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Studies of Leukotoxin from *Actinobacillus actinomycetemcomitans* Using the Promyelocytic HL-60 Cell Line

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The promyelocytic HL-60 cell line was examined for susceptibility to leukotoxin from *Actinobacillus actinomycetemcomitans*. Strains of *A. actinomycetemcomitans* which caused lysis of human peripheral blood polymorphonuclear leukocytes also lysed HL-60 cells as determined by release of intracellular lactate dehydrogenase. The killing of HL-60 cells by *A. actinomycetemcomitans* was dose dependent and temperature dependent, reached maximal levels after 45 min of incubation, and was inhibited by rabbit antisera to *A. actinomycetemcomitans*. Of 100 oral isolates of *A. actinomycetemcomitans* from 55 subjects, 16% from 11 healthy subjects, 43% from 13 adult periodontitis patients, 75% from 4 insulin-dependent diabetics, 66% from 2 generalized juvenile periodontitis patients, and 55% from 25 localized juvenile periodontitis patients produced leukotoxin. The same subject could harbor both leukotoxin-producing and -nonproducing isolates. The significantly higher proportion of leukotoxin-producing isolates in the disease groups compared with the healthy group is consistent with the hypothesis that leukotoxin from *A. actinomycetemcomitans* is an important virulence factor in the pathogenesis of certain forms of periodontal disease.

Actinobacillus actinomycetemcomitans is a gram-negative, capnophilic, fermentative coccobacillus which occurs in high numbers in most localized juvenile periodontitis lesions and much less frequently in adult periodontitis lesions (7, 10). This organism can elaborate a heat-labile, protease-sensitive factor which is toxic for human polymorphonuclear leukocytes (PMN) and monocytes in vitro but not toxic for human lymphocytes, platelets, or fibroblasts or for rabbit, rat, or mouse leukocytes (12, 14). Since PMN are key cells in defense against infectious agents, *A. actinomycetemcomitans* leukotoxin may be a significant virulence factor in localized juvenile periodontitis.

Detection and characterization of *A. actinomycetemcomitans* leukotoxin has been encumbered by the necessity of using fresh PMN from human peripheral blood. The collection of human PMN is a time-consuming procedure, only a limited number of PMN are available, and the PMN isolated from different individuals and from the same individual at different times may vary in leukotoxin susceptibility.

In the study of *A. actinomycetemcomitans* leukotoxin, continuously propagated myeloid cell lines may provide convenient target cells. The HL-60 cell line has been established from the peripheral blood of a patient with acute promyelocytic leukemia (4). These cells develop along the myelocytic lineage and maintain in culture many enzymatic and cell surface characteristics of mature granulocytes (4). We report here the feasibility of using the HL-60 line in the examination of *A. actinomycetemcomitans* leukotoxin and describe some factors which influence leukotoxin susceptibility. Also included is a study of the distribution of leukotoxin-producing *A. actinomycetemcomitans* in human periodontal disease.

MATERIALS AND METHODS

Bacterial strains. Test strains of *A. actinomycetemcomitans* included Y4, obtained from the Forsyth Dental Center, Boston, Mass., ATCC 29522, ATCC 29523, and ATCC 29524, obtained from the American Type Culture Collection (Rockville, Md.), and 100 human oral isolates (6 from oral soft tissue sites, 94 from microbial dental plaques, each from a single subgingival site) from our own culture collection. Strain Y4 has repeatedly been shown to produce leukotoxin to human PMN in vitro (2, 12, 14). Also included was *Haemophilus aphrophilus* ATCC 5908 as a representative of a closely related species which has been shown not to produce leukotoxin (2). All strains were grown in thioglycolate liquid medium (Difco Laboratories, Detroit, Mich.) to early stationary growth phase, harvested by centrifugation, washed three times in phosphate-buffered saline, pH 7.2, suspended in Hanks balanced salt solution (HBSS;

GIBCO Laboratories, Grand Island, N.Y.), and adjusted to an absorbance at 540 nm ranging from 0.3 to 1.0.

Target cell preparation. The HL-60 cell line was a gift of Robert Gallo, National Cancer Institute, Bethesda, Md. These cells were cultured in stationary flasks at 37°C under 5% CO_2–95% air, using RPMI 1640 medium (GIBCO) supplemented with 10% fetal bovine serum (Reheis Chemical Co., Phoenix, Ariz.), penicillin (50 IU/ml), and streptomycin (50 μg/ml). Before use, the HL-60 cells were washed three times in phosphate-buffered saline and resuspended in HBSS to a density of 10^7 cells per ml.

Fresh PMN were obtained from the peripheral blood of healthy human volunteers. After sedimentation in dextran and Ficoll-Hypaque density gradient centrifugation (15), the PMN were suspended in HBSS to a density of 10^7 cells per ml of HBSS. Final preparations contained 95 to 98% PMN; 95% of the PMN were viable as determined by trypan blue exclusion.

Assay for cytotoxicity. Bacterial cells were incubated in a water bath under constant shaking with HL-60 cells or PMN in HBSS at 37°C. The leukotoxic effect was suppressed by immersion on ice, and bacteria-target cell suspensions were centrifuged for 20 min at 600 × *g*. Controls included HL-60 cells, PMN, and bacteria, each incubated alone in HBSS.

The release of lactate dehydrogenase (LDH) was used to monitor cytotoxicity. As a first approximation, cytotoxic potency was considered to be directly proportional to the percentage of total cellular LDH released. LDH was assayed by a modification of the procedure of Berger and Broida (3), which depends on the formation of a colored phenylhydrazone as a measure of the amount of substrate (pyruvate) remaining in the standard assay procedure. Samples (50 μl) of serially diluted supernatants from cytotoxicity incubation mixtures were added to 0.1-ml aliquots of buffer-substrate, prepared according to Sigma Kit 500 (Sigma Chemical Co., St. Louis, Mo.), and incubated at 37°C

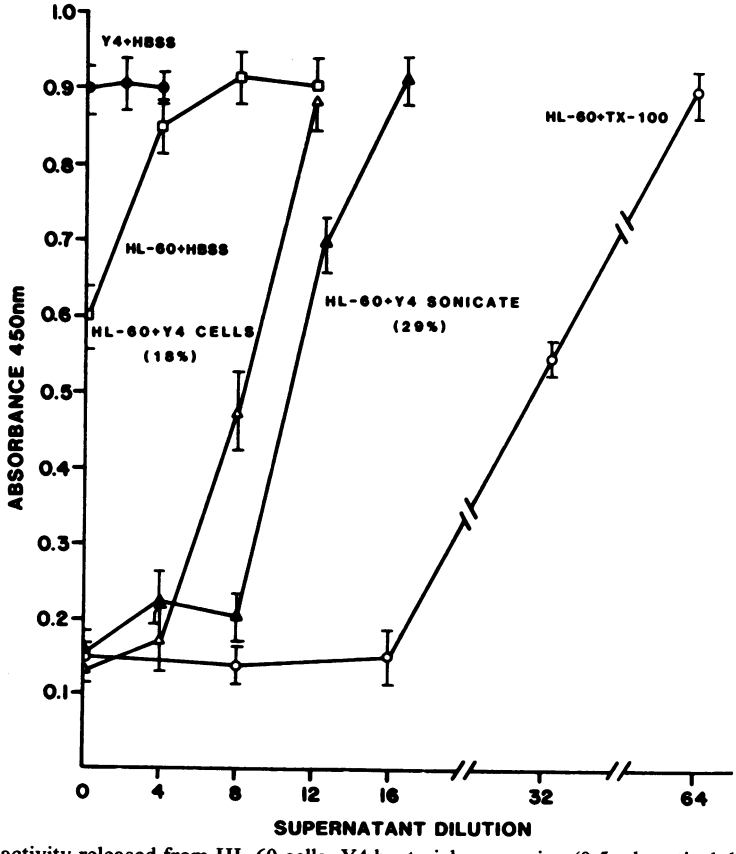

FIG. 1. LDH activity released from HL-60 cells. Y4 bacterial suspension (0.5 ml; optical density at 540 nm [OD_{540}] = 0.6) and 0.5 ml of Y4 bacterial sonic extract (1.0 mg [dry weight] per ml of HBSS) were both incubated with 0.5 ml of HL-60 cells (10^7 cells per ml) at 37°C for 60 min with constant shaking. The suspensions were chilled on ice and centrifuged at 600 × *g* for 20 min. Supernatant fluids were diluted with HBSS and assayed for LDH according to Berger and Broida (3), as described in the text. Total intracellular LDH was determined by incubating HL-60 cells in 0.1% Triton X-100 under the same conditions. Additional controls included Y4 bacterial cells incubated in HBSS and HL-60 cells incubated in HBSS; the absorbance at 450 nm of a buffer-substrate reference was 0.9. Values are expressed as the mean ± standard deviation of triplicate determinations. Percentages of total intracellular LDH released are shown in parentheses.

TABLE 1. Lactate dehydrogenase release from HL-60 cells or human polymorphonuclear leukocytes incubated with *A. actinomycetemcomitans* or *H. aphrophilus*

Organism	% LDH release from HL-60 cells (mean ± SD)[a]	% LDH release from human PMN (mean ± SD)[b]
A. actinomycetemcomitans		
Y4	32.4 ± 4.1[c]	24.7 ± 3.7[c]
ATCC 29522	29.7 ± 5.0[c]	23.3 ± 4.3[c]
ATCC 29524	30.3 ± 3.3[c]	24.7 ± 4.0[c]
ATCC 29523	7.8 ± 5.4	5.6 ± 4.4
67	4.5 ± 1.1	3.4 ± 2.9
H. aphrophilus		
ATCC 5908	3.3 ± 1.2	3.7 ± 3.1

[a] $n \geq 7$ experiments, optical density at 540 nm = 1.0 for bacterial suspensions.

[b] $n \geq 3$ experiments, optical density at 540 nm = 1.0 for bacterial suspensions.

[c] $P < 0.05$ compared with respective controls, as determined by Student's t test.

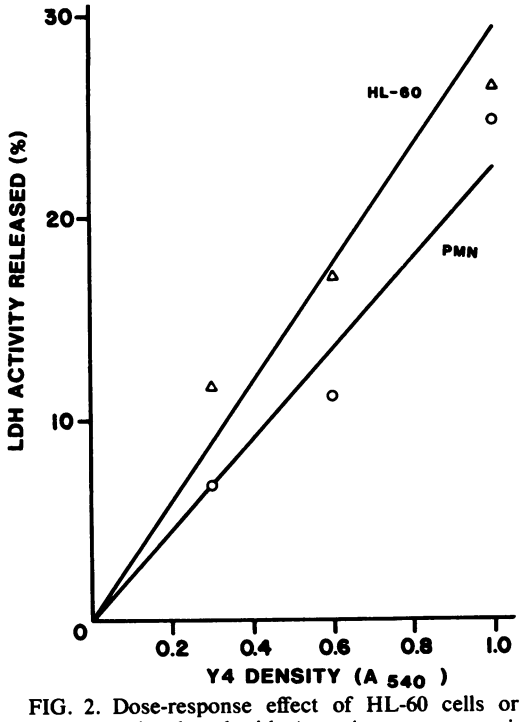

FIG. 2. Dose-response effect of HL-60 cells or human PMN incubated with *A. actinomycetemcomitans* Y4. A 0.5-ml volume of HL-60 cells or PMN (10^7 cells per ml in HBSS) was incubated with 0.5 ml of Y4 bacterial cells at an OD_{540} of 0.3, 0.6, or 1.0 for 60 min at 37°C with constant shaking. Total intracellular LDH was determined by incubation of HL-60 cells or PMN with 0.1% Triton X-100. The percentage of total LDH released was determined from dilutions necessary to reduce half of the pyruvate under standard assay conditions.

for 15 min. Then 100 μl of the Sigma Color Reagent (0.02% 2,4-dinitrophenylhydrazine in 1 N HCl) was added, and the mixture was held at room temperature for 20 min. A 1.0-ml volume of 0.4 M NaOH was added with rapid mixing, and the absorbance was read against a water blank at 450 nm. A reference blank containing buffer-substrate alone was included with each set of assays. To quantitate LDH release, serial dilutions of supernatant fluid from cytotoxicity tests were tested to determine the amount necessary to reduce about 50% of the pyruvate used in the standard assay. In this region, activity is proportional to dilution. Target cells incubated in 0.1% Triton X-100 served to indicate total cellular LDH. The percentage of total LDH released was determined by comparison of the supernatant dilution at the 50% activity level, from cells treated with bacteria to that from cells treated with 0.1% Triton X-100. Each experimental condition was run in triplicate, and the mean value and standard deviation were used in subsequent analyses.

Preparation of antiserum to *A. actinomycetemcomitans* Y4. Strain Y4 was grown to late logarithmic or early stationary phase in thioglycolate liquid medium (Difco), harvested by centrifugation, washed three times in phosphate-buffered saline, and suspended in sterile saline to a concentration of 10 mg (wet weight) per ml. Three female New Zealand white rabbits, each weighing approximately 4 kg, received intravenous injections of the Y4 suspension via the marginal ear vein, following the protocol of McCarty and Lancefield (8). Rabbit immune serum was obtained from the central artery of the ear, and antibody titer was determined by immunodiffusion. Once a satisfactory antibody titer had been obtained, the rabbits were exsanguinated by cardiac puncture. Y4 antisera were heated to 56°C for 30 min and stored in small aliquots at −70°C.

RESULTS

Figure 1 shows the cytotoxic effect of *A. actinomycetemcomitans* Y4, using HL-60 as a target cell and LDH release as a measure of leukotoxicity. An 8- to 10-fold dilution of supernatant from HL-60 suspensions treated with Y4 cells or sonic extract contained sufficient LDH to reduce about 50% of the pyruvate present. This may be compared with the release of LDH by Triton X-100, in which a 24-fold dilution of supernatant was required to reduce 50% of the substrate. Trypan blue staining of Y4-treated HL-60 cells revealed greater than 60% cell death, compared with less than 15% of untreated HL-60 cells. *A. actinomycetemcomitans* ATCC 29522 and ATCC 29524 also caused LDH release from HL-60 cells, whereas *A. actinomycetemcomitans* ATCC 29523 and 67 and *H. aphrophilus* ATCC 5908 did not result in significant LDH release. Similar results were obtained when these organisms were incubated with human PMN (Table 1). Heating *A. actinomycetemcomitans* leukotoxic strains to 56°C for 30 min abrogated the leukotoxic effect.

Figure 2 compares the effects of various cell

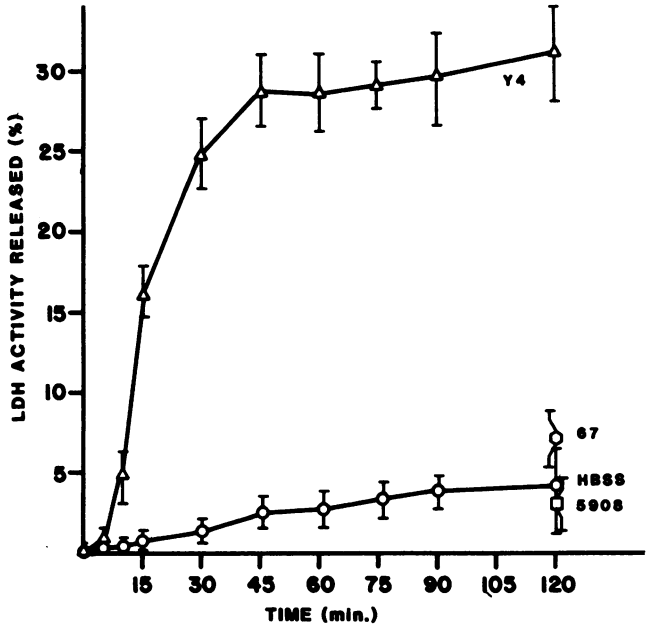

FIG. 3. Time course of the leukotoxic effect. A 0.5-ml volume of HL-60 cells (10^7 cells per ml in HBSS) was incubated at 37°C with 0.5 ml of Y4 bacterial cells (OD_{540} = 1.0) for 0 to 120 min. Total intracellular LDH was determined by incubation of HL-60 cells with 0.1% Triton X-100. Controls consisted of HL-60 cells incubated with HBSS and HL-60 cells incubated with the nonleukotoxic *A. actinomycetemcomitans* 67 and *H. aphrophilus* ATCC 5908. Values are expressed as the mean percentage of total LDH release ± standard deviation for triplicate determinations.

concentrations of Y4 on HL-60 and PMN. In the range tested, LDH release from both kinds of target cells was proportional to the number of Y4 cells. HL-60 cells tended to release greater amounts of LDH than did PMN; however, this difference was not statistically significant.

To examine the time course of LDH release, replicate mixtures of HL-60 and bacteria cells were incubated at 37°C, and the interaction was stopped at various time intervals. Interaction with Y4 resulted in maximal release of LDH within 45 min (Fig. 3). In contrast, *A. actinomycetemcomitans* 67 and *H. aphrophilus* ATCC 5908 did not release significant amounts of LDH even after 120 min of incubation.

Figure 4 shows the effect of temperature on the cytotoxic activity of a standard Y4 preparation. No LDH release was detected when HL-60 cells were incubated in HBSS alone or were treated with Y4 cells at 4°C; 11% of total cellular LDH was released when HL-60 cells were treated at 21°C; and 21% was released when HL-60 cells were treated at 37°C.

Figure 5 illustrates the effect of antiserum prepared against Y4 on cytotoxic activity. Addition of 5% rabbit antiserum nullified the cytotoxic effect of Y4 on HL-60 cells such that LDH was not released in amounts above that of a HBSS control. Serum (5%) from a localized juvenile periodontitis patient also had the same effect. Heating the antiserum (80°C, 30 min) destroyed its inhibitory effect. Neither normal rabbit serum nor serum from a disease-free human subject had any effect on the cytotoxic activity of Y4 in the test system.

The prevalence of leukotoxin-producing isolates of *A. actinomycetemcomitans* is shown in Table 2. One hundred fresh oral isolates from 55 subjects with various periodontal conditions were examined for leukotoxin production on up to three separate occasions. Leukotoxin-producing and -nonproducing isolates were found within each subject group, but the disease groups showed significantly higher proportions of leukotoxin-producing *A. actinomycetemcomitans* isolates than did the healthy group. Individuals were also identified who harbored both leukotoxin-producing and -nonproducing *A. actinomycetemcomitans*. In one localized juvenile periodontitis patient, for example, 5 of 10 isolates examined were found to produce leukotoxin.

DISCUSSION

We have here presented evidence that the cytotoxic activity of *A. actinomycetemcomitans* toward the promyelocytic HL-60 cell line is

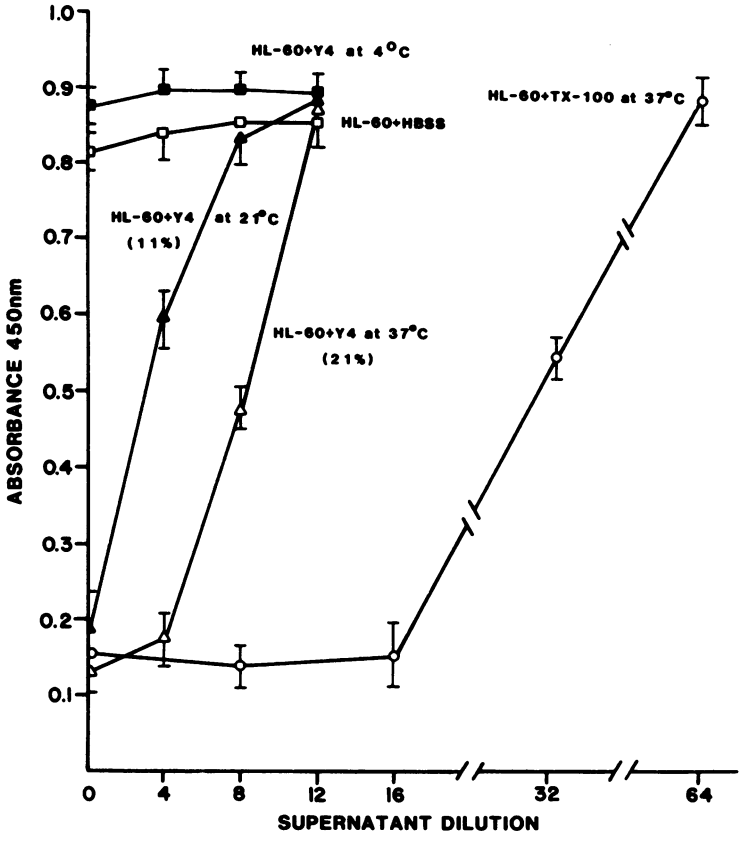

FIG. 4. Temperature effects on LDH release from HL-60 cells incubated with Y4. A 0.5-ml volume of Y4 bacterial cells (OD_{540} = 0.6) was incubated with 0.5 ml of HL-60 cells (10^7 cells per ml of HBSS) for 60 min at 4, 21 and 37°C. Controls consisted of HL-60 cells incubated in HBSS and in 0.1% Triton X-100. LDH was determine as described in the legend to Fig. 1. Values represent the mean ± standard deviation of triplicate determination. Percentages of total intracellular LDH released are shown in parentheses.

concordant with the leukotoxic activity of this organism toward human PMN, as reported by Baehni et al. (1, 2). *A. actinomycetemcomitans* ATCC 29522, ATCC 29524, and Y4 caused lysis of both HL-60 cells and PMN, whereas *A. actinomycetemcomitans* ATCC 29523 and 67 and *H. aphrophilus* ATCC 5908 did not lyse either HL-60 cells or PMN. HL-60 cells appeared to be slightly more sensitive than PMN to leukotoxic strains. For example, 1.5 to 2 times more cellular LDH was released from HL-60 cells exposed to Y4 than from PMN, as calculated from the percentage of pyruvate reduced (Fig. 6). The kinetics of LDH release from HL-60 cells incubated with leukotoxic *A. actinomycetemcomitans* is also similar to that reported for PMN. The maximal release of LDH from HL-60 cells incubated with *A. actinomycetemcomitans* whole cells occurred after 45 min, whereas maximal release from PMN exposed to *A. actinomycetemcomitans* cell sonic extracts takes place after 60 min (14) and from human monocytes after 120 min (12).

HL-60 cells and human PMN treated with Y4 cells showed similarities with respect to dose dependence and the effects of temperature and immune serum. An increasing number of *A. actinomycetemcomitans* Y4 cells caused increasing release of cellular LDH from HL-60 cells. Incubation at 37°C yielded greater LDH release than incubation at 21°C, and incubation at 4°C resulted in no appreciable release. Incubation of Y4 with rabbit anti-Y4 serum abrogated the cytotoxic effect. Similar results have previously been obtained with PMN exposed to *A. actinomycetemcomitans* whole cells (2) and with PMN and monocytes treated with Y4 sonic extracts (12, 13). Interestingly, incubation of HL-60 with Y4 in the presence of normal human serum did not result in enhanced leukotoxicity, as has been reported for PMN (13). This may indicate that certain PMN cell surface receptors for serum factors which are important in modulating leukotoxin-mediated PMN lysis are absent, or present in low numbers, on HL-60 cells. The absence of certain cell surface receptors on

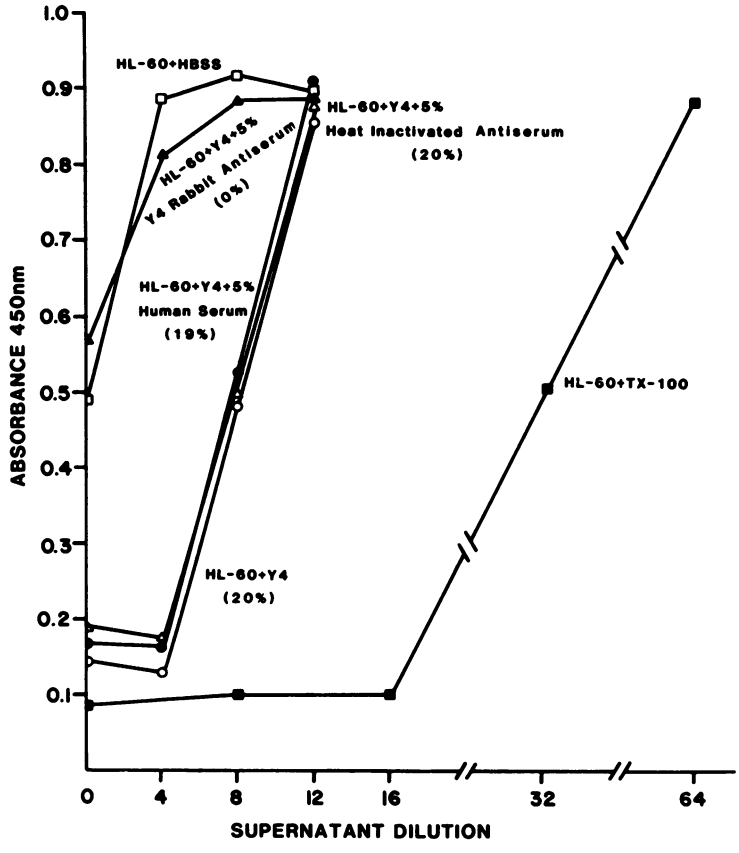

FIG. 5. Serum effects on LDH release from HL-60 cells incubated with Y4. A 0.5-ml volume of Y4 bacterial cells (OD_{540} = 0.6) was incubated with 0.5 ml of HL-60 (10^7 cells per ml of HBSS) at 37°C for 60 min with constant shaking. Reaction mixtures also contained (a) 5% rabbit antiserum to strain Y4, (b) 5% normal (nonimmune) human serum, or (c) 5% heat-inactivated rabbit antiserum (80°C for 30 min). Controls included HL-60 cells incubated in HBSS and in 0.1% Triton X-100. See legend to Fig. 1 for LDH assay. Percentages of total intracellular LDH released are shown in parentheses.

TABLE 2. Prevalence of leukotoxin-producing oral *A. actinomycetemcomitans*

Periodontal category	No. of patients	Total no. of isolates	% Leukotoxin-positive isolates
Healthy	11	13	6
Adult periodontitis	13	14	43[a]
Juvenile diabetic periodontitis	4	4	75[a]
Generalized juvenile periodontitis	2	3	66[a]
Localized juvenile periodontitis	25	66	55[a]

[a] Statistically significant, $P < 0.05$ compared with the healthy group as determined by chi-square analysis.

uninduced HL-60 cells has been demonstrated previously (4).

The finding that both healthy and periodontally diseased patients harbored leukotoxin-producing strains of *A. actinomycetemcomitans* does not necessarily indicate that this leukotoxin is unimportant in the pathogenesis of periodontal disease. It may be that the low numbers of *A. actinomycetemcomitans* in healthy patients (10) are insufficient to produce disease. On the other hand, the high numbers of leukotoxin-producing strains of *A. actinomycetemcomitans* in localized juvenile periodontitis may exceed the threshold level for disease. If so, leukotoxin could be a key pathogenic factor whose main function is to impair host defense cells and thereby permit microbial invasion of the gingival tissues. Indeed, microorganisms have been observed in the connective tissue of localized juvenile periodontitis lesions (5). A pathogenic mechanism involving tissue invasion by micro-

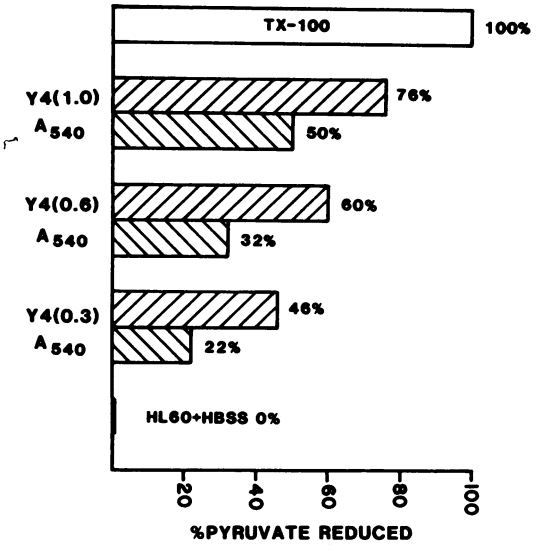

FIG. 6. LDH release as the percentage of pyruvate reduced. A 0.5-ml volume of HL-60 cells or human PMN (10^7 cells per ml of HBSS) was incubated with 0.5 ml of Y4 bacterial cells at an OD_{540} of 1.0, 0.6, or 0.3 for 60 min at 37°C with constant shaking. After centrifugation at 600 × *g* for 20 min, aliquots of culture supernatant were assayed for LDH release; the results are expressed as the percentage of pyruvate reduced. HL-60 or human PMN incubated with 0.1% Triton X-100 represent 100% pyruvate reduction. Symbols: ▨, HL-60 cells; ▧, PMN.

organisms has been previously hypothesized for staphylococcal leukocidin (16).

Additional similarities can be found between *A. actinomycetemcomitans* leukotoxin and staphylococcal leukocidin. Both leukotoxins are toxic for human PMN and monocytes but not for other cell types (16); they are both heat-labile, protease-sensitive factors which bind primarily to the cell membrane (12, 16); and they can both stimulate production of antitoxin antibodies. For staphylococci, the severity of infection is inversely proportional to serum levels of the antileukocidin antibody (6). Whether antibodies to *A. actinomycetemcomitans* leukotoxin exert a similar retarding effect on the progression of periodontal disease is a question which merits intensive investigation.

Circulating *A. actinomycetemcomitans* leukotoxin may alter granulocytic maturation and cause peripheral PMN to differ from normal PMN in a manner similar to that of staphylococcal leukocidin (11). This hypothesis may be supported by the findings of Shurin et al., which showed that a PMN chemotactic defect could be resolved after the extraction of periodontally affected teeth (9).

In conclusion, the promyelocytic HL-60 cell line appears to be susceptible to the leukotoxin produced by *A. actinomycetemcomitans*. The use of HL-60 cells should be helpful in the identification of leukotoxin-producing strains of *A. actinomycetemcomitans*, in the isolation and purification of this leukotoxin, and in determining its role in the etiology and pathogenesis of periodontal disease.

ACKNOWLEDGMENT

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