

## Bacterial Activation of Human Natural Killer Cells: Role of Cell Surface Lipopolysaccharide

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Culture of human peripheral blood lymphocytes with gram-negative bacteria associated with periodontal disease caused a rapid increase in the cytotoxic potential of natural killer (NK) cells. The NK cells were activated to kill NK-resistant targets, the peak cytotoxicity occurring on day 1 of culture. The addition of anti-Tac, anti-CD3, or anti-OKT-11 antibodies to block activation via the interleukin-2 (IL-2), T-cell, or E rosette receptors had a minimal effect on this inductive process. Anti-IL-2 antiserum was effective in blocking a significant amount, but not all, of the cytotoxicity in bacterium-activated cultures. Modest IL-2 production (5 to 6 National Institutes of Health units) was measured in lymphocyte cultures activated by bacteria, but proliferation was not induced during a 1-week period. When polymixin B sulfate was added to bind and block lipopolysaccharides, bacterium-induced cytotoxicity was completely abrogated for all activating bacteria. In addition, when culture supernatants from *Actinobacillus actinomycetemcomitans* were tested, activation still occurred. However, again, this activation was totally inhibited by polymixin B sulfate. Monocytes were also activated by bacteria to produce tumor necrosis factor (TNF). To exclude the possibility that TNF was responsible for cytotoxicity, an antiserum to TNF was added to cocultures of bacteria and lymphocytes with adherent cells removed. The antiserum had no effect on the inductive process. In addition, exogenous TNF did not kill M14 targets. These results suggest that bacterial cell surface lipopolysaccharides provide a major activation signal for NK cells to enhance cytotoxicity.

Considerable interest has been focused on activated cytotoxic human lymphocytes and their role as immunologic defense cells. Peripheral blood lymphocytes can be induced by interleukin-2 (IL-2) to develop into cytotoxic cells called lymphokine-activated killers capable of lysing a wide variety of tumor cell lines and fresh tumors (8). In addition to generation by lymphokines, activated killer cells have been generated from mixed lymphocyte culture (5, 27), in the presence of lectins (3), and from the stimulation of viruses (22).

Bacteria and bacterial products also potentiate killing by lymphocytes (11, 31). Recently, it was demonstrated that *Salmonella* strains which were fixed with glutaraldehyde enhance lymphocyte cytotoxicity against natural killer (NK)-sensitive and NK-resistant target cells in a manner comparable with induction by IL-2 (29). The cell types responding to the inductive effects of glutaraldehyde-fixed *Salmonella* strains were CD16<sup>+</sup> (Leu-11) and Leu-19<sup>+</sup> (28). Phillips and Lanier (23) have proposed that NK cells bearing these surface markers mediate a majority of lymphokine-activated killer activity against NK-resistant cell lines and fresh tumors.

Gram-negative bacteria associated with periodontal disease also mediate NK activation (R. A. Lindemann, K. T. Miyasaki, and L. E. Wolinsky, *J. Dent. Res.*, in press). This finding suggests that NK cells may play a role in antimicrobial defense. However, the mechanism or pathway whereby whole bacteria activate NK cells is not known. Tarkkanen et al. (28) demonstrated that protein synthesis, but not DNA synthesis, is required for lymphocyte activation by *Salmonella* bacteria and that both the induction and effector phases of activation are radioresistant. The same authors also concluded that the activation can be independent of interferon release, and no changes are observed in HLA-DR, transferrin receptor, or IL-2 receptor expression. Kinetics

studies with periodontal bacteria revealed that bacterial activation and IL-2 activation were significantly different over a 1-week period. Bacterium-activated lymphocyte cytotoxicity usually peaks at 1 to 2 days, while IL-2-activated lymphocyte cytotoxicity remains quite high after 7 days (Lindemann et al., in press).

It is well documented that lipopolysaccharides (LPS) from gram-negative bacteria are powerful immunostimulators (19). However, LPS extracted from *Salmonella* bacteria interfered with or blocked lymphocyte activation in the presence of whole bacteria (29). In contrast, LPS isolated from periodontal bacteria induced high levels of cytotoxicity from NK cells (Lindemann et al., in press). The purpose of this study was to determine the role of surface LPS on glutaraldehyde-fixed whole bacteria associated with periodontal disease in the activation of human lymphocytes. In addition, the relationship between bacteria and potential NK activation pathways, the IL-2 and E rosette receptors, was investigated.

### MATERIALS AND METHODS

**Isolation of lymphocytes.** Human peripheral blood lymphocytes from healthy volunteers were obtained by Ficoll-Paque (Pharmacia Fine Chemicals, Piscataway, N.J.) density gradient centrifugation. Lymphocytes containing adherent cells were tested in some experiments. For most activation experiments, lymphocytes with adherent cells depleted by plastic adherence (PBL) were used. PBL were suspended in RPMI 1640 medium with 10% human AB serum. The percentage of monocytes remaining was 1 to 3%, as determined by the universal rosetting reagent (13). Large granular lymphocytes (LGLs) were separated by a discontinuous Percoll (Pharmacia) gradient (30).

A panning technique was used to isolate lymphocyte subpopulations in conjunction with the monoclonal antibod-

TABLE 1. Bacteria tested

Organism	Strain (serotype)	Origin <sup>a</sup>	Source
<i>A. actinomycetem-comitans</i>	29523 (a)	ATCC	Blood
	Y4 (b)	FDC	Dental plaque
	67 (c)	SUNYAB	Dental plaque
<i>B. gingivalis</i>	35277	ATCC	Dental plaque
<i>H. aphrophilus</i>	19415	ATCC	Blood
<i>H. segnis</i>	HK316	RDCA	Dental plaque

<sup>a</sup> ATCC, American Type Culture Collection; FDC, Forsyth Dental Center; SUNYAB, State University of New York at Buffalo; RDCA, Royal Dental College at Aarhus.

ies Leu-11b (Becton Dickinson and Co., Mountain View, Calif.) and NKH-1A (Coulter Immunology, Hialeah, Fla.). Adherent cells were first removed by plastic adherence for 1 h. The nonadherent cells were then treated separately with the monoclonal antibodies ( $1 \mu\text{l}/10^6$  cells) and incubated on ice for 30 min. After the cells were washed twice,  $3 \times 10^6$  of the antibody-treated cells were placed on tissue culture plates previously coated with an affinity-purified goat anti-mouse immunoglobulin M antibody (Tago, Inc., Burlingame, Calif.). After a 2-h incubation at  $4^\circ\text{C}$ , the nonadherent cells were removed, and then the adherent cells were gently detached by a rubber policeman.

For monocyte preparation, PBL were cultured in 24-well microdilution plates (Costar, Cambridge, Mass.) at a concentration of  $2 \times 10^6$  per well in 1 ml of RPMI 1640 plus Nutridoma (Boehringer Mannheim Biochemicals, Indianapolis, Ind.), a serum- and endotoxin-free culture supplement. After cells were allowed to adhere for 1 h, nonadherent cells were vigorously washed off with warm medium. Approximately  $2 \times 10^5$  adherent cells remained per well and represented >90% of monocytes, as determined by immunoperoxidase staining with a macrophage-specific monoclonal antibody (Leu-M3; Becton Dickinson). After 24 h of culture, the medium was replaced, and test bacteria were added at a 10:1 ratio of bacteria to monocyte. After a further 24 h of culture, cell-free supernatants were retrieved and assayed for tumor necrosis factor (TNF).

**Bacteria.** The six bacteria tested are described in Table 1. All bacteria were provided by K. Miyasaki (University of California, Los Angeles), except for *Bacteroides gingivalis* which was provided by M. Newman (University of California, Los Angeles). *B. gingivalis* and the three *Actinobacillus actinomycetemcomitans* strains were chosen because they are clearly associated with human periodontal disease, and *Haemophilus aphrophilus* and *H. segnis* were chosen because they are not. Bacteria were grown at  $37^\circ\text{C}$  overnight under an atmosphere of 5%  $\text{CO}_2$ -95% air (*B. gingivalis* was grown under an atmosphere of 80%  $\text{N}_2$ -10%  $\text{CO}_2$ -10%  $\text{H}_2$ ) on plates consisting of Trypticase soy agar (BBL Microbiology Systems, Cockeysville, Md.) supplemented with 0.1% yeast extract (Difco Laboratories, Detroit, Mich.), 0.001% menadione (Sigma Chemical Company, St. Louis, Mo.), 0.05% equine hemin III (Sigma), and IsoVitaleX (BBL). Bacteria were collected, washed twice, and suspended in Dulbecco phosphate-buffered saline (pH 7.4; Whittaker M.A. Bioproducts, Walkersville, Md.).

**Glutaraldehyde fixation of bacteria.** Washed bacteria were suspended in Dulbecco phosphate-buffered saline to an optical density of 0.3 at 540 nm. A 25% (vol/vol) solution of glutaraldehyde (Eastman Kodak Co., Rochester, N.Y.) was added slowly to achieve a final concentration of 0.25%. The suspension was incubated overnight at  $20^\circ\text{C}$  and subse-

quently stored at  $4^\circ\text{C}$ . Prior to use, bacteria were washed four times and resuspended in Dulbecco phosphate-buffered saline at  $2 \times 10^8$  cells per ml.

**Target cells.** The NK-sensitive human erythroleukemia cell line, K562, and the NK-resistant cell line, UCLA SO-M14 (M14, melanoma), were used as targets in the cytotoxicity assays. Target cells ( $5 \times 10^6$  in 1 ml of RPMI 1640 with 10% fetal calf serum) were labeled with  $250 \mu\text{Ci}$  of  $^{51}\text{Cr}$  for 1 h at  $37^\circ\text{C}$ .

**Activation of cytotoxicity by bacteria and culture supernatants.** PBL were incubated for 24 h at  $37^\circ\text{C}$  with glutaraldehyde-fixed bacteria at a 5:1 bacterium-to-lymphocyte ratio in RPMI 1640 with 10% AB serum. Culture supernatants were collected and stored at  $-4^\circ\text{C}$ . The lymphocytes were then washed twice and resuspended in RPMI 1640 with 10% AB serum prior to cytotoxicity assays. Viability, as tested by trypan blue exclusion, was >85%. The culture supernatants (100  $\mu\text{l}$ ) were also separately added to PBL for a 24-h incubation period prior to cytotoxicity assays.

**Antibodies.** Anti-Tac (1:1,000; provided by Thomas Waldmann, National Cancer Institute) reacts with the human IL-2 receptor. Anti-IL-2 antiserum was provided by Amgen (Thousand Oaks, Calif.). A 1:1,000 dilution of this antiserum was shown to inhibit NK cytotoxicity induced by 100 U of ala-125 rIL-2 (Amgen) per ml against M14 targets. Anti-CD3 (1:1,000; OKT-3; Ortho Diagnostics Inc., Raritan, N.J.) reacts with the T-cell receptor. OKT-11 (1:1,000; Ortho) reacts with the sheep erythrocyte receptor protein found on peripheral blood T lymphocytes and NK cells. Antibodies at the above-mentioned dilutions were incubated during the 24-h bacterial induction period to assess their ability to inhibit lymphocyte activation. To some cultures, anti-human TNF alpha polyvalent antibody (Endogen, Boston, Mass.) was added at a dilution of 1:1,000. The specific activity of this antibody was 10,000 neutralizing anti-human TNF alpha units per ml in the standard L929 TNF cytotoxicity assay. A preimmune rabbit control immunoglobulin G was tested in parallel.

**IL-2 production.** The number of National Institutes of Health units of IL-2 produced in culture supernatants was measured by a kit (InterTest 2; Genzyme, Boston, Mass.). This kit utilizes an enzyme-linked immunosorbent assay system (7) to quantitate natural and recombinant human IL-2s. Briefly, microdilution plates were coated with monoclonal mouse anti-IL-2 overnight and washed after each step. Culture supernatants and IL-2 standards were dispensed into wells for 3 h. Polyvalent rabbit anti-IL-2 was added for 1 h. An alkaline phosphatase-conjugated goat anti-rabbit immunoglobulin was dispensed for 90 min, and finally, *p*-nitrophenyl phosphate substrate was added.  $A_{405}$  was measured after 60 min by a spectrophotometer (Titertek Multiskan; Flow Laboratories, Inc., McLean, Va.). A standard curve was plotted with absorbance values of standards, and absorbance values of test samples were compared to this curve to determine IL-2 units.

**DNA synthesis.** Effector cells ( $10^5$  cells per well in 200  $\mu\text{l}$  of RPMI 1640 with 10% AB serum) were seeded in microwells after 24 h of culture with medium only, bacteria, or IL-2. Each well was pulsed with  $1 \mu\text{Ci}$  of [*methyl*- $^3\text{H}$ ]thymidine (New England Nuclear Corp., Boston, Mass.) for 5 h, and cells were then harvested onto filter paper with a harvester (Ph.D.; Cambridge Technology, Cambridge, Mass.). The paper was processed for liquid scintillation counting. Each assay was done in triplicate.

**PB.** To analyze the effect of bacterial cell surface LPS on lymphocyte activation, polymixin B sulfate (PB; Sigma) was added to cultures at 100  $\mu\text{g}/\text{ml}$  to bind LPS.

**Cytotoxicity assay.** K562 or M14 target cells ( $5 \times 10^3$ ) were mixed with effector cells (at 50:1, 25:1, and 12.5:1 ratios of effector cells to target cells) in round-bottom microdilution plates containing 200  $\mu\text{l}$  of RPMI 1640 with 10% AB serum. The plates were centrifuged at  $120 \times g$  for 4 min to initiate cell-to-cell contact and then incubated for 4 h at  $37^\circ\text{C}$  in a humidified incubator with 5%  $\text{CO}_2$ . At the end of the assay, plates were centrifuged at  $275 \times g$  for 8 min; 100  $\mu\text{l}$  of supernatant was harvested from each well, and the  $^{51}\text{Cr}$  released from target cells was counted. Each assay was performed in quadruplicate. Cytotoxicity was defined as the percentage of specific  $^{51}\text{Cr}$  released or  $([\text{experimental release} - \text{spontaneous release}]/[\text{maximal release} - \text{spontaneous release}]) \times 100$ . Experimental release was defined as the counts per minute of  $^{51}\text{Cr}$  released from target cells caused by effector cells. Maximal release was defined as the counts per minute of  $^{51}\text{Cr}$  released from target cells induced by 2% Nonidet P-40 detergent. Spontaneous release was defined as the counts per minute of  $^{51}\text{Cr}$  from target cells incubated in medium alone.

**TNF assay.** The TNF bioassay was a modification of previously described methods (25). Murine L929 cells were chromated with  $^{51}\text{Cr}$  (0.25 mCi;  $5 \times 10^6$  cells in 0.5 ml of culture medium for 1 h at  $37^\circ\text{C}$  in a shaking water bath), washed three times, and plated in 96-well U-bottom plates (Costar) at  $5 \times 10^4$  cells per well in the presence of actinomycin D (5  $\mu\text{g}/\text{ml}$ ). Test samples were added in triplicate wells to a total volume of 0.2 ml and incubated in a humidified  $\text{CO}_2$  incubator for 18 h. Specific  $^{51}\text{Cr}$  release was calculated by determining supernatant counts by the following equation:  $([\text{experimental release} - \text{spontaneous release}]/[\text{maximal release} - \text{spontaneous release}]) \times 100$ . Experimental release was defined as the counts per minute of  $^{51}\text{Cr}$  released from target cells caused by test samples. Maximal release and spontaneous release are as described above for cytotoxicity assay. A specific rabbit antiserum to human TNF (as described above) was added to certain wells at a final dilution of 1:1,000 to verify that the cytotoxic activity was due to TNF and not to lymphotoxin or another cytotoxin.

**Effect of TNF on M14 targets.** TNF (1,000 U/ml; Cetus Corp., Emeryville, Calif.) was incubated with M14 target cells in microdilution plates for 24 h. Microdilution wells were assayed for thymidine incorporation (as described above). TNF was also incubated with chromated M14 targets for 24 h, and the spontaneous release of  $^{51}\text{Cr}$  was counted (as described above).

**Statistics.** The significance of cytotoxicity assays was determined by first converting the three effector-to-target ratios into lytic units (24). A lytic unit is defined as the number of cells required to cause a specified amount of target lysis (in this case, 30%) and is usually expressed as lytic units per  $10^6$  lymphocytes. This method allows a more accurate comparison between lymphocyte donors. The paired *t* test (two tailed) was then applied to determine the significance of lytic unit values for 30% target lysis (LU 30% in figures).

## RESULTS

**Bacterial activation of Percoll fractions.** To determine the cell or cells activated by bacteria, density gradient centrifugation was performed. Tarkkanen et al. (29) had demon-

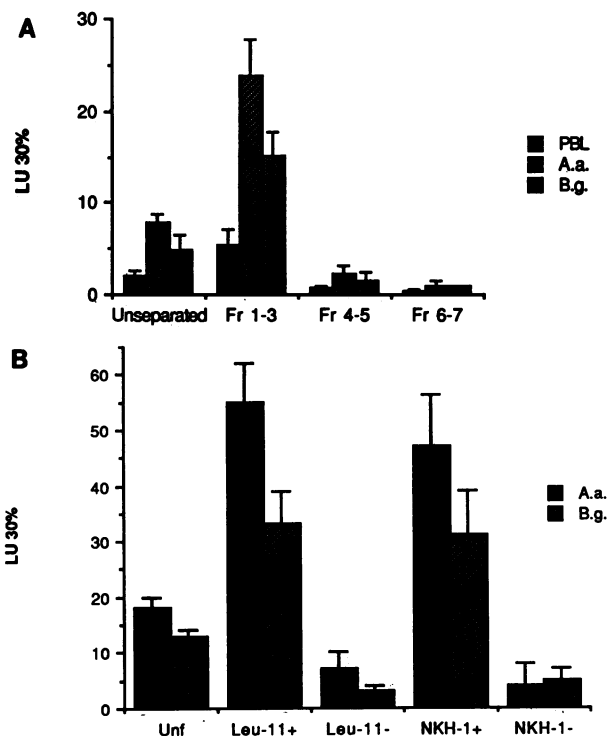


FIG. 1. (A) Cytotoxicity of lymphocytes with different densities activated by bacteria. After Percoll fractionation, bacteria were added at a 10:1 bacterium-to-lymphocyte ratio, and cytotoxicity was tested after 24 h against M14 targets. (B) Cytotoxicity of different lymphocyte subpopulations activated by bacteria. After separation, bacteria were added at a 10:1 bacterium-to-lymphocyte ratio, and cytotoxicity was tested after 24 h against M14 targets. A.a., *A. actinomycetemcomitans* Y4; B.g., *B. gingivalis*; Fr, fraction; Unf, unfractionated. Cytotoxicity is expressed in lytic units (LU 30%). Bars indicate standard deviations from the means.

strated that *Salmonella* bacteria activate primarily low-density fractions of PBL. To evaluate whether oral pathogens behaved similarly and activated LGL-rich fractions, bacteria associated with human periodontal disease were tested. Low-density fractions containing LGL (Fig. 1A; fractions 1 to 3) were highly enriched for cells activated by periodontal bacteria. Low-density fractions contained most of the NK activity. These fractions have been shown to contain a high percentage of cells bearing NK-associated antigens (29). In contrast, fractions 4 to 7 showed minimal cytotoxic activity against M14 targets after 24 h of incubation with bacteria.

**Phenotypic characterization of the effector cell.** To determine the cell or cells activated by bacteria, panning experiments were performed to isolate lymphocyte subpopulations. Tarkkanen et al. (28) had demonstrated that *Salmonella* bacteria activate primarily Leu-11<sup>+</sup> and Leu-19<sup>+</sup> (NKH-1) lymphocyte subpopulations. To evaluate whether oral pathogens behaved similarly and activated the same cells, bacteria associated with human periodontal disease were tested. After 24 h of incubation, the vast majority of cytotoxicity against M14 targets was contained within the Leu-11<sup>+</sup> and NKH-1<sup>+</sup> populations (Fig. 1B).

**Results of antibody inhibition assays.** To evaluate the role of NK surface receptors in the bacterial activation process, monoclonal antibodies directed against the IL-2 receptor and the E rosette receptor were tested separately. The ability of these antibodies to block cytotoxic activation by bacteria

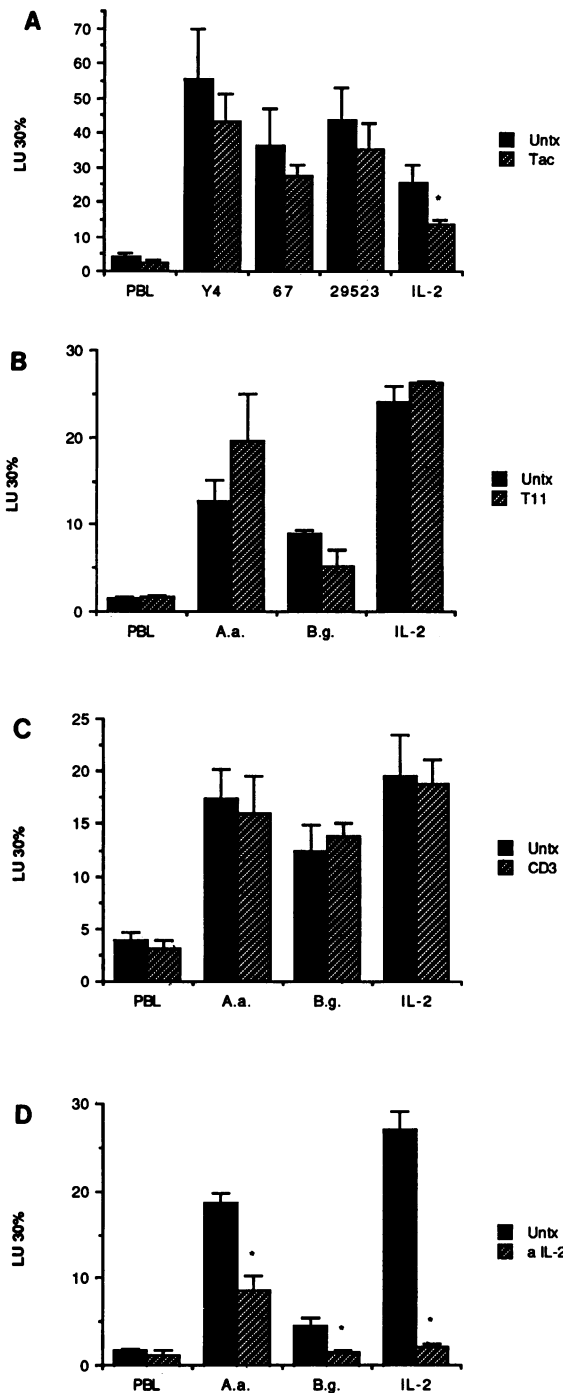


FIG. 2. Effect of antibodies cultured with bacteria for 24 h on killing of M14 targets. Representative experiments are presented. Cytotoxicity is expressed in lytic units (LU 30%). Bars indicate standard deviations from the means. Asterisks indicate significant differences from untreated (untx) samples at  $P < 0.05$  by the paired  $t$  test. IL-2 was added at 100 U/ml. (A) Anti-IL-2 receptor (anti-Tac) antibody (Tac) cultured with three serotypes of *A. actinomycetemcomitans*. (B) Anti-E rosette receptor (OKT-11) antibody (T11) cultured with *A. actinomycetemcomitans* Y4 and *B. gingivalis*. (C) Anti-CD3 (OKT-3) antibody (CD3) cultured with *A. actinomycetemcomitans* Y4 and *B. gingivalis*. (D) Anti-IL-2 antiserum (aIL-2) cultured with *A. actinomycetemcomitans* Y4 and *B. gingivalis*.

was measured in three or four subjects. All subjects responded similarly in the antibody-blocking experiments; however, there were individual differences in the levels of cytotoxic enhancement (Fig. 2). Anti-IL-2 receptor antibody (anti-Tac) had a minimal effect on the bacterial induction of cytotoxicity by three pathogenic *A. actinomycetemcomitans* strains (Fig. 2A). Antibodies against the E rosette receptor (OKT-11), which is found on some NK cells, had no effect on the cytotoxicity generated after culture with *A. actinomycetemcomitans* or *B. gingivalis* (Fig. 2B). The possibility that bacteria activated T cells via the T-cell receptor, which would have contributed to cytotoxicity, was examined. Anti-CD3 antibody had no effect on cytotoxic development in bacterium-activated cultures (Fig. 2C). To determine the role of IL-2 in the activation process, an antiserum to IL-2 was incubated in cocultures of bacteria and lymphocyte. This anti-IL-2 antiserum substantially blocked bacterial activation by two activating bacteria, although *A. actinomycetemcomitans* cultures still retained cytotoxicity above control values (Fig. 2D). This antiserum was also effective in binding and blocking the cytotoxicity induced by exogenously added IL-2. Dilutions of antiserum above 1:1,000 had no additional effect on cytotoxicity (data not shown).

**Units of IL-2 produced by activated lymphocytes.** Because IL-2 antibodies substantially decreased the activation by bacteria, IL-2 was tested as a potential activation signal. IL-2 production was measured in culture supernatants by a modified enzyme-linked immunosorbent assay. Modest but significant IL-2 production above control cultures (approximately 6 National Institutes of Health units) was measured in lymphocyte cultures activated by bacteria (Table 2). However, the cytotoxicity that developed in bacteria-lymphocyte cultures was often equivalent to 50 to 100 U of IL-2 per ml (Fig. 2).

**DNA synthesis.** As IL-2 was measured in culture supernatants, its potential effects on proliferation were studied. Tarkkanen et al. (28) had demonstrated that *Salmonella* bacteria does not induce lymphocyte proliferation. Lymphocytes cultured in the presence of periodontal bacteria did not proliferate at any time during a 1-week period (Table 3). Cytotoxicity, measured in conjunction with proliferation, peaked on day 1 of culture and subsequently declined. By day 7, the cytotoxicity of bacterium-treated lymphocytes was negligible compared with that of the IL-2 culture.

**Effect of PB.** Because IL-2 did not appear to be responsible for all the bacterium-activated cytotoxicity, the effect of LPS, a known immune-activating component of bacterial cell walls, was evaluated. The antibiotic PB was tested because of its ability to bind and neutralize the effects of LPS. PB totally inhibited activation by all bacteria tested

TABLE 2. Bacterium-activated IL-2 production measured at 24 h postculture

Subject no.	IL-2 production (NIH units) <sup>a</sup>		
	Untreated	<i>A. actinomycetemcomitans</i> 67	<i>B. gingivalis</i>
1	1.2	5.6	5.6
2	1.1	6.4	5.4
3	2.0	5.4	5.4

<sup>a</sup> NIH, National Institutes of Health. The means  $\pm$  standard deviations were  $1.4 \pm 0.5$ ,  $5.8 \pm 0.5$ , and  $5.5 \pm 0.1$  for control and cultures treated with *A. actinomycetemcomitans* 67 and *B. gingivalis*, respectively. Mean values for bacterium-treated cultures were significantly different from those of control ( $P = 0.05$ ; paired  $t$  test).

TABLE 3. Effect of bacterial activation on cytotoxicity and DNA synthesis

Time of analysis and treatment <sup>a</sup>	Mean cytotoxicity (LU) ± SD	Mean thymidine uptake (cpm) ± SD
<b>Day 1</b>		
Untreated	16.13 ± 6.85	141 ± 25
<i>A. actinomycetemcomitans</i> 67	55.57 ± 14.44	439 ± 160
<i>B. gingivalis</i> ATCC 35277	42.67 ± 12.60	412 ± 50
IL-2	68.68 ± 24.39	539 ± 128
<b>Day 4</b>		
Untreated	1.40 ± 0.72	200 ± 10
<i>A. actinomycetemcomitans</i> 67	25.88 ± 0.33	548 ± 70
<i>B. gingivalis</i> ATCC 35277	5.82 ± 2.98	365 ± 36
IL-2	80.19 ± 3.14	19,571 ± 1,523
<b>Day 7</b>		
Untreated	3.28 ± 0.25	220 ± 39
<i>A. actinomycetemcomitans</i> 67	12.37 ± 0.13	293 ± 70
<i>B. gingivalis</i> ATCC 35277	3.38 ± 0.04	348 ± 27
IL-2	126.88 ± 19.17	17,233 ± 1,423

<sup>a</sup> PBL were used in untreated cultures. IL-2 was used at 100 U/ml. Targets were K562 cells for day 1 and M14 cells for days 4 and 7.

(Fig. 3). Both pathogenic and nonpathogenic periodontal bacteria activated lymphocytes to kill M14 targets. PB had no effect on cytotoxicity when cultured with PBL alone or with PBL activated by IL-2.

**Culture supernatants.** To determine the effect of IL-2 on bacterial wall components released in culture, supernatants were collected and added to autologous lymphocytes, and the mixture was incubated for 24 h. Supernatants from *A. actinomycetemcomitans* Y4 cultures activated PBL significantly above control cytotoxicity values (Fig. 4). This activation was abolished by the addition of PB. Supernatants from *B. gingivalis* had no significant activating effect on lymphocytes.

**Bacterial activation of monocytes and TNF production.** Because a recent report had suggested that TNF augmented NK cytotoxicity (21), the role of TNF in bacterial activation was studied. As LPS is a known monocyte activator, the possibility that contaminating monocytes released TNF and enhanced NK cytotoxicity or killed targets directly was considered. Monocytes were also activated by whole bacteria and released significant amounts of TNF, as measured by

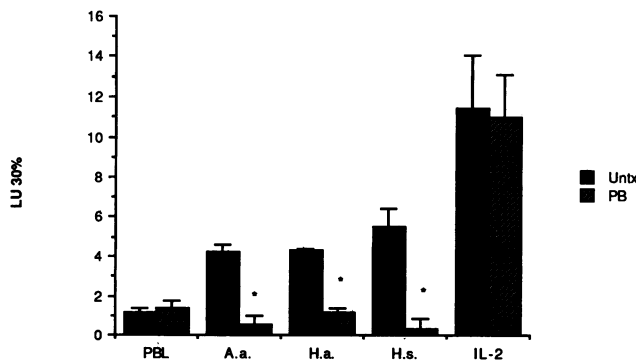


FIG. 3. Inhibition of bacterial activation of lymphocytes by PB (100 µg/ml) at 24 h (M14 targets). A.a., *A. actinomycetemcomitans* Y4; H.a., *H. aphrophilus*; H.s., *H. segnis*. Bars indicate standard deviations from the means. Asterisks indicate significant differences from untreated (Untx) sample at  $P < 0.05$  by the paired  $t$  test.

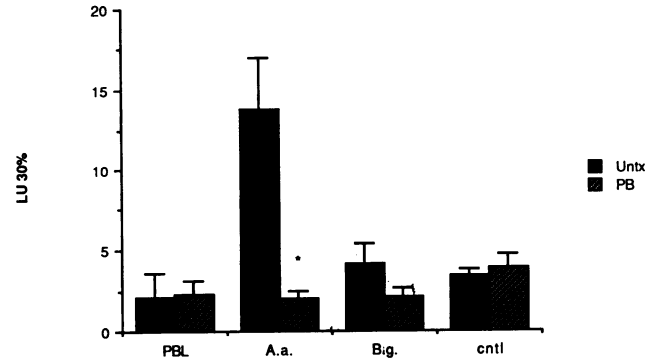


FIG. 4. Activation of lymphocytes by bacteria-lymphocyte culture supernatants. Culture supernatant (100 µl) was added to lymphocytes and cultured for 24 h. The activation of lymphocytes was inhibited by PB (100 µg/ml). A.a., *A. actinomycetemcomitans* Y4; B.g., *B. gingivalis*; cntl, untreated PBL supernatant; Untx, untreated. Bars and asterisks are as defined in the legend to Fig. 3.

a cytotoxicity assay (Fig. 5). Addition of an antiserum to TNF completely abrogated the cytotoxicity in this assay. To evaluate monocyte participation in the bacterial activation of lymphocytes, an antiserum to TNF was incubated with bacteria and lymphocytes in culture. The antiserum had no effect on activation of lymphocytes by any bacteria tested (data not shown).

To rule out the possibility that TNF was not completely inhibited by TNF antiserum, the direct effects of TNF on M14 targets were assessed. Incubation of TNF with M14 targets for 24 h had no effect on proliferation (Table 4). In addition, M14 targets were resistant to exogenously added TNF at 1,000 U/ml.

DISCUSSION

Previous studies have indicated that gram-negative bacteria rapidly activate the cytotoxic capacity of human PBL. Bacterium-enhanced PBL cytotoxicity at 24 h was comparable with IL-2-activated cytotoxicity. However, bacterial activation peaked rapidly after 24 h of incubation, a result which contrasts markedly with the kinetics of IL-2 activation.

The results of this investigation demonstrate that low-density LGL fractions were substantially enriched for cells capable of activation by periodontal bacteria. Phenotypi-

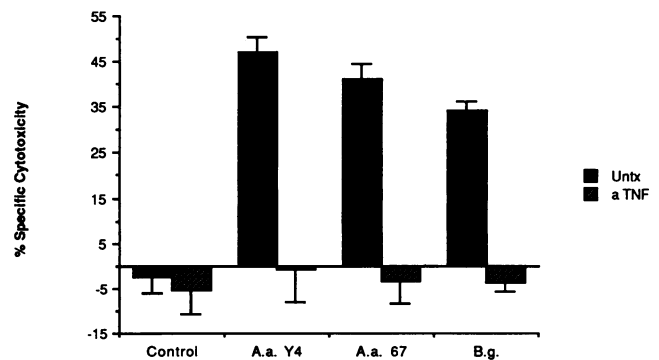


FIG. 5. Bacterial activation of monocytes to produce TNF. Bacteria were added in a 10:1 bacterium-to-monocyte ratio. Antiserum to TNF (a TNF; 1:1,000) abrogated cytotoxicity. Abbreviations and symbols are as defined in the legend to Fig. 4.

TABLE 4. Effects of TNF on M14 targets at 24 h

Treatment	Mean cytotoxicity (% <sup>51</sup> Cr release) ± SD	Mean thymidine uptake (cpm) ± SD
Control	24.55 ± 1.29	71,710 ± 1,949
TNF (1,000 U/ml)	24.92 ± 0.86	64,862 ± 4,197

cally, Leu-11<sup>+</sup> and NKH-1<sup>+</sup> cells mediated the vast majority of cytotoxicity after activation. These data agree with those of Tarkkanen et al. (28), who found that NK cells made up the lymphocyte subpopulation primarily activated by *Salmonella* bacteria. Therefore, I sought to delineate the mechanism whereby bacteria activate NK cells.

Despite differences in IL-2 activation and bacterial activation kinetics, the autocrine effect of rapid IL-2 release after bacterial contact is a plausible explanation for the enhanced NK cytotoxicity against cell targets. Tarkkanen et al. (28) did not find increases in IL-2 receptor expression during 96 h of lymphocyte culture with *Salmonella* bacteria; therefore, other methods were utilized to assess the role of IL-2 in the activation process. The effects of antibodies against IL-2 and its receptor were measured. Anti-Tac had a minimal effect on the blocking of bacterial activation, indicating that this molecule does not participate in recognition between NK cells and bacteria. However, this hypothesis does not exclude the possibility that IL-2 participates in the activation process. Ortaldo et al. (20) demonstrated that the Tac antigen is not involved in the augmentation of LGL cytolysis induced by IL-2. The finding that anti-IL-2 antiserum was effective in partially inhibiting activation, while anti-Tac was not, supports this observation and suggests that although bacteria did not activate via the Tac antigen, IL-2 was released as a result of contact between NK cells and bacteria. It remains possible, however, that IL-2 reacts with a second human IL-2-binding protein lacking reactivity with anti-Tac, as recently described on LGL (6).

When IL-2 units in culture supernatants were measured by enzyme-linked immunosorbent assay, higher levels were quantitated in supernatants from bacterial cultures than those from untreated cultures. These levels were 5 to 6 National Institute of Health units per culture. Although bacteria induced low levels of IL-2 in the supernatants, the induction did not appear to be responsible for the entire activation, because the level of activation was usually much higher than what 6 U/ml would have caused (Fig. 2). In addition, the anti-IL-2 antiserum was effective in neutralizing approximately 100 U of exogenously added IL-2, yet not all cytotoxicity was blocked in bacterium-activated cultures.

The possibility that bacteria stimulated T cells via the T-cell receptor was tested. Antibody against the T-cell receptor was added during culture to block activation via this molecule. Activation was not inhibited, which suggested that T cells did not participate in the activation phenomenon. However, in T cells, an alternative pathway of activation via the sheep erythrocyte receptor protein (T11) has also been demonstrated (15). It has been suggested that this molecule is responsible for the polyclonal activation of T lymphocytes by a variety of bacteria and viruses (15). As the T11 molecule is also expressed on some NK cells, its role in bacterial activation was investigated. A monoclonal antibody directed against T11 did not block activation by any bacteria tested. It remains possible, although unlikely, that OKT-11 antibody binds a T11 epitope that was not stimulated by the bacterial antigens selected in this study.

The present data support the observations of Tarkkanen et al. (28), who demonstrated that DNA synthesis was not required for activation. The addition of glutaraldehyde-fixed periodontal bacteria did not induce the proliferation of PBL at any time during a 1-week culture period. Cytotoxicity peaked on day 1 of culture, although modest but significant cytotoxicity was noted on day 3 with some bacteria. By day 7, cytotoxicity of cultures with bacteria was 10-fold less than that of IL-2 cultures. This finding suggests that cytotoxic effectors on day 3 did not have to proliferate to be cytolytic and contrasts with the observations of Grimm and Wilson (9), who demonstrated that activation of lymphokine-activated killers requires proliferation. These data also contrast with the findings of Banck and Forsgren (2), who demonstrated that many bacteria, including gram-negative bacteria, were primarily mitogenic for B cells and unseparated PBL. However, they found a maximal response at 3 to 4 days and did not test any of the bacteria used in this study. Miller et al. (16) also found that only non-T cells are stimulated by LPS from gram-negative bacteria, but in their study, peak stimulation occurred at 7 to 9 days of culture.

LPS (endotoxins) isolated from gram-negative periodontal bacteria, including some of the bacteria tested in this study, have been shown to be potent activators of NK activity (Lindemann et al., in press). Therefore, surface-bound LPS on glutaraldehyde-fixed bacteria was considered a likely candidate to provide the activation stimulus to lymphocytes in culture. It has been demonstrated that the antibiotic PB readily binds to LPS and can neutralize endotoxin toxicity (17). It has been shown that PB modifies the human neutrophil response to endotoxin (4). The addition of 100 µg of PB per ml per bacteria-lymphocyte culture abrogated the inductive effects of all bacteria tested. Culture supernatants collected after 24 h of incubation with *A. actinomycetemcomitans* Y4 also activated autologous PBL; however, PB eliminated this response completely. These data again suggest that IL-2 measured in supernatants was not totally responsible for NK activation and that the major activation signal was provided by LPS. However, there may be a direct LPS enhancement plus secondary cytolytic activation by IL-2. Also, whole LPS or LPS moieties are apparently shed by some fixed bacteria into the culture media, and these LPS or LPS moieties can elicit a similar activation response. Transmission electron microscopy has demonstrated that large numbers of LPS vesicles are found in the external environment of *A. actinomycetemcomitans* (12). This finding is contrary to the observations of Tarkkanen et al. (29) with LPS from *Salmonella* bacteria. They found that LPS blocks activation by whole bacteria. However, there are differences between enteric and oral bacteria that could be responsible for the apparent contradiction. *A. actinomycetemcomitans* is relatively low in 3-deoxy-D-manno-2-octulosonic acid, and its O-antigen chain length exhibits far less heterogeneity than that from enteric bacteria does (10; C. I. Hoover and S. J. Fisher, J. Dent. Res. 64:371, 1985).

The mechanism whereby LPS activates NK cells is unknown. Salata et al. (26) observed that LPS enhance NK activity by increasing the binding of NK cells to tumor targets and by promoting more efficient killing. Kang et al. (Y. H. Kang, M. Carl, P. Grimley, L. Watson, and L. Yaffe, J. Histochem. Cytochem. 34:1356, 1986) postulated that LPS may induce NK interferon release, which would have a subsequent autocrine effect on NK cells. Tarkkanen et al. (29) found that bacterial activation may occur by an interferon-independent mechanism. In the present study, activation occurred with both pathogenic (*A. actinomycetemcomi-*

*tans* and *B. gingivalis*) and nonpathogenic (*H. aphrophilus* and *H. segnis*) periodontal bacteria, which suggests that other mechanisms may modify host tissue responses. Additionally, it remains unclear why activation is terminated so quickly despite the continued presence of bacterial surface LPS during 1-week culture periods. Perhaps the fact that IL-2 receptors are not induced by bacteria (28) prevents further autocrine activation, or IL-2 levels are too low to induce proliferation.

Activation occurred in the presence or absence of monocytes. Although it appears that cell surface LPS directly activates NK cells, the possibility that LPS stimulated monocytes contaminating PBL preparations was considered. A recent report has demonstrated that the monocyte product, TNF, increases the lytic capacity of NK cells and enhances the capacity of NK cells to be activated by IL-2 (21). It has been shown that whole bacteria stimulate monocyte TNF production, and TNF in turn may act on NK cells or directly on target cells. Therefore, the effects of a TNF antiserum on bacterial activation were tested. This antiserum had no effect on the activation process, suggesting that target killing was mediated by NK cells and not by monocytes. In addition, if monocytes secreted another factor in culture which augmented NK cells, the effects of that factor should have been manifested when culture supernatants were tested. Although supernatants from *A. actinomycetemcomitans* Y4 activated PBL, no such augmentation was observed when supernatant cultures were treated with PB. Finally, when TNF was added to M14 cell cultures, it was not directly cytotoxic to targets nor did it inhibit proliferation.

The significance of bacterial activation has not been completely established. The bactericidal activity of NK cells or bacterium-activated cells against enteric bacteria was not found to be significant (29). It is likely that modest direct killing of bacteria occurs after NK activation (18), but certainly not to the degree that neutrophils kill bacteria. Perhaps NK cells provide a secondary defense against microbial agents. It is intriguing to speculate on the negative aspects of NK activation. Bacterium- or virus-activated NK cells may kill innocent bystander host cells by the release of cytotoxic factors (32) or by direct binding and killing (14). Additionally, as it is proposed that NK cells regulate immune responses by eliminating antigen-presenting cells (1), activation of NK cells may impair an effective antibody response.

In conclusion, this study has demonstrated that gram-negative whole bacteria fixed with glutaraldehyde activated NK cells by bacterial cell surface LPS. This activation induced the modest release of IL-2 which was not shown to be responsible for the majority of cytotoxicity.

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