

## Release of Leukotriene B<sub>4</sub> from Human Neutrophils after Interaction with Nontypeable *Haemophilus influenzae*

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**Opsonization of nontypeable *Haemophilus influenzae* with antibody is critical for the interaction between the organism and human polymorphonuclear leukocytes (PMNs). Nontypeable *H. influenzae* opsonized in fresh antibody-positive serum induced the release of  $42.5 \pm 17.9$  ng of leukotriene B<sub>4</sub> 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<sub>4</sub> by the PMNs. Simultaneous determinations of phagocytosis demonstrated similar patterns of response. A small amount ( $26.7 \pm 7.6\%$ ) of unopsonized nontypeable *H. influenzae* was phagocytosed by PMNs during 20 min of incubation at 37°C. In contrast,  $89.3 \pm 2.0\%$  of nontypeable *H. influenzae* opsonized in fresh antibody-positive serum was phagocytosed 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 *H. influenzae* plays an important role in the inflammatory process and may contribute to the production of middle ear effusions in otitis media.**

Nontypeable *Haemophilus influenzae* is a common respiratory pathogen. It is carried in the nasopharynx 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 *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 *H. influenzae*, local infection is frequent. In fact, nontypeable *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 *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 *H. influenzae*. Specific anti-nontypeable *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 *H. influenzae* (2). Leukotriene B<sub>4</sub> (LTB<sub>4</sub>) 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, LTB<sub>4</sub> may modulate T-cell activity (26, 27) and promote fluid extravasation by increasing vascular permeability (5).

The present study was designed to investigate the inter-

action between human neutrophils and nontypeable *H. influenzae* and to measure the release of LTB<sub>4</sub> 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  $500 \times g$  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 *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<sub>2</sub>. 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<sub>2</sub>. 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  $1,200 \times g$  for 15 min, separated, and washed three times in HBSS-gel.

**LTB<sub>4</sub> assay.** One milliliter of a PMN suspension ( $3 \times 10^7$  PMNs) was preincubated at 37°C for 5 min and then mixed with 1 ml ( $3 \times 10^8$  to  $12 \times 10^8$  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<sub>2</sub> (PGB<sub>2</sub>) was added as an internal standard for high-performance liquid chromatography (HPLC) analysis. The resultant mixture was centrifuged at  $1,200 \times g$  for 15 min at 4°C.

The supernatant was applied to a C<sub>18</sub> reversed-phase extraction cartridge (Sep-Pak C18 from Waters Associates-Millipore Corp., Milford, Mass.) which had been previously

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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-Schweiz, Switzerland), and the residue was redissolved in 100  $\mu$ l of 30% methanol and stored at  $-70^{\circ}\text{C}$  until HPLC was performed.

The samples were injected into a  $\text{C}_{18}$  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  $\text{LTB}_4$  in the samples, different mixtures of purified  $\text{LTB}_4$  and  $\text{PGB}_2$  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 ( $\text{LTB}_4/\text{PGB}_2$ ) as a function of the  $\text{LTB}_4$  concentrations. The ratio of the absorption peak height of  $\text{LTB}_4$  to that of  $\text{PGB}_2$  in each of the reversed-phase HPLC samples was referred to the calibration curve to quantify the  $\text{LTB}_4$ . 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  $5 \times 10^4$  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  $56^{\circ}\text{C}$  for 30 min to remove complement. Hypogammaglobulinemic serum served as an exogenous source of complement and had been pretested 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  $37^{\circ}\text{C}$  in 5%  $\text{CO}_2$ . One hundred microliters was then plated onto chocolate agar and incubated overnight at  $37^{\circ}\text{C}$  in 5%  $\text{CO}_2$ . 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*- $^3\text{H}$ ]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  $37^{\circ}\text{C}$  in 10% human pooled serum known to contain nontypeable *H. influenzae*-specific antibody. Opsonized bacteria were resuspended in HBSS-gel at a concentration of  $5.0 \times 10^7$  CFU/ml. PMNs were prepared at a concentration of  $5 \times 10^6$  cells per 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  $37^{\circ}\text{C}$ . 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  $160 \times g$  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  $1,400 \times g$ .

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  $37^{\circ}\text{C}$  and immediately centrifuged at  $1,400 \times g$ . 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)  $\times 100$ , 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  $\mu\text{g}$  of hemin (Kodak, Rochester, N.Y.) per ml and 10  $\mu\text{g}$  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  $\text{PGB}_2$  were purchased from Sigma Chemical Co., St. Louis, Mo. HPLC solvents were obtained from Aldrich Chemical Co., Milwaukee, Wis. Purified  $\text{LTB}_4$  was a generous gift from J. Rokach, Merck Frost, Montreal, Quebec, Canada.

**Statistical analysis.** The amounts of leukotriene released were expressed as the means  $\pm$  standard errors of the means (SEMs) for three to four different experiments. The percent of phagocytosis was expressed as the mean  $\pm$  SEMs for three experiments. Means were compared by using two-tailed Student's *t* test.

## RESULTS

**$\text{LTB}_4$  release.** (i) **Effect of AA.** Incubation of PMNs with opsonized nontypeable *H. influenzae* in the presence of AA produced  $\text{LTB}_4$  as the major metabolite detectable at 280 nm of UV absorption. The amount of  $\text{LTB}_4$  released at 20 min of incubation correlated directly with the concentration of AA. In the absence of AA or in concentrations less than 50  $\mu\text{M}$ , no chromatographic peaks with the retention time of synthetic  $\text{LTB}_4$  were detected. On the other hand, concentrations higher than 400  $\mu\text{M}$  AA did not augment further the release of  $\text{LTB}_4$ . In the absence of organisms, increasing concentrations of AA resulted in less than 12 ng of  $\text{LTB}_4$  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  $\text{LTB}_4$  release from  $3 \times 10^7$  human granulocytes after 20 min of incubation was on the order of  $3 \times 10^8$  CFU (bacteria/PMNs, 10:1). Increasing concentrations of nontypeable *H. influenzae* stimulated greater release of  $\text{LTB}_4$ .

(iii) **Kinetics (Fig. 2).** Release of  $\text{LTB}_4$  began immediately upon addition of nontypeable *H. influenzae*. Peak production occurred at 15 min. Release of  $\text{LTB}_4$  returned to baseline at 60 min.

(iv) **Effect of opsonization (Fig. 3).** Unopsonized bacteria generated little or no  $\text{LTB}_4$  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  $\text{LTB}_4$  per ml. Inactivation of the complement component had minimal effect on  $\text{LTB}_4$  release (no significant difference). Similarly, fresh antibody-negative serum stimulated the release of  $\text{LTB}_4$  in amounts similar to unopsonized bacteria ( $P < 0.05$ ).

**Phagocytosis.** To study the physical interaction between

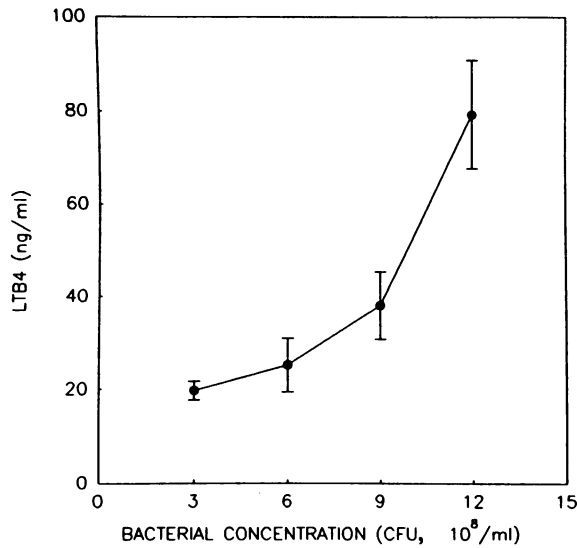


FIG. 1. Effect of bacterial concentration on LTB<sub>4</sub> release. PMNs (3 × 10<sup>7</sup>) were incubated with the organisms at 37°C for 15 min. Results are means ± SEMs for four experiments.

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

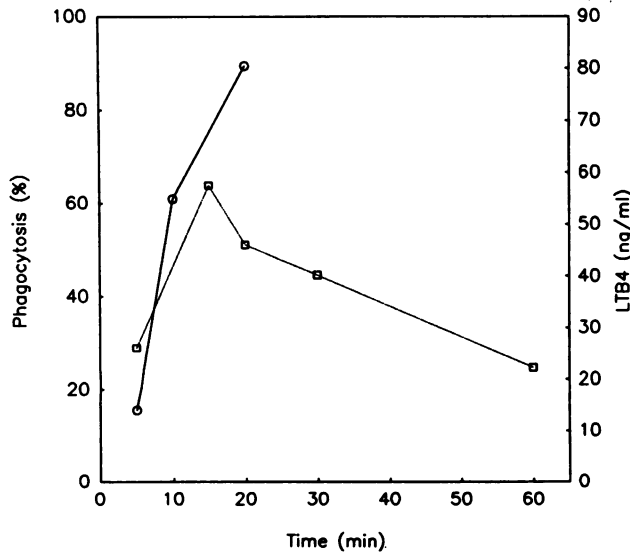


FIG. 2. Kinetics of LTB<sub>4</sub> release and phagocytosis. PMNs (3 × 10<sup>7</sup>) were incubated with 12 × 10<sup>8</sup> bacteria at 37°C for 10, 20, 30, and 60 min. Results are means ± SEMs for four experiments. □, LTB<sub>4</sub>; ○, phagocytosis.

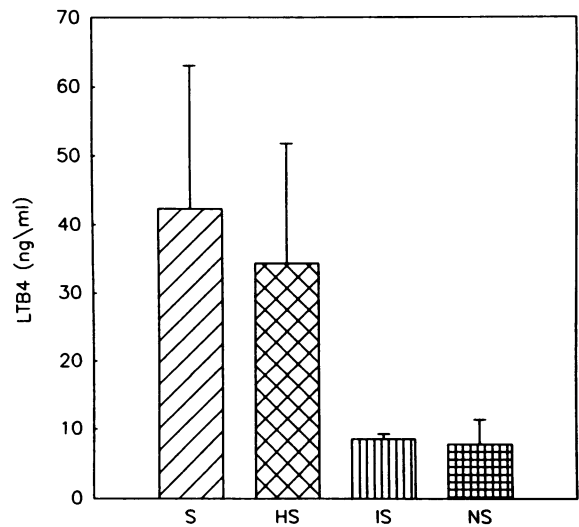


FIG. 3. Effect of antibody and complement on the release of LTB<sub>4</sub>. PMNs (3 × 10<sup>7</sup>) were incubated with 12 × 10<sup>8</sup> bacteria at 37°C for 20 min. Results are means ± SEMs for four experiments. Abbreviations: S, antibody and complement; HS, antibody alone; IS, complement alone; NS, no opsonization. HS versus S, no significant difference; IS versus S,  $P < 0.05$ ; NS versus S,  $P < 0.05$ .

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

LTB<sub>4</sub> is the major product of AA metabolism produced by the lipoxygenase pathway in human phagocytes. In vitro, LTB<sub>4</sub> promotes chemotaxis of neutrophils, eosinophils, and

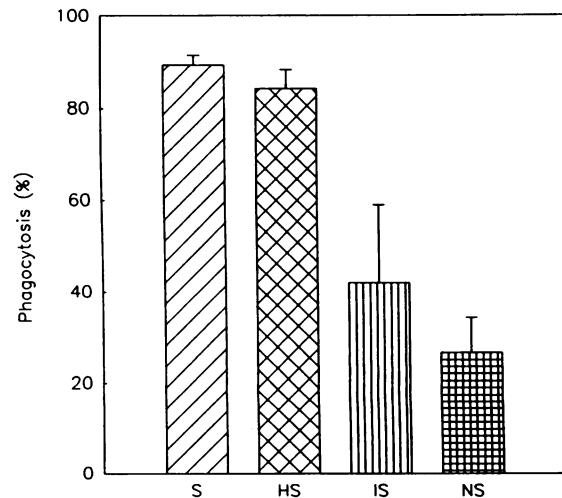


FIG. 4. Effect of antibody and complement on the phagocytosis of nontypeable *H. influenzae*. PMNs (3 × 10<sup>7</sup>) were incubated with 12 × 10<sup>8</sup> bacteria at 37°C for 20 min. Results are means ± SEMs for three experiments. Abbreviations are the same as for Fig. 3. HS versus S, no significant difference; IS versus S,  $P < 0.05$ ; NS versus S,  $P < 0.001$ .

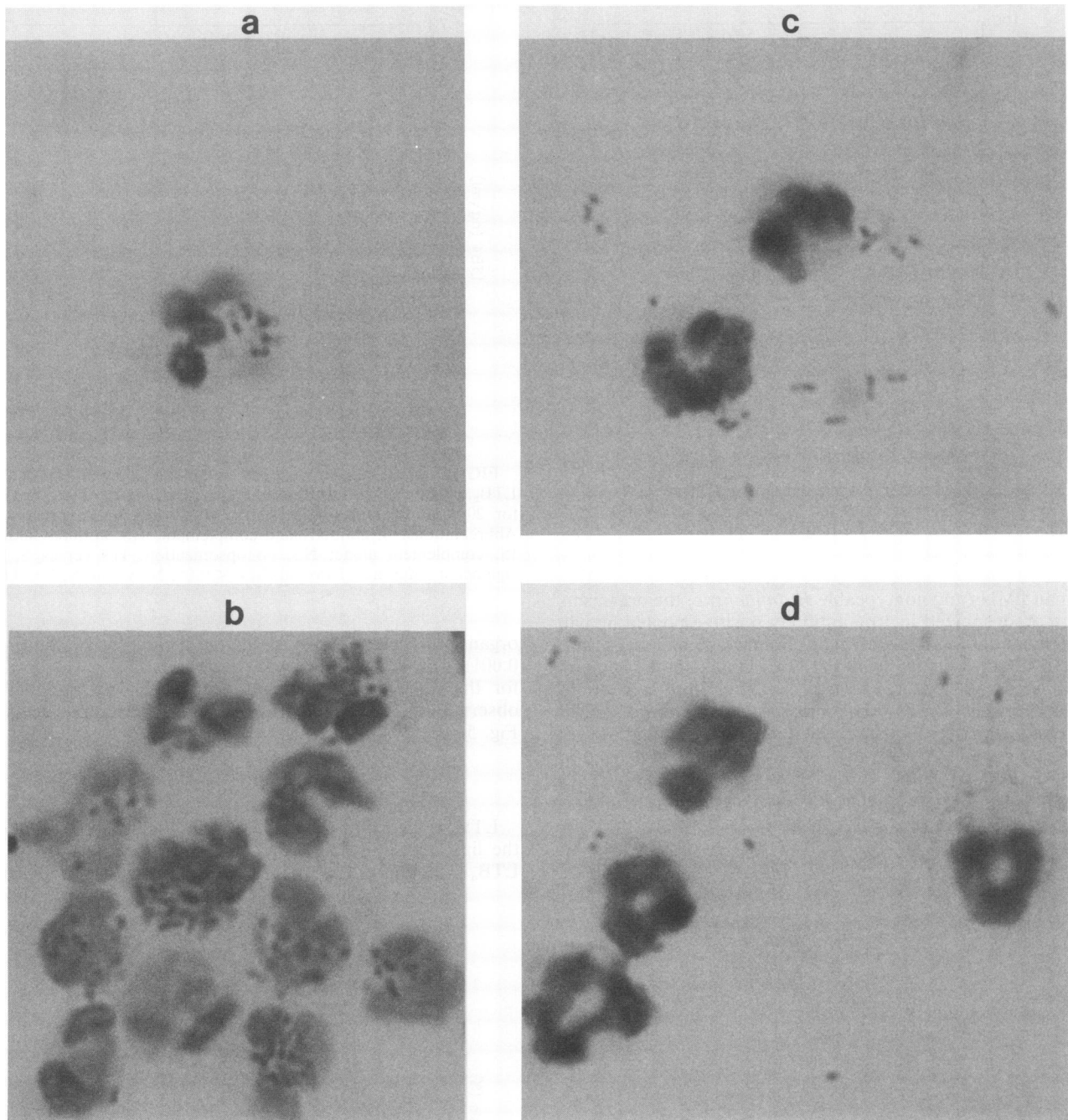


FIG. 5. Light microscopy of the phagocytic process with nontypeable *H. influenzae*. PMNs ( $3 \times 10^7$ ) were incubated with  $12 \times 10^8$  bacteria at  $37^\circ\text{C}$  for 20 min. (a) Antibody and complement; (b) antibody alone; (c) complement alone; (d) no opsonization.

macrophages (30). Recently,  $\text{LTB}_4$  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  $\text{LTB}_4$  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  $\text{LTB}_4$  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,  $\text{LTB}_4$  release by PMNs paralleled phagocytosis of *H. influenzae*. The present study also demonstrated the importance of antibody in the phagocytosis of nontypeable *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 *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 LTB<sub>4</sub> (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 pathophysiological local (tissue destruction) and systemic (increase of 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 LTB<sub>4</sub>. After active LTB<sub>4</sub> 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 LTB<sub>4</sub> in our assay, the kinetics of the LTB<sub>4</sub> 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 LTB<sub>4</sub> and the importance of strain variation and structural components of the bacterium need to be investigated.

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