We addressed this possibility by the experiments shown in Fig. 4. Here, we compared the effects of LTX and a lysosomotropic agent (36), leucine methyl ester (LME), on lymphocyte responsiveness. Exposure of HPBLs to either LTX or LME resulted in depletion of all monocytes and depression of lymphocyte responsiveness. The proliferative response of LME-treated cells was totally restored by the addition of monocytes; in contrast, the proliferative response of LTX-treated cells remained impaired. These results suggest that the LTX may have direct, but nonlethal, effects on lymphocytes themselves.

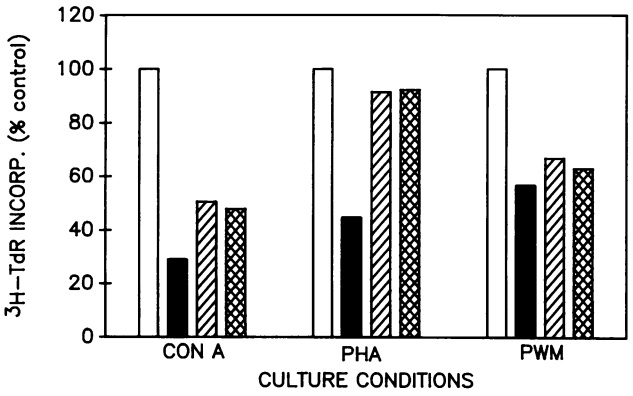

FIG. 3. Effect of readdition of monocytes on inhibition of HPBL proliferation by LTX. HPBLs were exposed to LTX as described and cultured with the indicated mitogen. Control cells exposed to medium only (□), cells exposed to LTX (■), cells exposed to LTX which then received 10% monocytes (▨), and cells exposed to LTX which then received 20% monocytes (▩) are shown. Data are expressed as a percentage of the [^{3}H]TdR incorporation observed in control cultures; each point represents the mean of quadruplicate cultures of a typical experiment. Standard errors were within 5% of the mean.

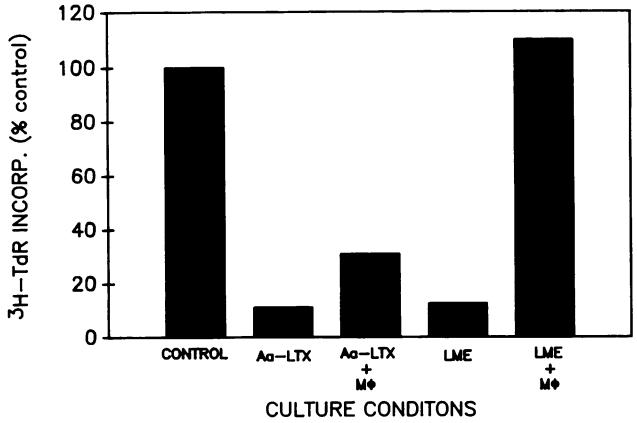

FIG. 4. Comparison of the effects of LTX (Aa-LTX) and LME on lymphocyte responsiveness. HPBLs were exposed to LTX or LME (10 mM), washed, and then cultured with ConA. Macrophages (Mϕ) were added back to 20% of the total cells. The results represent the mean of quadruplicate cultures of a typical experiment.

DISCUSSION

Microbial virulence may be the consequence of their ability to resist, escape, or subvert host defense mechanisms. In regards to the last, several microorganisms are capable of suppressing the immune response through various products, including toxins, enzymes, cell wall components, and metabolites (for a review, see reference 22). Furthermore, several suspected periodontal pathogens are also capable of suppressing lymphocyte responses in vitro; these include *Treponema denticola* (27), *Fusobacterium nucleatum* (25), *Capnocytophaga ochracea* (5), *Centipeda periodontii* (24), several *Bacteroides* spp. (B. J. Shenker and J. Slots, J. Dent. Res. **66**:275, 1987), and *A. actinomycetemcomitans* (28, 29). *A. actinomycetemcomitans*, in particular, produces several factors that can subvert host defense mechanisms. For example, we have previously demonstrated that *A. actinomycetemcomitans* produces a factor capable of activating T suppressor cells, which in turn down-regulates lymphocyte responsiveness (29). Taichman and colleagues (1, 2, 16, 32, 37) have also demonstrated that many strains of *A. actinomycetemcomitans* produce a toxin capable of killing human PMNs and monocytes. These potential virulence factors have been shown to be separate entities, based on the following. (i) The monoclonal antibodies made against one factor are unable to modify the activity of the other factor. (ii) Both factors can be chromatographically separated. (iii) The immunosuppressive factor is found in all strains of *A. actinomycetemcomitans*, whereas LTX is only found in some strains. In this report, we have demonstrated that the LTX is capable not only of killing human monocytes but also of impairing lymphocyte responsiveness. Although part of this impairment is the result of monocyte depletion, the LTX also appears to have nonlethal effects on lymphocytes as well. LTX inhibition involves both B and T cells and is reflected in altered DNA, RNA, and protein synthesis, as well as immunoglobulin production. Lymphocyte viability was not altered. Only partial responsiveness was restored when autologous monocytes were added to LTX-treated cells. Furthermore, effects on lymphocytes do not appear to be the result of products released from dying monocytes and, thereby, influencing innocent bystander cells. This was demonstrated by comparing the effects of LTX and the lysosomotropic agent, LME; this agent has previously been shown to selectively kill monocytes, rendering lymphocytes unresponsive to B- and T-cell stimulants (36). Although both agents killed virtually all monocytes present in our HPBL populations and decreased lymphocyte proliferation, the responsiveness of the LME-treated cells was fully restored by the addition of monocytes, whereas cells exposed to LTX remained impaired. It is interesting that PHA responses were minimally affected by LTX treatment and were fully restored by the addition of monocytes. Although PHA and ConA may stimulate slightly different human T-cell subpopulations, the responses to both mitogens have been shown to be dependent upon monocytes (36). These findings, therefore, suggest

that LTX does not directly affect all lymphocytes but rather a subset of these cells. The latter finding also indicates that these observations are not due to the carry-over of LTX into the cultures, resulting in killing of the replenished monocytes. At this time, it is premature to speculate on the mode of action by which LTX alters lymphocyte responsiveness.

Microbial products represent an important source of immunoregulatory agents, some of which may affect the immune response via several different mechanisms that interfere with either the induction or expression of immunity. For example, streptococcal products inhibit bone marrow stem cell maturation (10), whereas cholera toxin interacts with mature cells (42). The latter results in suppression of T-cell effector populations in vitro and in vivo and is associated with the activation of adenylate cyclase and intracellular accumulation of cyclic AMP (30). On the other hand, products of *Corynebacterium parvum* and *T. denticola* affect macrophages, provoking the release of mediators capable of inhibiting lymphocyte function (17, 27). T regulatory cells are also a potent target for microbial modulation of the immune response. *A. actinomycetemcomitans* has been shown to selectively, but nonspecifically, activate human T suppressor cells (28, 29), whereas human immunodeficiency virus infects and destroys T helper cells (45). Other bacterial products have been implicated in impairing macrophage function and blocking or preventing receptor expression (43).

In summary, recent studies suggest a strong association between *A. actinomycetemcomitans* infection and the etiology of juvenile periodontitis (46). Although the immunologic mechanism(s) involved in periodontal disease is not clearly defined, there is substantial evidence that impaired host defense mechanisms may contribute to the disease process. *A. actinomycetemcomitans*, in particular, may be able to upset antibacterial defense mechanisms in the gingival crevice. Several in vitro studies, including the present investigation, indicate that this organism can kill human peripheral blood and gingival crevice PMNs and monocytes, as well as adversely affect immune functions of human lymphocytes. Hence, bacterium-derived immunosuppressive and leukotoxic factors may contribute to the pathogenesis of periodontal disease. Such factors could lead to a state of hyporesponsiveness that favors colonization by the initiating organism or by other opportunistic organisms.

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