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Immunosuppressive Effects of *Centipeda periodontii*: Selective Cytotoxicity for Lymphocytes and Monocytes

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We have examined soluble sonic extracts prepared from several strains of *Centipeda periodontii* for their ability to alter human lymphocyte function. These organisms were isolated from subgingival plaque of patients with periodontal disease. We found that sonicates from several, but not all, strains of *C. periodontii* caused a dose-dependent inhibition of lymphocyte responsiveness to concanavalin A, phytohemagglutinin, pokeweed mitogen, and formalinized *Staphylococcus aureus*. Inhibition was associated with a concomitant decrease in cell viability assessed by trypan blue exclusion, ^{51}Cr release, and electron microscopy. The maximal number of dead cells was observed 20 to 24 h after exposure to the sonic extract. Susceptible cells include human lymphocytes (both B and T), monocytes, and erythrocytes, whereas polymorphonuclear cells, murine L-929 fibroblasts, and sheep erythrocytes were not affected. Preliminary characterization of the cytotoxic activity indicates that it is heat labile and trypsin sensitive and has an M_r of 60,000. It has been proposed that impaired host defense may play a pivotal role in the pathogenesis of periodontal diseases. The data presented in this paper suggest that immunosuppression (local or systemic or both) could be initiated by *C. periodontii*. This immunosuppression may alter the nature and consequences of host-parasite interactions, thereby enhancing the pathogenicity of *C. periodontii* itself or some other opportunistic organism.

Microbial virulence may sometimes be the consequence of their ability to resist, escape, or subvert host defense mechanisms. The ability of microorganisms 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 references 40 and 58). Such modulation of the immune response may be a critical event in the outcome of numerous infections including measles (2), rubella (18, 31), influenza (16), leprosy (14, 26), candidiasis (9, 35), leishmaniasis (33, 41), trypanosomiasis (23), cryptococcosis (28), tuberculosis (17, 19), and syphilis (29, 50), among others. Perhaps the most prominent example of this relationship between host and pathogen is acquired immune deficiency syndrome, where the etiologic agent, human immunodeficiency virus, infects and destroys a subpopulation of T lymphocytes.

Although immunologic dysfunction may be associated with the development of many diseases, the nature of the contribution of the immune system to the pathogenesis of periodontal disease is poorly understood. For example, several studies suggest that the immune response may have undesirable effects, contributing to the disease process by several different immunopathologic mechanisms (3, 8, 12, 25, 32, 53). On the other hand, there is a vast literature supporting the view that the immune system plays a beneficial role in protecting or limiting bacterial infection (7, 15, 21, 38, 39). As a logical extension of the latter view, it is possible and reasonable to propose that immune dysfunction (local or systemic or both) may contribute to the susceptibility to and progression of periodontal disease. It is in this context that we have initiated a series of investigations to evaluate the immunomodulatory potential of suspected periodontal pathogens (43, 45–47). In this study we have examined *Centipeda periodontii* for its ability to alter human lymphocyte responsiveness in vitro. *C. periodontii* is a large, anaerobic, motile, rod-shaped, gram-negative bacterium with numerous flagella that was recently isolated and characterized from subgingival lesions of patients with chronic periodontitis and juvenile periodontitis (20, 24). Although little is known about the pathogenic potential of this organism, it is frequently obtained from samples taken from diseased sites and not from samples of healthy sites or from healthy individuals. We have found that soluble sonic extracts of several strains, but not all, inhibit human peripheral blood lymphocyte (HPBL) activation to both mitogens and antigens. More importantly, inhibition appears to be the result of cytotoxic events that occur over a relatively long period of time (20 to 24 h) and are limited to killing and lysis of lymphocytes, monocytes, and human erythrocytes. It is reasonable to propose that if this organism acts in vivo as it does in vitro, subversion of the immune system could result in the enhanced pathogenicity of *C. periodontii* itself or that of other opportunistic organisms.

MATERIALS AND METHODS

Bacterial strains and culture conditions. Isolates were obtained from subgingival plaque samples of patients with adult periodontitis and localized juvenile periodontitis. The following strains were kindly provided by C. H. Lai at the University of Pennsylvania: LL2381, LL2383 (ATCC 35019), LL2465, LL2467, LL2021, LL2022, and LL2441. The isolation and characterization of these strains have been previously described (20). All strains were maintained by weekly subculture on blood agar plates. Mass cultures were grown anaerobically in supplemented brain heart infusion (24). Cells were harvested by centrifugation and washed two times in saline.

Bacterial fractions were prepared as previously reported (43). Briefly, bacterial cells harvested from 1-liter cultures were washed, suspended in 20 ml of phosphate-buffered saline, and disrupted by sonication at 4°C. Unbroken cells

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were removed by centrifugation at 12,000 × *g* for 10 min, and the membrane fraction was sedimented at 85,000 × *g* for 60 min. The protein that remained in suspension after high-speed centrifugation was designated the soluble extract (SE) and contained both cytoplasmic and periplasmic proteins. The SE was extensively dialyzed against phosphate-buffered saline followed by RPMI 1640, and the protein concentration was determined by the Bio-Rad assay (Bio-Rad Laboratories, Richmond, Calif.) with bovine serum albumin as a standard (5).

Isolation and preparation of blood cells. HPBL were prepared as described previously (43). Briefly, HPBL 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.). HPBL were then isolated by buoyant density centrifugation on Ficoll-Hypaque (LSM, Litton-Bionetics, Kensington, Md.) by the method of Boyum (4). The HPBL were washed twice with HBSS and diluted to 2×10^6 or 5×10^6 viable cells per ml of culture medium consisting of RPMI 1640 (GIBCO Laboratories, Grand Island, N.Y.) with 10 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid buffer (Calbiochem, La Jolla, Calif.), 2% heat-inactivated pooled human AB serum, 2 mM L-glutamine, 100 U of penicillin per ml, and 100 μg of streptomycin per ml. Viable cell counts were performed by assessing trypan blue exclusion.

Erythrocytes were obtained by collecting 5 ml of blood as described above. The blood was centrifuged at 300 × *g* for 10 min, and the buffy coat was removed. This was repeated two times, and the cells were suspended to 10^8 cells per ml of phosphate-buffered saline.

Purified populations of lymphocytes and monocytes were obtained by counterflow centrifugal elutriation as described by Wahl et al. (57) and modified in our laboratory. Briefly, mononuclear cells (lymphocytes and monocytes) were obtained as described above. Samples of 5×10^8 to 10×10^8 cells were placed in 20 ml of HBSS (Ca^{2+} and Mg^{2+} free) containing 0.5% bovine serum albumin and pumped into a Beckman elutriator rotor (JE-6 rotor equipped with a standard chamber; Beckman Instruments, Inc., Fullerton, Calif.) at a flow rate of 7 ml/min with a rotor speed of 1,960 rpm. This initial flow rate allows for stratification of the mononuclear cells in the horizontal chamber according to size and density while any contaminating platelets are eluted. Cells were then eluted by sequential increases in the flow rate; one fraction of 150 to 200 ml was eluted at each flow rate. Lymphocytes were eluted at flow rates of 7 to 12 ml/min, whereas monocytes were eluted at 12.5 to 14 ml/min. All fractions were counted and monitored for their size profile on a model ZBI Coulter Counter connected to an IBM-PC computer with appropriate hardware and software (Personal Computer Analyzer; Nucleus Inc., Oak Ridge, Tenn.) to allow for size distribution analysis. Cell purity was assessed by (i) morphologic appearance, (ii) nonspecific esterase stain, and (iii) insolubilized monoclonal antibodies (Quantigen assay; Bio-Rad). The lymphocyte preparations were routinely 96 to 98% pure and the monocytes were 85 to 95% pure by these criteria.

Purified populations of B cells and T cells were obtained by E-rosette formation as described by Madsen et al. (22). Briefly, sheep erythrocytes were washed and treated with 0.14 M 2-aminoethylisothiouronium bromide (pH 9) for 15 min. After four washes, the sheep erythrocytes were incubated with 1.0×10^7 lymphocytes (obtained as described above by counterflow centrifugal elutriation) for 60 min as a cell pellet. The cells were then gently suspended and separated in Ficoll-Hypaque as described above. Nonrosetted cells found at the interface were 90 to 95% B cells, and the rosetted cells found in the pellet were lysed to remove sheep erythrocytes and found to contain >95% T cells.

Assays of mitogen- and antigen-induced proliferation. HPBL suspension (0.1 ml) containing 2×10^5 cells was placed into each well of flat-bottomed microculture plates (Falcon 3072; Becton Dickinson Labware, Oxnard, Calif.). Cultures received 0.1 ml of medium (as described above) or various concentrations of the *C. periodontii* SE diluted in medium and sterilized through a 0.22-μm filter (Schleicher & Schuell Co., Keene, N.H.). The cells were then incubated for 15 min (or as indicated) at 37°C, at which time the cultures received an optimal mitogenic dose of either concanavalin A (ConA, 1 μg per culture; Calbiochem), phytohemagglutinin (2 μg per culture; Wellcome Research Laboratories, Beckenham, England), pokeweed mitogen (2 μg per culture; Calbiochem), or formalinized *Staphylococcus aureus* (0.25 μg of cells per culture, Pansorbin; Calbiochem) in a volume of 25 μl. The cells were incubated for 96 h (120 h for *S. aureus*) at 37°C in humidified air containing 5% CO_2.

Assays for antigen-induced proliferation were set up as described above, except that cultures received 10^6 cells and 10 ng of tetanus toxoid and were incubated for 6 days.

HPBL proliferation (DNA synthesis) was assessed by the incorporation of tritiated thymidine ([^{3}H]TdR). Cell cultures were incubated as described above, and 0.25 μCi of [^{3}H]TdR (specific activity, 42 Ci/mmol; Amersham Corp., Arlington Heights, Ill.) was added for the last 4 h. The cultures were then harvested onto glass fiber filter paper with an automated cell harvester (model PhD; Cambridge Technology, Cambridge, Mass.). [^{3}H]TdR incorporation was determined by counting in a Beckman LS 3800 liquid scintillation spectrometer. Incorporation into cells exposed to bacterial extract was expressed as a percentage of incorporation observed in control cultures receiving mitogen or antigen alone.

Cell viability. Viable cell counts were performed by assessing trypan blue exclusion in cells incubated, as indicated above, for various periods of time. Viability in cultures exposed to SE is expressed as a percentage of the viability in the appropriate control cultures.

Chromium release assay. SE cytotoxicity was assessed in several cells, including B cells, T cells, monocytes, polymorphonuclear cells (PMN), L-929 cells, and human and sheep erythrocytes, by determining extracellular release of ^{51}Cr from labeled cells. PMN and the murine L-929 cell line were prepared as previously described (44, 46). Cells (1×10^7 to 5×10^7) were labeled (50 min, 37°C) with 0.5 mCi of ^{51}Cr (as sodium chromate; New England Nuclear Corp., Boston, Mass.) in a total volume of 1.0 ml of HBSS, washed two times in HBSS (with 10-min incubations at 37°C between washes) to remove free label and then adjusted to 10^6 cells per ml in RPMI 1640. Cells (0.1 ml) were placed into each well of a 96-well round-bottomed microculture plate followed by the addition of 0.1 ml of RPMI 1640 (control) or SE. The plates were incubated for 24 h (or as indicated) at 37°C. At the end of the incubation period, the plates were centrifuged (250 × *g*, 5 min) and 0.1 ml of culture supernatant was removed, placed in a glass tube (10 by 75 mm), and counted in a Beckman Gamma 5500 spectrometer. ^{51}Cr release in cells exposed to SE was expressed as a percentage of the control, i.e., the amount released from cells exposed

to 0.1% Triton X-100. Background release from labeled control cells exposed to RPMI 1640 ranged from 5 to 15%.

Electron microscopy. Cells examined in this study by transmission electron microscopy were fixed with 2% (wt/vol) osmium tetroxide in Tyrode solution. After fixation for 1 h, the cells were washed and rinsed in Tyrode solution for 30 min (4°C); the cell pellet was dehydrated in ethanol and then embedded in Epon (Luft 196X). Ultrathin sections were prepared with an LKB Ultrotome III (LKB, Bromma, Sweden) and placed on Formvar-carbon coated one-hole copper grids. The sections were double contrasted with uranyl acetete (6) and lead citrate (34). All preparations were examined in a Philips EM300 electron microscope operated at 80 kV.

Ion-exchange and gel-filtration chromatography. SE prepared from *C. periodontii* LL2383 were applied to a 1.5- by 15-cm DEAE-Sephacel (Pharmacia, Uppsala, Sweden) column equilibrated in 50 mM Tris (pH 7.4). After extensive washing the column was eluted with a linear NaCl gradient (10 mM to 1 M NaCl). Alternate fractions were tested for both cytotoxic and lymphocyte-suppressive activities as described above. Active fractions were pooled, concentrated on Diaflo PM-10 membranes (Amicon Corp., Lexington, Mass.), and applied to an Ultrogel AcA-34 column (2.5 by 85 cm; LKB) that was equilibrated in 50 mM Tris containing 100 mM NaCl (pH 7.4) at 4°C. Alternate fractions were assayed for their ability to inhibit lymphocyte proliferation as well as kill lymphocytes (^{51}Cr release).

RESULTS

Effect of *C. periodontii* SE on HPBL responsiveness. SE of several strains of *C. periodontii* were first examined for their ability to alter ConA-induced lymphocyte activation. *C. periodontii* LL2383, LL2381, LL2465, and LL2467 caused a dose-dependent inhibition of ConA-induced [^{3}H]TdR incorporation (Fig. 1); doses causing a 50% reduction in [^{3}H]TdR incorporation (ID_{50}s) were as follows (micrograms of protein per milliliter): LL2021, 257.7; LL2381, 72.9; LL2383, 64.5; LL2465, 156.0; LL2467, 72.9. These values were determined

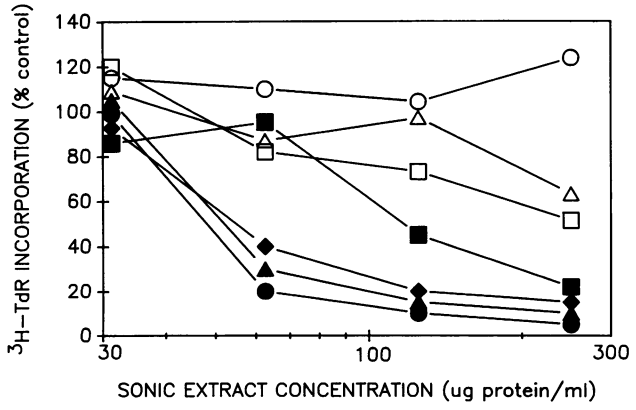

FIG. 1. Effect of *C. periodontii* SE on the proliferative response of HPBL to ConA. HPBL were incubated with SE prepared from several strains of *C. periodontii*: LL2021 (□), LL2022 (△), LL2381 (◆), LL2383 (●), LL2441 (○), LL2465 (■), and LL2467 (▲). After a 15-min incubation with SE, an optimal mitogenic dose of ConA was added. Results are plotted as percentages of [^{3}H]TdR incorporation observed in control cultures receiving mitogen alone (45,260 cpm). Each point represents the mean value of three experiments each performed in quadruplicate culture; standard errors were within 5% of the mean.

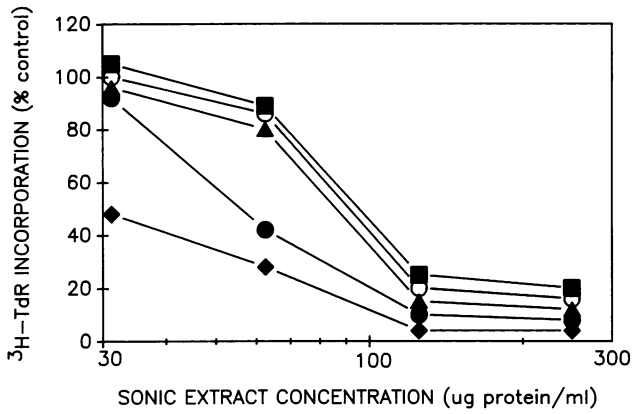

FIG. 2. Effect of *C. periodontii* on the proliferative response of HPBL to both mitogens and antigens. HPBL were incubated with SE of strain LL2383 for 15 min followed by the addition of an optimal mitogenic dose of ConA (●), phytohemagglutinin (▲), pokeweed mitogen (■), *S. aureus* (◆), or an optimal antigenic dose of tetanus toxoid (○). Results are plotted as percentages of [^{3}H]TdR incorporation in control cultures receiving mitogen or antigen alone. Each point represents the mean value of quadruplicate cultures in each of three experiments; standard errors were within 5% of the mean. Net incorporation in control cultures was 34,603 cpm (ConA), 65,258 cpm (phytohemagglutinin), 50,506 cpm (pokeweed mitogen), 8,541 cpm (*S. aureus*), and 7,592 cpm (tetanus toxoid).

by linear regression analysis of the data in Fig. 1. In strains LL2022 and LL2441 the inhibition, if any, was too minimal to calculate an ID_{50}. *C. periodontii* LL2021 and LL2022 demonstrated only marginal suppression, whereas strain LL2441 exhibited no inhibitory effect at comparable SE concentrations. Similar suppression was observed on ConA-induced protein ([^{3}H]leucine incorporation) and RNA ([^{3}H]uridine incorporation) synthesis (data not shown). Because strain LL2383 demonstrated the most potent inhibitory activity, SE of this strain along with the noninhibitory strain LL2441 (as a control) were employed in the majority of subsequent experiments.

We wanted to determine whether the lymphocyte-suppressive effect was limited to ConA activation of HPBL or extended to other mitogenic as well as antigenic responses. Figure 2 shows the inhibition by strain LL2383 SE of HPBL activation by ConA (T-cell), phytohemagglutinin (T-cell), pokeweed mitogen (T- and B-cell), and formalinized *S. aureus* (B-cell) mitogens. The responses to ConA, phytohemagglutinin, and pokeweed mitogen were similarly inhibited in a dose-dependent fashion, with inhibition ranging from 5 to 85%. *S. aureus* responsiveness was consistently more sensitive to this inhibitory effect (50 to 95% inhibition); this suggests a difference in sensitivity between B and T lymphocytes. Inhibition was observed regardless of the concentration of the mitogen (optimal, suboptimal, or supraoptimal; data not shown). Additionally, pokeweed mitogen-induced production of both immunoglobulin G and immunoglobulin M were equally depressed at comparable concentrations of SE (data not shown). SE from strain LL2441 failed to inhibit any of these responses. Because there is some controversy as to the immunologic relevance of mitogen-induced blastogenesis, we also tested strain LL2383 SE for its ability to alter specific antigenic responses. Responsiveness to tetanus toxoid was suppressed in a dose-dependent fashion, with values similar to those observed with the mitogens (Fig. 2). In addition to soluble

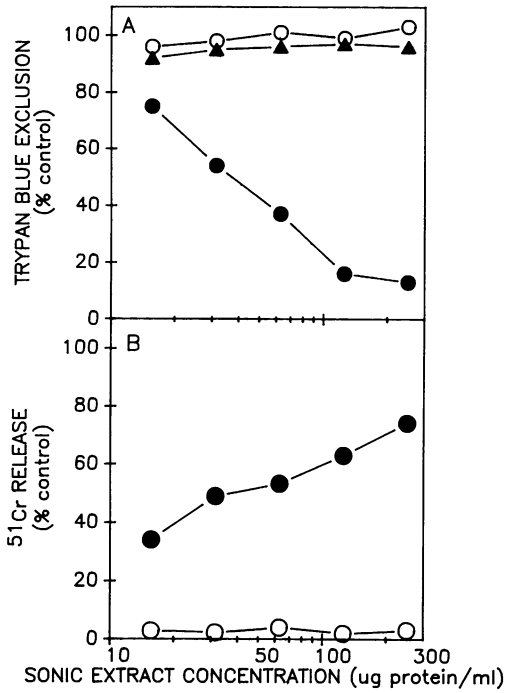

FIG. 3. Cytotoxic effect of *C. periodontii* on HPBL. HPBL were incubated with various doses of *C. periodontii* SE (in the absence of mitogen) and then evaluated for cytotoxic effects. (A) Effects on trypan blue exclusion after exposure to LL2383 SE for 1 h (▲) and 24 h (●) and to LL2441 SE for 24 h (○). The data are expressed as percentages of the viability observed in control cultures receiving medium only. (B) Effects on ^{51}Cr release after exposure to strains LL2383 SE (●) and LL2441 SE (○) for 24 h. The data are expressed as percentages of the total available ^{51}Cr released by 0.1% Triton X-100. Each point represents the mean value of quadruplicate cultures of a representative experiment. Standard errors were within 5% of the mean.

antigens, proliferation in response to allogeneic stimulation (mixed lymphocyte reaction) was also depressed (data not shown).

Cytotoxic effects of *C. periodontii* SE. The results reported above clearly indicate that both B- and T-cell responses are depressed by some strains of *C. periodontii* regardless of the mode by which the cells are stimulated. Therefore, any mode of action accounting for these effects would most likely involve target sites or pathways common to activation of both classes of lymphocytes. We first wanted to ascertain that the inhibitory effects were not due to altered cell viability. When cells were exposed to LL2383 SE for 1 h, there was no effect on cell viability as assessed by trypan blue exclusion (Fig. 3A). However, we had also carried out a series of kinetic studies to determine the conditions of maximal suppression of [^{3}H]TdR incorporation. To summarize these results (data not shown), we found that SE had to be present for the first 24 h and, further, that the effect was reversible at 1 and 3 h but irreversible at 24 h. Based on these results, we repeated the viability assays after 24 h of exposure to SE; there was a dose-dependent reduction in cell viability at this time (Fig. 3A). The dose dependency was similar to that observed for inhibition by [^{3}H]TdR incorporation. Strain LL2441 had no effect on cell viability. We confirmed the cytopathic effect by utilizing a ^{51}Cr release assay in which HPBL were preloaded with the radioisotope and then exposed to SE; release of ^{51}Cr was then measured and expressed as a percentage of the total release (with Triton X-100). As observed with trypan blue, a dose-dependent release of ^{51}Cr was observed at 24 h in the presence of LL2383 SE but not with LL2441 SE (Fig. 3B). Furthermore, 49.6 μg of LL2383 SE caused a 50% reduction in cell viability as measured by the ability to exclude trypan blue, and 59.4 μg resulted in 50% ^{51}Cr release; these values are comparable to the ID_{50} observed for the inhibition of [^{3}H]TdR incorporation (see above). We also examined the time course of ^{51}Cr release; maximal release occurred between 20 and 24 h (Fig. 4). Control cultures exposed to media or strain LL2441 failed to cause significant release of ^{51}Cr at this time. This finding is in agreement with our kinetic analyses for inhibition of HPBL proliferation. Therefore, it is reasonable to assume that the immunoinhibitory effect of *C. periodontii* is most likely due to the cytopathic ability of this organism.

We also verified these cytopathic effects by electron microscopy. Cells exposed to strain LL2383 SE displayed various stages of disintegration (Fig. 5). These include a lack of mitochondria, ruptured cytoplasmic membranes, vacuolization of the cytoplasm, and finally complete disintegration of the cell. The number of cells exhibiting these cytopathic alterations as well as the severity of destruction increased with exposure time. After 8 h of exposure, few affected cells could be found (Fig. 5A), whereas at 20 and 24 h practically all cells exhibited these alterations (Fig. 5B). These results paralleled our observations with ^{51}Cr release. Control HPBL (exposed to either medium or strain LL2441 SE) appeared healthy at all times, with intact solitary ribosomes, mitochondria, and cytoplasmic and nuclear membranes.

We also examined *C. periodontii* LL2383 SE for target cell specificity. Since our standard HPBL preparations contain not only lymphocytes but also monocytes (approximately 15 to 30%, depending on the individual donor and preparation), we first compared purified lymphocytes and monocytes for susceptibility to *C. periodontii*-mediated ^{51}Cr release. The susceptibilities to the cytotoxic effects of strain LL2383 of both purified populations of cells (lymphocytes and monocytes) were similar to that of the unfractionated HPBL preparation (Fig. 6). We also compared the sensitivity of purified B- and T-cell preparations (Fig. 6B). As observed

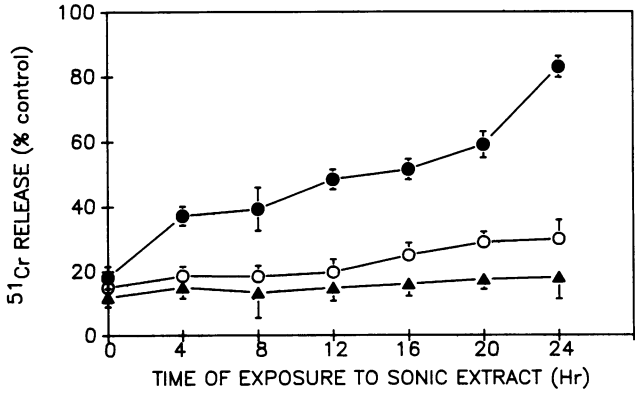

FIG. 4. Kinetics of ^{51}Cr release. HPBL were exposed to 100 μg of strain LL2383 SE (●) or strain LL2441 SE (○) per ml or to medium (▲) for various periods of time and then evaluated for ^{51}Cr release. The data are expressed as percentages of the total available ^{51}Cr released in the presence of 0.1% Triton X-100. Each point represents the mean ± standard deviation of quadruplicate cultures of a representative experiment.

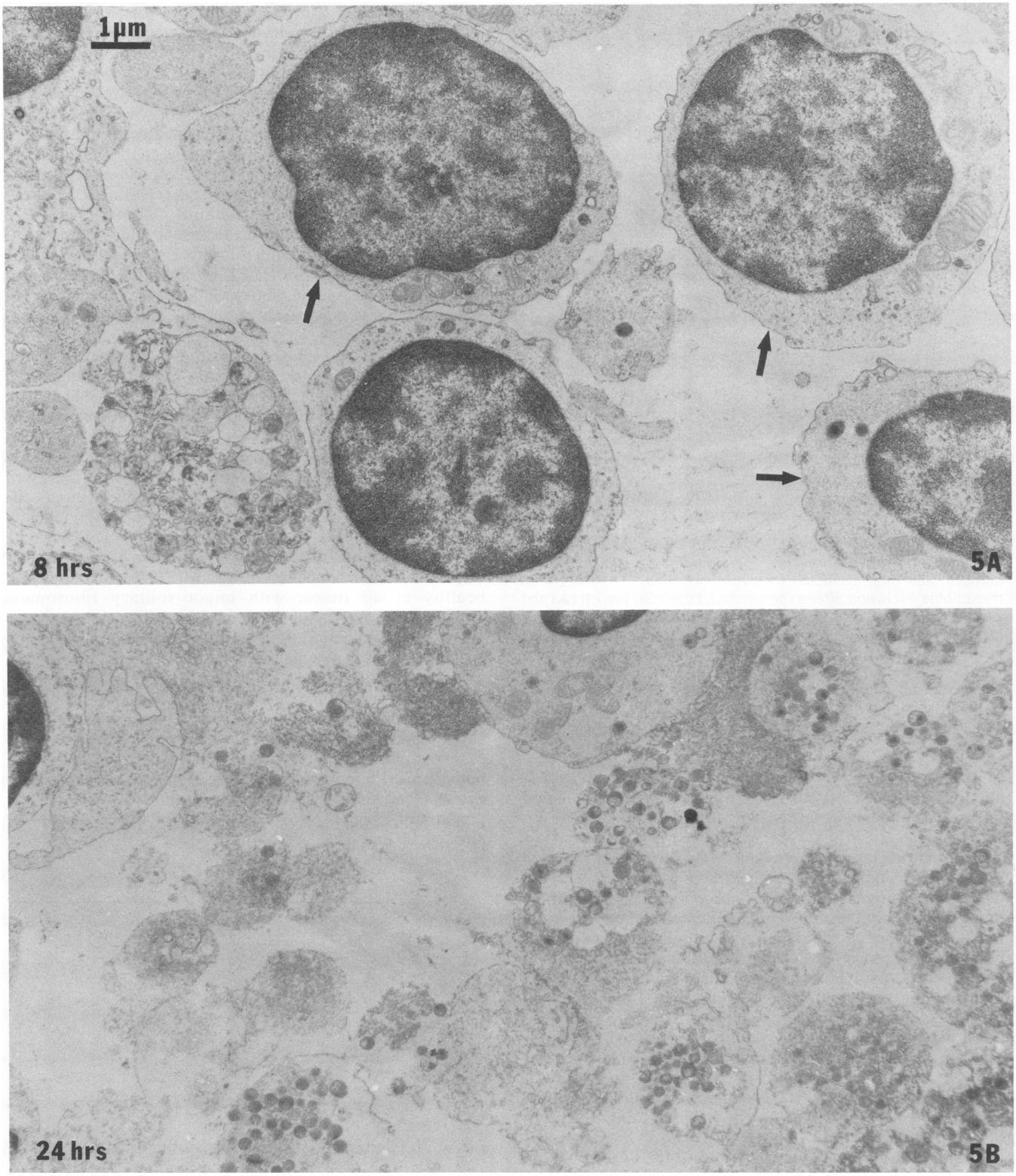

FIG. 5. Electron microscopic evaluation of HPBL exposed to *C. periodontii* LL2383 SE (100 μg/ml) for 8 h (A) and 24 h (B). Magnification, ×10,000. At 8 h, most HPBL show a healthy ultrastructural morphology (arrows). After 24 h of exposure, the vast majority of the cells show extensive cytopathic alterations, with few healthy cells present.

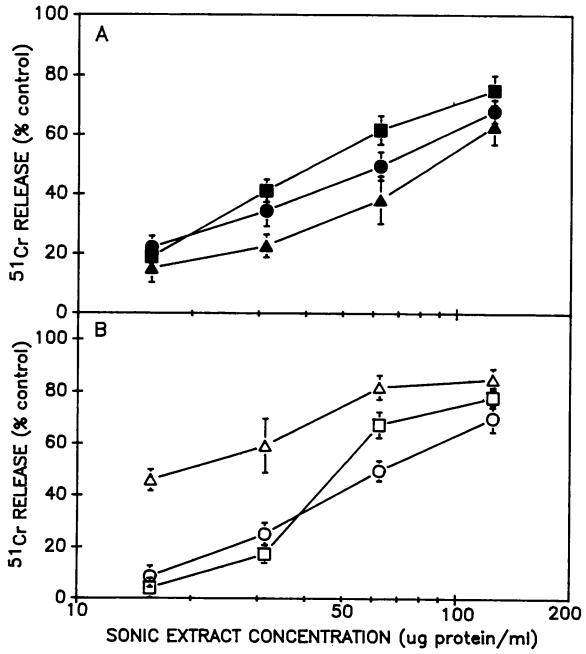

FIG. 6. Effect of *C. periodontii* on purified cell populations. Different cell populations were preloaded with ^{51}Cr and then compared for susceptibility to the cytotoxic effect of *C. periodontii* LL2383 SE after 24 h. (A) Results obtained with unfractionated HPBL (●), purified lymphocytes (▲), and monocytes (■). (B) Results from purified lymphocytes (○), purified T cells (□), and purified B cells (△). Each point represents the mean ± standard error of quadruplicate cultures of a representative experiment.

with the effects on cell proliferation (Fig. 2), B cells appear to be more sensitive to the cytotoxic effects of strain LL2383 SE than do T cells. Strain LL2441 SE failed to demonstrate any cytotoxicity on any of these cells. On the other hand, strain LL2383 SE failed to alter the viability of either human PMN or the murine L-929 fibroblast cell line (Table 1), although it did inhibit proliferation of the latter. Furthermore, human erythrocytes, but not sheep erythrocytes, were lysed by LL2383 SE at concentrations comparable to those that kill lymphocytes.

Partial characterization of the cytotoxic factor. Preliminary studies have been carried out to determine the physicochemical characteristics of the *C. periodontii* cytotoxic factor. The cytotoxic activity was sensitive to temperature, with partial activity destroyed at 56°C and total activity destroyed at 75°C after a 30-min exposure. Additionally, the cytotoxic activity was destroyed after exposure to trypsin. The crude SE was fractionated sequentially by ion-exchange (DEAE-Sephacel) and gel-filtration (ACA-34) chromatography. All the activity bound to the DEAE-Sephacel column and could be eluted with NaCl (250 mM). Figure 7 shows the elution profile from the ACA-34 column; cytotoxic activity appeared in a well-defined peak corresponding to an M_r of approximately 60,000. This partial purification resulted in a 50-fold increase in specific activity over the crude SE. It should be noted that both activities, i.e., cytotoxicity and suppression of HPBL proliferation, were found to coelute from both columns; this is further confirmation of the relationship between these two biologic phenomena. Further purification of the cytotoxic factor is currently being investigated.

TABLE 1. Effects of *C. periodontii* sonic extract on nonlymphoid cells

SE concn (μg/ml)	^{51}Cr release[a]			
	PMN	L-929	hRBC	sRBC
31.2	4.9 ± 0.1	0.5 ± 0.1	62.0 ± 2.3	5.4 ± 2.0
62.5	4.5 ± 0.2	0.5 ± 0.1	78.2 ± 3.6	2.8 ± 2.3
125.0	4.5 ± 0.1	1.0 ± 0.3	82.1 ± 3.9	1.3 ± 0.5
250.0	4.0 ± 0.4	3.8 ± 0.4	85.7 ± 2.7	4.9 ± 1.7

[a] Cell cultures were established as described in Materials and Methods. Cells were incubated with and without various concentrations of SE for 24 h and then assessed for ^{51}Cr release. The data are expressed as a percentage of the total release caused by Triton X-100 and represent the means ± standard errors of quadruplicate cultures of a representative experiment. hRBC and sRBC, Human and sheep erythrocytes, respectively.

DISCUSSION

Enhanced susceptibility to infectious disease is a commonly acknowledged complication in patients with overt primary and secondary immunodeficiencies (11, 36, 37). Less widely appreciated, however, are the observations of immunologic dysfunction as sequalae to microbial infection in an otherwise healthy person. There are several mechanisms by which microbial pathogens evade or compromise the immune response of the host. These may include, for

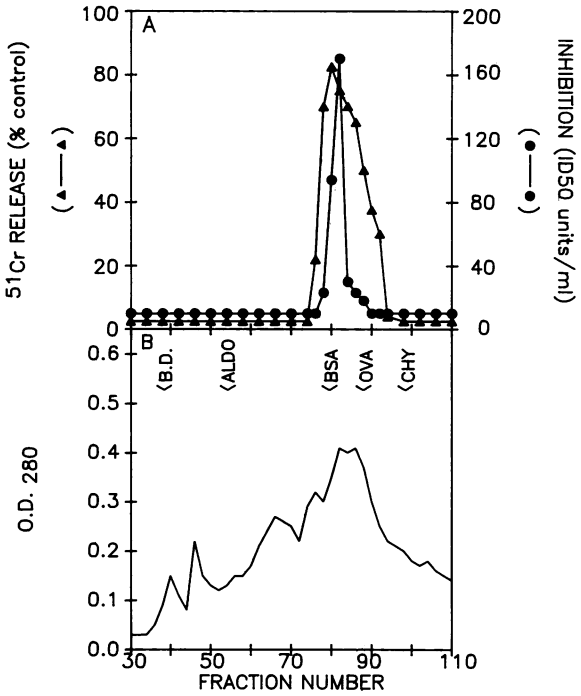

FIG. 7. Gel filtration chromatography of strain LL2383 SE. SE was prepared from 1 liter of strain LL2383 and first chromatographed on a DEAE-Sephacel column as described in Materials and Methods. The active fractions were pooled, concentrated, and chromatographed by gel filtration chromatography (ACA 34). (A) Alternate fractions were assayed for their ability to inhibit HPBL proliferation in response to ConA (●) and to cause ^{51}Cr release (▲). The ^{51}Cr release data are plotted as percentages of the total release caused by Triton X-100. Inhibition data are plotted as ID_{50} units per ml of column fraction, where an ID_{50} unit is defined as the amount (microliters) required to cause a 50% reduction in [^{3}H]TdR incorporation. (B) Profile of optical density at 280 nm along with M_r markers: B. D., blue dextran; ALDO, aldolase; BSA, bovine serum albumin; OVA, ovalbumin; and CHY, chymotrypsin.

example, antigenic drift, which results in antigenic variants that can reinfect or persist within a person. Alternatively, microbial pathogens may produce virulence factors that aggressively subvert the immune response by causing an immunosuppressed state in the host. Such immunosuppression may occur via subtle mechanisms such as the production of immunoglobulin proteases which destroy "antibacterial" antibodies (10). At the other extreme, microorganisms may produce factors that interfere or perturb immunoregulatory functions (40, 58). These factors ultimately prevent the development or maturation of an immune response. The net result of these interactions is to provide the organism (or possibly other opportunistic organisms) with an ecological advantage in colonization.

In the present study, we have evaluated *C. periodontii* for its ability to alter HPBL responsiveness. We have demonstrated that several strains of this organism (four out of seven) can suppress HPBL proliferative responses to both mitogens and antigens as well as immunoglobulin production. The suppression is dose dependent and involves altered DNA, RNA, and protein synthesis. More importantly, suppression was associated with altered cell viability. Cell death was assessed by both trypan blue exclusion and ^{51}Cr release; the cytopathic effects were confirmed by electron microscopy. Maximum cell death occurred 20 to 24 h after exposure to the bacterial extract. In addition to lymphocytes (both B and T) and monocytes being susceptible to this cytotoxic factor(s), human erythrocytes underwent hemolysis after 24 h of exposure, but not after 1 to 3 h. Human PMN, murine L cells, and sheep erythrocytes were not susceptible to these toxic effects. Preliminary characterization of this cytotoxic activity indicates that it has an M_r of approximately 60,000, is trypsin sensitive, and is heat labile with activity being destroyed at 75°C.

Many microbial species have been shown to produce protein toxins capable of killing susceptible host cells. Although the cellular mechanisms of action for many of these toxins have not been elucidated, there are some general patterns of activity that allow for classification into two groups (reviewed in references 1 and 27). First, there are those bacterial toxins that may be grouped as membrane-damaging cytolysins. These toxins damage cell membranes so that leakage of intracellular constituents occurs, and this ultimately results in cell lysis and death. Examples of bacteria that produce cytolytic toxins are *Clostridium perfringens*, *Staphylococcus aureus*, and *Streptococcus pyogenes*. Generally, membrane damage appears to be due to one of the following: (i) hydrolysis of membrane phospholipids, (ii) binding and sequestration of cholesterol, or (iii) detergentlike action (1). The second group of bacterial protein toxins act intracellularly. These toxins, generally, bind to specific receptors on the plasma membrane of susceptible cells, translocate across the membrane barrier, and then interact with an intracellular target site (27). Examples of bacteria producing this type of toxin are *Corynebacterium diphtheriae*, *Pseudomonas aeruginosa*, *Shigella* sp., *Vibrio cholerae*, *Escherichia coli*, and *Bordetella pertussis*. Many of these toxins are ADP-ribosyl transferases (27). Although the intracellular target site varies, ADP ribosylation by many of these toxins results in the inhibition of protein synthesis and eventual cell death. The membrane-damaging cytolytic toxins usually cause cell death rapidly (within minutes to 1 h after exposure), whereas the intracellular acting toxins cause a slower death (4 to 24 h), although inhibition of protein synthesis may be first observed within minutes after toxin exposure. Although we have no direct evidence on the mechanism by which the *C. periodontii* toxin acts to kill cells, we do believe that its behavior is consistent with that of a toxin acting intracellularly. First, we do not observe extensive cell death until 16 to 24 h after exposure. Second, we have observed inhibition of protein synthesis in activated lymphocytes. This suppression not only reflects inhibition of activated or mitogen-induced protein synthesis, but also results in a reduction of protein synthesis ($[^3H]$leucine incorporation) to values below that observed in resting or control cells. This, however, does not explain the hemolytic effect on human erythrocytes. Further study is required to evaluate the association between toxin-induced inhibition of protein synthesis and cell death as to possible cause-and-effect relationships. These findings are in contrast to those with a leukotoxin produced by another suspected periodontal pathogen, *Actinobacillus actinomycetemcomitans* (51, 52). This leukotoxin has an M_r of 115,000 and is also trypsin and temperature sensitive (56). However, it kills human and monkey monocytes and PMN but not lymphocytes or erythrocytes. Furthermore, the *A. actinomycetemcomitans* toxin kills cells rapidly (within 30 min) and is inhibited by phospholipids, suggesting that it may act by mechanisms associated with that of membrane-damaging toxins (13).

Although *C. periodontii* is not a well known organism, it has been repeatedly isolated from subgingival lesions of patients with chronic and juvenile periodontitis and not from healthy persons or healthy sites of diseased persons (20). As reported in this paper, this organism produces a potential virulence factor that, if active in vivo, could have untoward effects on host defense mechanisms. Although the role of the immune system in periodontal disease is poorly defined, recent studies strongly suggest a role for altered host defenses as a possible underlying mechanism (30, 42, 48, 49, 54, 55). In this regard, bacterium-derived immunosuppressive or lymphotoxic factors may contribute to the disease process. Such bacterially derived immunosuppressive factors could lead to a state of hyporesponsiveness (perhaps only in the early stages of infection) that favors colonization by the initiating organism or by other opportunistic organisms. However, we do know that periodontal patients eventually develop antibodies to many (but not all) suspected oral pathogens. Perhaps, this is due to (i) the immune response of the host becoming refractory to the suppressive agents, (ii) changes in the microbial flora to other strains or species that do not produce such inhibitory agents, or (iii) the host developing the ability to neutralize these factors. In any case, once the immunoinhibitory agents are eliminated, a state of immunologic deficiency could then be followed by a vigorous but delayed immune response to microbial antigens; this may be a consequence of specific antigenic stimulation or of polyclonal cell activation. This active immune response may (i) eliminate the microorganisms and result in remission of disease, (ii) further contribute to the progression of disease through immunopathologic mechanisms, or (iii) because of antigenic overload be unable to adequately eliminate and protect the host. Therefore, the duration and extent of the delay in onset of a protective immune response may be critical to the establishment of infection and the progression of disease. Furthermore, this may help explain the contradictory findings of persistent infection in patients with peridontal disease in the face of an apparently strong immune response. In summary, although the exact role of the immune system to the pathogenesis of periodontal disease is not known, current findings suggest that its role is dynamic and may change with time.

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