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Activation of the Neutrophil Bactericidal Activity for Nontypable *Haemophilus influenzae* by Tumor Necrosis Factor and Lymphotoxin

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ABSTRACT

Previous studies have suggested that, *in vivo*, activated T lymphocytes and neutrophils are important in immunity to nontypable *Haemophilus influenzae*. We now extend this work by showing that neutrophils pretreated with products of activated T lymphocytes or activated macrophages show significantly enhanced killing of nontypable *H. influenzae*. Lymphotoxin, a product of activated T lymphocytes, significantly enhanced the neutrophil-mediated killing of nontypable *H. influenzae*, and tumor necrosis factor, produced by activated T lymphocytes as well as macrophages stimulated by activated T lymphocytes, also significantly increased the bactericidal activity of neutrophils. These cytokine-induced effects were seen with short pretreatment times of neutrophils and were maximal by 30 min. The killing of *H. influenzae* by neutrophils required the presence of heat-labile opsonins. In the absence of these opsonins, both tumor necrosis factor and lymphotoxin were unable to promote the killing of the bacteria by neutrophils. Furthermore, the results showed that tumor necrosis factor-primed neutrophils displayed significantly increased expression of CR3 and CR4 that was associated with increased phagocytosis of complement-opsonized nontypable *H. influenzae*. These cytokines may play an important role in immunity toward nontypable *H. influenzae* by stimulating neutrophil bactericidal activity. (***Pediatr Res* 37: 155–159, 1995**)

Abbreviations

TNF, tumor necrosis factor
LT, lymphotoxin
CR3, complement receptor type 3 (CD18/CD11b)
CR4, complement receptor type 4 (CD18/CD11c)
HBSS, Hanks' balanced salt solution

Nontypable *Haemophilus influenzae* is a common respiratory pathogen. It is carried in the nasopharynx of 70–80% of healthy children (1). Colonization rates increase during respiratory illness (2). In particular, colonization of the nasopharynx with nontypable *H. influenzae* may exceed 95% at the time of otitis media (3), when the organism may compose 50% of the total bacterial flora (4). Although invasive disease, such as bacteremia, is uncommon with nontypable *H. influenzae*, local infection is frequent. Nontypable *H. influenzae* is the second leading cause of acute otitis media and sinusitis and the leading cause of recurrent or chronic otitis media in children (5–8).

The protective role of antibody against nontypable *H. influenzae* respiratory infections in both humans and rat models is now questionable. High levels of antibody to this organism are present in serum, saliva, and sputum of patients with chronic bronchitis (9, 10), and in one study the level of antibody in these fluids correlated directly with the incidence of infection and mortality observed in these patients (10). This is despite the fact that bactericidal antibody to nontypable *H. influenzae* has been demonstrated and has been shown to be protective in nontypable *H. influenzae*-related otitis media (11–13). Patients with chronic bronchitis can support high colonization rates of nontypable *H. influenzae* in the presence of significant levels of bactericidal and opsonic activity in the serum (14). It has been suggested that the IgA anti-*Haemophilus* antibody in bronchopulmonary secretions blocks the bactericidal and opsonizing effects of antibody to the bacteria (15).

Using a rat model and immunization by intra-Peyer's patch injection with nontypable *H. influenzae*, it was shown that immunity could be transferred to naive recipients by T lymphocytes, but a primary protective role for antibody could not be established. Protection by T lymphocytes was manifested by increased clearance of bacteria from the lungs (16–18). No clear correlation was found between local or systemic antibody and bacterial clearance, and it was concluded that bacterial clearance is accelerated by factors that induce alterations in phagocytes (17). Immunized nude rats were less capable of

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accelerated clearance of nontypable *H. influenzae* compared with normal rats (16). These findings suggest that mechanisms for clearance of *H. influenzae* from the respiratory tract other than local or systemic antibody need to be investigated.

Recent evidence supports the concept that T lymphocytes are involved in recruiting and activating neutrophils at sites of infection (19). Czuprynski *et al.* (20) showed that immunologically specific T lymphocytes, in an antigen- and dose-dependent manner, recruited neutrophils to a specific site. The recruited neutrophils, specific for *Listeria monocytogenes*, expressed high levels of bactericidal activity. Appelberg and Silva (21) demonstrated that T cells were important for inducing a persisting neutrophilia during intraperitoneal infection with *Mycobacterium avium*. Injection of antigen- pulsed spleen cells from *M. avium*–infected mice into the peritoneal cavity of normal mice caused a neutrophil accumulation at this site. Studies with a pathogenic free-living amoeba, *Naegleria fowleri*, demonstrated that neutrophils play an important role in immunity to *Naegleria* (22). Mice immune to this amoeba after immunization with killed antigen generated neutrophils that showed enhanced responses to the tripeptide f-Met-Leu-Phe (23) when challenged a second time with the antigen. Other studies have suggested that cell-mediated immune responses may be crucial for the activation of neutrophil-mediated killing of *Nocardia asteroides* in mice (24).

We have previously found that human T lymphocytes when activated by phytohemagglutinin–phorbol myristate acetate in culture were able to activate the neutrophil oxygen-dependent respiratory burst (25). In these studies, it was demonstrated that it was necessary for T lymphocytes to directly bind the neutrophils to induce these effects and that T cells actually primed the neutrophils for an increased response to f-Met-Leu-Phe and phorbol myristate acetate (25). Some evidence also has been presented suggesting that macrophages regulate the accumulation of neutrophils during inflammation (26).

Cytokines produced exclusively or preferentially by stimulated T lymphocytes have been shown to activate a range of neutrophil functions (27). These include LT (28–31), interferon-γ (also a major product of natural killer cells) (29–34), and IL-2 (35, 36).

Other cytokines of interest that may have an effect on neutrophil activation are produced primarily by macrophages but can also be released by stimulated T cells. These include TNF (30, 33, 37–40, 41), IL-1 (27, 29), granulocyte-colony-stimulating factor (34, 42), and IL-8 (43).

All of these cytokines can, in various ways, regulate the inflammatory response by acting on the neutrophil. They have the ability to increase neutrophil adhesion to the endothelium, to attract cells to infection foci via chemotaxis, to retain leukocytes at inflammatory sites by their migration inhibitory properties, and to activate the neutrophils with a subsequent increase in antimicrobial activity. In this study, the ability of the two related cytokines TNF and LT to alter the neutrophil-mediated killing of nontypable *H. influenzae* has been investigated. The results show that both cytokines are effective in priming neutrophils to increase their bactericidal activity for nontypable *H. influenzae*.

METHODS

Bacteria. A nontypable *H. influenzae* was used in all experiments. The bacteria were grown on chocolate agar plates at 37°C for 24 h, harvested, and suspended in HBSS. The bacterial concentration was quantitated immediately before use in a hemacytometer chamber. At the same time, an inoculum was made and spread on chocolate agar for verification of numbers of viable bacteria by colony formation.

Neutrophils. Neutrophils were prepared from the blood of healthy volunteers by the rapid-single-step method (44). The blood was mixed with heparin and layered onto a high density Ficoll-Hypaque medium (1.114 g/L) and centrifuged at room temperature for 30 min at 200–600 $\times$ *g*. After centrifugation, the mononuclear leukocytes appeared in a band at the interface, the neutrophils formed a band 1 cm below, and the red cells centrifuged to the bottom of the tube. The neutrophils in the second band were harvested and washed three times in HBSS. The neutrophils were of >96% purity and >99% viability by trypan blue dye exclusion.

The study was approved by the Ethics Committee of the Women's and Children's Hospital. Informed consent was obtained from the volunteers.

Bactericidal activity. Neutrophil bactericidal activity for nontypable *H. influenzae* was carried out essentially as described previously for *Staphylococcus aureus* (45). To 100 μL of neutrophils (5×10^6) were added either 100 μL (100 U) of TNF, 200 U of LT, or 100 μL of HBSS, and the mixtures were incubated at 37°C for 30 min. After incubation, 1×10^6 nontypable *H. influenzae* were added and the volume made up to 500 μL with HBSS. Because of the sensitivity of nontypable *H. influenzae* to serum complement-mediated bactericidal activity, human serum deficient in the sixth component of complement (C6D serum) was used in the assay as an opsonin at 4% (vol/vol) final concentration. C6D serum was prepared by depleting C6 from human serum by immunoadsorption (Sigma Chemical Co., St. Louis, MO). Controls were added in which the cytokine diluent (HBSS) was used in place of cytokines. In other experiments, the cytokine preincubation time and also the cytokine concentrations were varied. The tubes were gassed with 5% $C0_2$–air mixture and incubated at 37°C with end-to-end mixing. Samples were taken at 0 and 30 min, diluted in water, and plated on chocolate agar for determination of viable bacterial count.

Flow cytometric analysis of integrin expression. The expression of integrins on neutrophil surfaces was examined as previously described (45). Neutrophils (5×10^5) were incubated at 4°C/30 min in the presence of saturating concentrations of MAb. After incubation, the cells were washed and stained at 4°C/30 min with fluorescein-conjugated second antibody (goat anti-mouse IgG, Organon Teknika Corp., West Chester, PA) in the presence of 25% (vol/vol) autologous plasma. The cells were washed and fixed with 1% (wt/vol) paraformaldehyde. Fluorescence distribution was measured in a Becton Dickinson FACS analyzer (Mountain View, CA). Fluorescence values were corrected by subtraction of values for an isotype-matched negative control. Anti-CD11a (HB 202, IgG1) was TSI/22 (45), anti-CD11b (IOM-1, IgG) was from

Immunotech, Marseilles, France, and anti-CD11c (kB90, IgG1) was from Dakopatts, Glostrup, Denmark.

Phagocytosis. Phagocytosis of nontypable *H. influenzae* was measured by a previously reported assay using FITC-labeled bacteria and flow cytometry analyses (46). The bacteria were labeled with FITC (Sigma) as previously described (46) and adjusted to 1×10^9/mL in HBSS. The bacteria (1×10^8) were then mixed with TNF-primed or diluent-treated neutrophils (5×10^6) in a total volume of 500 μL that included 4% human C6D serum. After a 20-min incubation at 37°C a 50-μL sample was taken, added to 500 μL of cold HBSS, and then treated with 0.065% trypan blue to quench fluorescence from adhered bacteria. The fluorescence due to internalized bacteria was measured on a FACSCAN (Lysis II program, Becton Dickinson, Sydney, Australia).

Cytokines. Human recombinant TNF was produced by Genentec Inc. (San Francisco, CA) and was kindly provided by Dr. G. R. Adolf, Ernst-Behringer Institute, Vienna, Austria. The recombinant TNF was produced in *Escherichia coli*, purified, and had a sp act of 6×10^7 U/mg as assayed by the supplier (cytotoxicity on actinomycin D-treated murine connective tissue cell line L929). Human recombinant LT was also from Genentec (provided by Dr. Adolf) and contained a sp act of 1.2×10^8U/mg. The preparations were >99% pure and estimated to contain <0.125 endotoxin units/mL of endotoxin.

Statistics. The data are presented as percentage of bacterial survival and analyzed using the *t* test for paired data.

RESULTS

Treatment of human neutrophils with TNF significantly increased the ability of the cells to kill nontypable *H. influenzae* when serum opsonins were present (Fig. 1). TNF induced a 5-fold decrease in the survival of the bacteria ($p < 0.01$). When serum recognition factors were inactivated by heating at 56°C/30 min, TNF failed to stimulate this activity (data not presented). The effects of TNF were evident at a TNF concentration as low as 1 U/5 $\times$ 10^6 cells ($p < 0.001$) (Fig. 2).

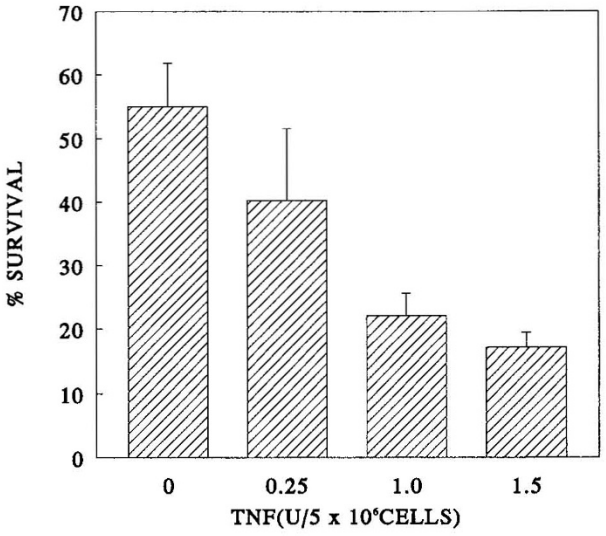

Figure 2. TNF concentration-related effects on the neutrophil bactericidal activity. Neutrophils were pretreated with the indicated concentrations of TNF and then tested for bactericidal activity. The results are the mean ± SEM of three experiments each with cells from different individuals.

The related cytokine LT was also effective in enhancing neutrophil bactericidal activity. Neutrophils pretreated with LT showed significantly enhanced bactericidal activity (Fig. 3) ($p < 0.01$). Bacterial survival was decreased approximately 4-fold by treatment with LT. Studies on the concentration-related effects showed that 50 U of LT were required to induce significant enhancement ($p < 0.05$) of bactericidal activity (Fig. 4).

Preincubation times of 30 to 60 min with TNF were most effective in enhancing neutrophil bactericidal activity (Fig 5). The degree of enhancement was gradually reduced as the preincubation time was reduced to less than 30 min, although the simultaneous addition (0 time) of TNF and bacteria still resulted in a significant ($p < 0.05$) enhancement of the neutrophil bacterial killing.

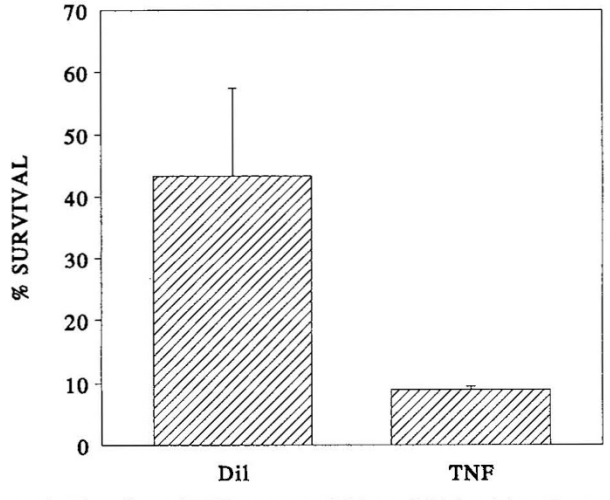

Figure 1. The effect of TNF on neutrophil bactericidal activity. Neutrophils were pretreated with either diluent (*Dil*) or 100 U of TNF and then examined for bactericidal activity. The results are presented as means ± SEM of our experiments each with cells from a different individual.

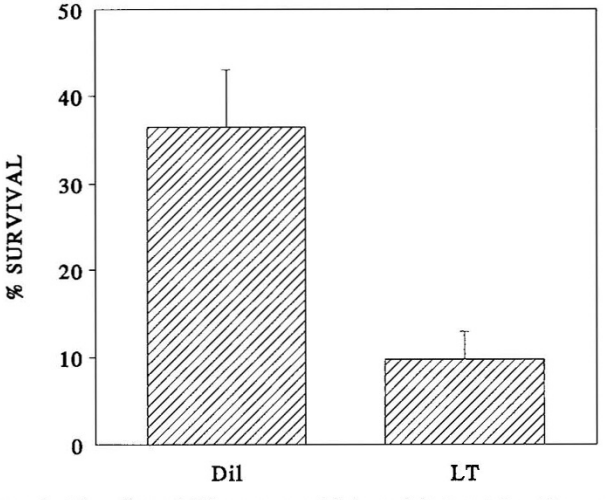

Figure 3. The effect of LT on neutrophil bactericidal activity. Neutrophils were treated with either 250 U of LT or diluent (*Dil*) and then tested for bactericidal activity. The results are presented as the mean ± SEM of four experiments each conducted with cells from different individuals.

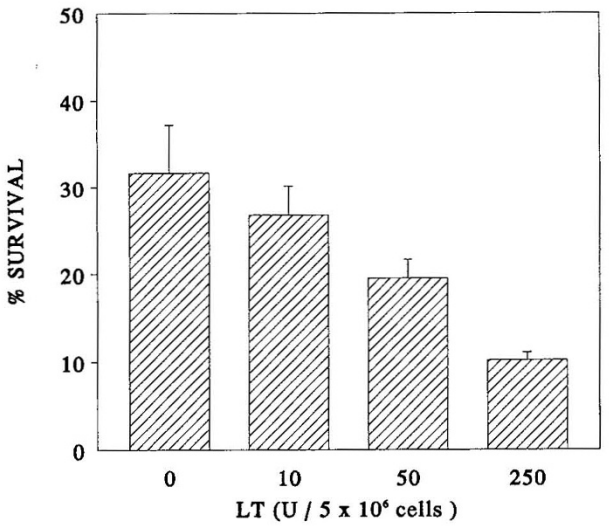

Figure 4. LT concentration-related effects on the neutrophil bactericidal activity. Neutrophils were pretreated with varying concentrations of LT and then tested for bactericidal activity. The results are mean ± SEM of three determinations each with cells from the same individual.

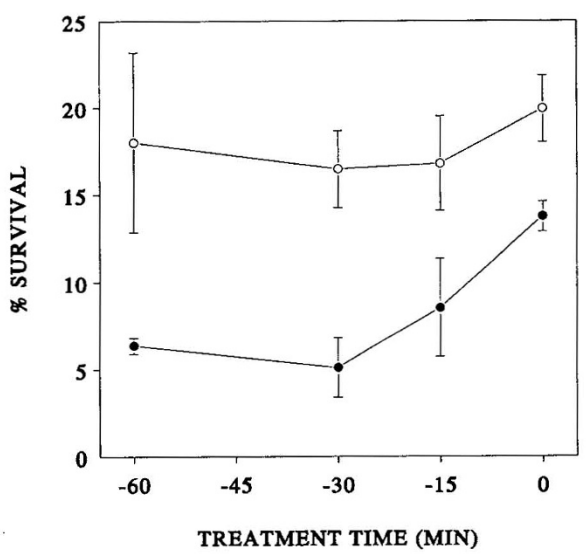

Figure 5. The effect of different TNF pretreatment times on neutrophil activation. Neutrophils were pretreated for 60, 30, 15, and 0 min with either TNF (●) or diluent (○). The results are presented as mean ± SEM of three experiments.

Additional studies showed that, under these conditions, TNF enhanced the surface expression of the neutrophil integrin receptors CR3 ($p < 0.001$) and CR4 ($p < 0.01$) (Fig. 6). However, TNF was unable to cause a significant change in the expression of the other integrin receptor, leukocyte functional antigen-1 (CD18/CD11a) (data not presented). Associated with this change in CR3 and CR4 expression, the TNF-primed neutrophils showed significantly enhanced ($p < 0.05$) phagocytosis of nontypable *H. influenzae* (Fig. 6).

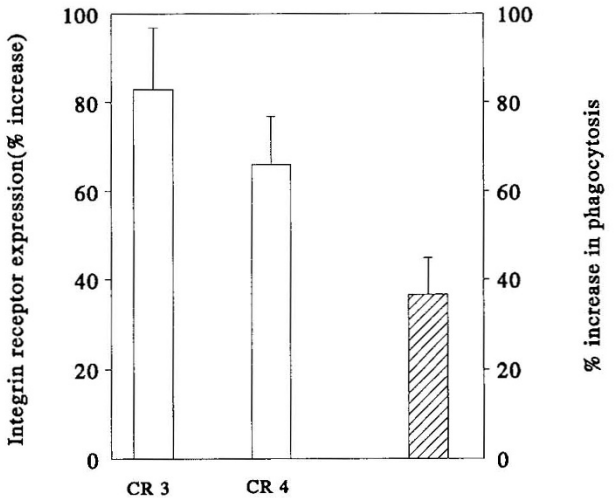

Figure 6. TNF-induced changes in CR3 and CR4 expression and in phagocytosis of *H. influenzae*. Expression of CR3 and CR4 was evaluated by flow cytometry using antibodies to CD11b and CD11c (α-chain), respectively. The results are presented as the percentage increase in CR3/CR4 expression (□) and as the percentage increase in bacterial phagocytosis (▨) due to TNF-neutrophil priming. The data are presented as mean ± SEM of four experiments.

DISCUSSION

The results demonstrate that preexposure of human neutrophils to TNF or LT significantly increases their ability to kill nontypable *H. influenzae*. The concentrations of cytokines required to achieve enhancement of neutrophil bactericidal activity were within the physiologic concentrations achieved *in vivo* (46). Inasmuch as both TNF and LT were effective in this system, the results raise the possibility that macrophages, the major producers of TNF, and T lymphocytes, responsible for producing LT, may play an important role in immunity to nontypable *H. influenzae*. Our results may explain previous findings that showed that activated T lymphocytes and neutrophils were important for increasing the clearance of *H. influenzae* in rat lungs (16). From our studies, we suggest that these T lymphocytes enhance the neutrophil bactericidal activity against *H. influenzae* both by the release of LT and the activation of macrophages for release of TNF.

The effects of TNF and LT were seen in the presence of heat-labile serum factors. These were required for neutrophil killing of *H. influenzae*. When these factors were absent, neither TNF- nor LT-treated neutrophils showed any bactericidal activity. This is similar to our previous findings with *S. aureus* (45). Interaction of TNF and LT with neutrophils leads to increased expression of CR3 (45, 48), which allows the interaction of neutrophils with the C3bi opsonized bacteria. We have confirmed that TNF increases the expression of both CR3 and CR4 and now demonstrate that this enhancement of expression of CR3 and CR4 leads to enhanced phagocytosis of complement-coated *H. influenzae*. The increase in the number of bacteria phagocytosed and the well-established associated increase in the ability of the TNF/LT-primed neutrophils to show increased release of oxygen-derived reactive species and release of lysosomal enzymes (27) are most likely responsible for the enhanced microbial killing seen.

The role of antibody in immunity and protection from nontypable *H. influenzae* infection remains unknown (16–18), and it appears that T lymphocytes and production of neutrophil-activating cytokines are important in immunity against this organism (16–18). T lymphocytes are important in recruiting macrophages and neutrophils in the lungs and, as demon-

strated by our studies, they activate neutrophils by releasing lymphokines, leading to increased neutrophil killing of nontypable *H. influenzae* and possibly clearance of the bacteria from the lungs. Our data suggest that identification of *H. influenzae* antigens that stimulate T cells to produce cytokines such as LT could prove to be useful in the search for appropriate vaccine antigens.

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