Release of Leukotriene B\(_4\) from Human Neutrophils after Interaction with Nontypable \textit{Haemophilus influenzae}

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Opsonization of nontypeable \textit{Haemophilus influenzae} with antibody is critical for the interaction between the organism and human polymorphonuclear leukocytes (PMNs). Nontypeable \textit{H. influenzae} opsonized in fresh antibody-positive serum induced the release of 42.5 ± 17.9 ng of leukotriene \(B_4\) per ml from PMNs after 20 min of incubation at 37°C. On the other hand, opsonization of the organisms in fresh antibody-negative serum stimulated the release of significantly smaller amounts of leukotriene \(B_4\) by the PMNs. Simultaneous determinations of phagocytosis demonstrated similar patterns of response. A small amount (26.7 ± 7.6%) of unopsonized nontypeable \textit{H. influenzae} was phagocyted by PMNs during 20 min of incubation at 37°C. In contrast, 89.3 ± 2.0% of nontypeable \textit{H. influenzae} opsonized in fresh antibody-positive serum was phagocyted during the same incubation period (\(P < 0.001\)). Removal of complement through heat inactivation at 56°C for 30 min did not significantly affect phagocytosis. These data suggest that the humoral immune response to nontypeable \textit{H. influenzae} plays an important role in the inflammatory process and may contribute to the production of middle ear effusions in otitis media.

Nontypeable \textit{Haemophilus influenzae} is a common respiratory pathogen. It is carried in the nasopharynges of 10 to 35% of healthy children (15, 22), but the use of a more selective medium allows detection of the organism in 70 to 80% of nasopharyngeal samples from healthy children (19). Colonization rates increase during respiratory illness (14). In particular, colonization with nontypeable \textit{H. influenzae} may exceed 95% at the time of otitis media (8), when the organism may compose 50% of the total bacterial flora (21). Although invasive disease, such as bacteremia, is uncommon with nontypeable \textit{H. influenzae}, local infection is frequent. In fact, nontypeable \textit{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 (1, 3, 12, 20).

Serum antibody against nontypeable \textit{H. influenzae} is absent in the early phase of an ear infection and develops during convalescence (6). The immune response appears to be strain specific (18, 26). Less is known about the local immune response to nontypeable \textit{H. influenzae}. Specific anti-nontypeable \textit{H. influenzae} immunoglobulins G, M, and A have been detected in middle ear effusions of children with otitis media, but the functional properties of these antibodies are not known at this time (7, 27).

The presence of inflammatory mediators has also been detected in studies of middle ear disease due to nontypeable \textit{H. influenzae} (2). Leukotriene \(B_4\) (LT\(B_4\)) is a primary product of arachidonic acid (AA) metabolism detected in middle ear effusions (17). It is released from human polymorphonuclear leukocytes (PMNs), induces chemotaxis and chemokinetic migration of PMNs, and stimulates PMNs to degranulate, release lysosomal enzymes, and produce radicals of oxidative metabolism (9). In addition, LT\(B_4\) may modulate T-cell activity (26, 27) and promote fluid extravasation by increasing vascular permeability (5).

The present study was designed to investigate the interaction between human neutrophils and nontypeable \textit{H. influenzae} and to measure the release of LT\(B_4\) during phagocytosis.

**MATERIALS AND METHODS**

Preparation of PMNs. Human leukocytes were obtained from 20 to 40 ml of heparinized venous blood from healthy adult volunteers after signed informed consent was obtained. The volunteers were not on any medications for 1 week prior to donation of blood. After dextran-Hypaque sedimentation of the erythrocytes, the PMNs were separated by differential density centrifugation on Ficoll-Hypaque at 500 × g for 20 min. Erythrocyte contamination was removed by sequential washing with hypotonic and hypertonic saline solutions. PMNs suspended in Hanks' balanced salt solution (HBSS)-gel were more than 95% pure and 90% viable.

**Bacteria.** Nontypeable \textit{H. influenzae} (P.W. 3943) was isolated from the middle ear fluid of a child with otitis media with effusion. The bacterium was grown on chocolate agar plates at 37°C in 5% \(CO_2\). Several colonies were placed in 4.5 ml of brain heart infusion (BHI) broth with hemin and NAD and incubated overnight at 37°C in 5% \(CO_2\). The following day, 0.5 ml was transferred to fresh BHI broth and incubated under the same conditions until the mid-log phase of growth was reached. Bacteria were then centrifuged at 1,200 × g for 15 min, separated, and washed three times in HBSS-gel.

**LT\(B_4\) assay.** One milliliter of a PMN suspension (3 × 10\(^7\) PMNs) was preincubated at 37°C for 5 min and then mixed with 1 ml (3 × 10\(^8\) to 12 × 10\(^8\) CFU) of the bacteria in the presence of AA and incubated at 37°C in a shaking water bath. The reaction was stopped by the addition of 1.5 ml of ice-cold methanol. Twenty nanograms of prostaglandin \(B_2\) (PGB\(_2\)) was added as an internal standard for high-performance liquid chromatography (HPLC) analysis. The resultant mixture was centrifuged at 1,200 × g for 15 min at 4°C.

The supernatant was applied to a C\(_18\) reversed-phase extraction cartridge (Sep-Pak C18 from Waters Associates-Millipore Corp., Milford, Mass.) which had been previously...
washed with 10 ml of distilled HPLC-grade water and activated with 10 ml of HPLC-grade methanol. After the sample was placed on the extraction cartridge, the cartridge was washed with 10 ml of distilled water and the leukotrienes were eluted with 10 ml of 70% methanol. The methanol fraction was evaporated under vacuum in a rotary evaporator (Rotavapor; Buchi Laboratoriums-Technik AG, Flawil, Switzerland), and the residue was redissolved in 100 µl of 30% methanol and stored at −70°C until HPLC was performed.

The samples were injected into a C18 reversed-phase column (Beckman, San Ramon, Calif.) and eluted isocratically at a flow rate of 1 ml/min in an HPLC system (Pharmacia, Piscataway, N.J.) with a solvent system of methanol-water-acetic acid (70:28:2, vol/vol/vol) adjusted to pH 5.5 by ammonium hydroxide. The eluate was monitored at 280 nm by a wavelength spectrometric detector (Pharmacia). For quantitation of the concentration of LTB₄ in the samples, different mixtures of purified LTB₄ and PGB₂ were prepared in HBSS-gel and processed as described above. In this way, the calibration curve was made by charting the ratio of peak heights (LTB₄/PGB₂) as a function of the LTB₄ concentrations. The ratio of the absorption peak height of LTB₄ to that of PGB₂ in each of the reversed-phase HPLC samples was referred to the calibration curve to quantify the LTB₄. Each sample was tested in duplicate.

**Bactericidal antibody assay.** The organism to be used in the assay was grown on chocolate agar and transferred to a BHI broth as described above. The suspension containing the organism was diluted in Gey’s balanced salt solution with 10% bovine serum albumin (BSA) to yield 5 × 10⁸ organisms per ml. The test mixture contained 0.02 ml of bacterial suspension, 0.02 ml of heat-inactivated serum, 0.02 ml of complement source (serum), and 0.04 ml of Gey’s balanced salt solution-BSA. Serum was heat inactivated at 56°C for 30 min to remove complement. Hypogammaglobulinemic serum served as an exogenous source of complement and had been pretreated to prove the absence of any bactericidal activity. Controls included bacteria suspended in Gey’s balanced salt solution-BSA alone and bacteria prepared with antibody-negative serum. The lowest dilution of serum tested in the assay was 1:5. The preparation was incubated for 1 h at 37°C in 5% CO₂. One hundred microliters was then plated onto chocolate agar and incubated overnight at 37°C in 5% CO₂. The number of colonies appearing on the plate was counted and compared with the bacterial control in order to determine the percent of organisms killed. The reciprocal of the highest dilution of serum that killed 50% of the organisms was the titer of antibody.

**Phagocytosis assay.** The radioisotope [methyl-³H]thymidine was added to BHI broth in which nontypeable *H. influenzae* organisms were to be grown overnight. Radiolabeled bacteria were washed three times in HBSS-gel and opsonized for 15 min at 37°C in 10% human pooled serum known to contain nontypeable *H. influenzae*-specific antibody. Opsonized bacteria were resuspended in HBSS-gel at a concentration of 5.0 × 10⁸ CFU/ml. PMNs were prepared at a concentration of 5 × 10⁶ cells/ml in HBSS-gel. PMNs (0.5 ml) were added to 0.5 ml of the bacterial suspension in polypropylene vials (Bio-Vial; Beckman Instruments, Chicago, Ill.). The mixture was rocked at 37°C. At 3, 10, and 20 min of incubation, 3 ml of cold HBSS-gel was added to stop the reaction and the mixture was centrifuged at 160 × g for 5 min in order to pellet the PMNs and separate the cells from the extracellular organisms. This procedure was repeated twice before the mixture was finally centrifuged at 1,400 × g.

The supernatant fluid was decanted, and the cell pellet was dissolved in 0.3 ml of Protosol and 3 ml of Liquifluor (New England Nuclear, Boston, Mass.). The preparation was counted in a beta liquid scintillation counter (LS6000; Beckman Instruments). The control specimen, 0.5 ml of PMNs and 0.5 ml of opsonized bacteria, was incubated for 20 min at 37°C and immediately centrifuged at 1,400 × g. The pellet was then processed as above. The percentage of bacteria phagocytosed by the PMNs was calculated by the following formula: percent uptake = (cpm in sample cell pellet/cpm in control cell pellet) × 100, where cpm is counts per minute.

**Reagents.** HBSS buffered to pH 7.4 with 10 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES) buffer and containing 0.1% gelatin (HBSS-gel) was used to wash and to suspend cells. Bacteria were grown in BHI broth (BBL, Cockeysville, Md.) containing 10 µg of hemin (Kodak, Rochester, N.Y.) per ml and 10 µg of NAD per ml. Ficoll-Hypaque and dextran-Hypaque were prepared according to the method of Boyum (9). AA (5.8,11,14-eicosatetraenoic acid from porcine liver) and PGB₂, were purchased from Sigma Chemical Co. St. Louis, Mo. HPLC solvents were obtained from Aldrich Chemical Co., Milwaukee, Wis. Purified LTB₄ was a generous gift from J. Rokach, Merck Frost, Montreal, Quebec, Canada.

**Statistical analysis.** The amounts of leukotriene released were expressed as the means ± standard errors of the means (SEMs) for three to four different experiments. The percent of phagocytosis was expressed as the mean ± SEMs for three experiments. Means were compared by using twotailed Student’s *t* test.

**RESULTS**

**LTB₄ release.** (i) Effect of AA. Incubation of PMNs with opsonized nontypeable *H. influenzae* in the presence of AA produced LTB₄ as the major metabolite detectable at 280 nm of UV absorption. The amount of LTB₄ released at 20 min of incubation correlated directly with the concentration of AA. In the absence of AA or in concentrations less than 50 µM, no chromatographic peaks with the retention time of synthetic LTB₄ were detected. On the other hand, concentrations higher than 400 µM AA did not augment further the release of LTB₄. In the absence of organisms, increasing concentrations of AA resulted in less than 12 ng of LTB₄ release per ml (*P < 0.001*). One hundred micromolar AA was chosen for use in subsequent experiments.

(ii) Effect of bacterial concentration (Fig. 1). The smallest number of bacteria able to stimulate LTB₄ release from 3 × 10⁸ human granulocytes after 20 min of incubation was on the order of 3 × 10⁶ CFU (bacteria/PMNs, 10:1). Increasing concentrations of nontypeable *H. influenzae* stimulated greater release of LTB₄.

(iii) Kinetics (Fig. 2). Release of LTB₄ began immediately upon addition of nontypeable *H. influenzae*. Peak production occurred at 15 min. Release of LTB₄ returned to baseline at 60 min.

(iv) Effect of opsonization (Fig. 3). Unopsonized bacteria generated little or no LTB₄ compared with fully opsonized bacteria (*P < 0.05*). Nontypeable *H. influenzae* opsonized in fresh antibody-positive serum stimulated the release of more than 40 ng of LTB₄ per ml. Inactivation of the complement component had minimal effect on LTB₄ release (no significant difference). Similarly, fresh antibody-negative serum stimulated the release of LTB₄ in amounts similar to unopsonized bacteria (*P < 0.05*).

**Phagocytosis.** To study the physical interaction between
human PMNs and nontypeable *H. influenzae*, the organisms were preopsonized in 10% antibody-positive pooled human serum, antibody-negative hypogammaglobulinemic serum, or HBSS-gel alone for 30 min at 37°C. Uptake of the bacteria by PMNs was measured at 3, 10, and 20 min of incubation. As seen in Fig. 2, 15% of the organisms were phagocytosed at 3 min, 61% were phagocytosed at 10 min, and 89% were phagocytosed at 20 min when organisms were opsonized in fresh antibody-positive serum. Elimination of complement through heat inactivation had no effect on phagocytosis (Fig. 4). Opsonization of nontypeable *H. influenzae* in fresh antibody-negative serum resulted in a lesser degree of phagocytosis (*P* < 0.05). Less than 30% of unopsonized organisms were taken up during the incubation period (*P* < 0.001). These data suggest that antibody alone was critical for the phagocytosis of nontypeable *H. influenzae*. The observations are depicted in the light micrographs seen in Fig. 5a to d.

**DISCUSSION**

LTB₄ is the major product of AA metabolism produced by the lipoxygenase pathway in human phagocytes. In vitro, LTB₄ promotes chemotaxis of neutrophils, eosinophils, and
FIG. 5. Light microscopy of the phagocytic process with nontypeable \textit{H. influenzae}. PMNs (3 \times 10^7) were incubated with 12 \times 10^8 bacteria at 37°C for 20 min. (a) Antibody and complement; (b) antibody alone; (c) complement alone; (d) no opsonization.

macrophages (30). Recently, LTB\textsubscript{4} instilled into subsegments of normal human lung was shown to induce PMN migration in vivo without causing changes in protein permeability of the respiratory epithelial surface (24). These findings provide a role for LTB\textsubscript{4} in the host response to infections through the recruitment of neutrophils from the bloodstream into the tissue.

Clinical studies of otitis media, conducted by us as well as by others, have documented the presence of LTB\textsubscript{4} in middle ear effusions of more than 50% of children with active disease and in concentrations that approximate those detected in the present study (2, 17). As seen in the present report, LTB\textsubscript{4} release by PMNs paralleled phagocytosis of \textit{H. influenzae}. The present study also demonstrated the importance of antibody in the phagocytosis of nontypeable \textit{H. influenzae}. Although complement augmented bacterial uptake, it was not essential. This observation is consistent with the findings of Musher et al. (25), who additionally observed some variability of complement requirement among a small number of \textit{H. influenzae} isolates. Animal models have
corroborated the importance of antibody eradicating infection with nontypeable *H. influenzae*. In particular, antibody markedly enhanced clearance of the organism from the lungs of infected mice (11).

It is interesting to address the question of whether there are specific phospholipid sources of AA used for leukotriene biosynthesis in human neutrophils during phagocytosis. The requirement for exogenous AA seen in the present study suggests that PMNs incorporate free AA released at the inflammatory site by other cells and metabolize it to LTB₄ (31). It also has been reported that PMNs release AA from membrane phospholipids in response to phagocytosis (32) and chemotactic factors (13). Furthermore, the recent demonstration of active transfer of AA between different types of cells (4, 10, 23) has particular interest in light of the pleocytosis seen during acute and convalescent phases of middle ear disease (16). Although the release of inflammatory mediators is a physiological function of PMNs, the uncontrolled release of these potent factors at the site of infection provokes pathological local (tissue destruction) and systemic (increase in vascular permeability, vasodilatation, and shock) reactions. Therefore, the concentration of active inflammatory mediators must be regulated. The human neutrophil has the capacity to both synthesize and catabolize LTB₄. After active LTB₄ is bound by PMNs, it is internalized and inactivated by omega oxidation (9). Although we did not measure the concentration of omega oxidation metabolites of LTB₄ in our assay, the kinetics of the LTB₄ concentrations at different times suggested a decreased synthesis and/or release over 30 min. The influence of nontypeable *H. influenzae* with regard to release and subsequent inactivation of LTB₄ and the importance of strain variation and structural components of the bacterium need to be investigated.

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REFERENCES


