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Quantitative Flow Cytometric Analysis of Opsonophagocytosis and Killing of Nonencapsulated *Haemophilus influenzae* by Human Polymorphonuclear Leukocytes

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Since nonencapsulated *Haemophilus influenzae* persists in the lower respiratory tracts of patients with chronic bronchitis despite the presence of specific antibodies, complement, and polymorphonuclear leukocytes (PMNs), opsonophagocytosis of *H. influenzae* was analyzed. Nonencapsulated *H. influenzae* isolated from the sputa of chronic bronchitis patients was labeled with fluorescein isothiocyanate and incubated with human PMNs in the presence of complement and antibodies for 30 min at 37°C. *Candida albicans* was added to each sample as an internal standard, and the reduction of the number of bacteria was determined by flow cytometry. Fluorescence quenching with ethidium bromide was used to discriminate between intracellular and extracellular bacteria. Opsonophagocytosis of viable *H. influenzae* d1 was 17% ± 29% in the presence of complement and human pooled sera containing high titers of strain-specific antibodies. Opsonophagocytosis of six other *H. influenzae* strains was also poor. Under the same conditions, opsonophagocytosis of *Staphylococcus aureus* was 90% ± 5%, and opsonophagocytosis of *C. albicans* was 55% ± 23%. About half of the number of *H. influenzae* bacteria associated with PMNs was internalized. Opsonophagocytosis of heat-killed *H. influenzae* d1 (41% ± 20%) was higher than that of viable bacteria of the same strain ($P < 0.05$). This result suggests that the accessibility of epitopes on *H. influenzae* for opsonizing antibodies is better on killed than on viable bacteria. We conclude that viable nonencapsulated *H. influenzae* is poorly opsonophagocytized in the presence of strain-specific antibodies and complement.

Nonencapsulated (nontypeable) *Haemophilus influenzae* causing serious respiratory tract infections in patients with chronic bronchitis persists in the lower respiratory tract (14, 21, 27). The persistence of *H. influenzae* may be associated with the occurrence of variants (12, 13), but the mechanisms involved are unknown. Phagocytosis of opsonized bacteria, an important defense mechanism, is active in the bronchial tree (8). Despite an abundance of polymorphonuclear leukocytes (PMNs) and specific antibodies, *H. influenzae* is not eradicated from the lower respiratory tract (8, 12, 16, 21). Therefore, we investigated the opsonophagocytosis of nonencapsulated *H. influenzae*. Flow cytometry was chosen to measure the opsonophagocytosis and killing of *H. influenzae* by PMNs (5, 7, 18, 19, 25). With this method, the reduction in the number of bacteria and the association of bacteria with PMNs are measured simultaneously by using the fluorescence of the bacteria labeled with fluorescein isothiocyanate (FITC) (7, 18). Fluorescent labeling with propidium iodide (PI), which binds to DNA, allows the quantitative detection of killed bacteria in the bacterial suspension since PI penetrates only into killed bacteria (6). Internalization of bacteria in PMNs was determined by fluorescence microscopy after quenching the fluorescence of the extracellular bacteria with ethidium bromide (10).

We compared the opsonophagocytosis of several *H. influenzae* strains and other microorganisms in the presence of antibodies and complement. Our findings, indicating a very ineffective opsonophagocytosis of nonencapsulated *H. influenzae*, offer insight into one of the possible mechanisms involved in the persistence of this microorganism in the lower respiratory tracts of chronic bronchitis patients.

MATERIALS AND METHODS

Microorganisms and growth conditions. Nonencapsulated *H. influenzae* d1 and d3 were isolated from the sputum of one chronic bronchitis patient (T [13]). Strain d3 is an antigenic variant of nonencapsulated strain d1. *H. influenzae* A920044, A920045, and A920047 were isolated from sputum samples of three other chronic bronchitis patients. Strains G946 and G947 were isolated from the throats of healthy adults, and *H. influenzae* type b strain 760705 was isolated from the cerebrospinal fluid of a patient with meningitis. The opsonophagocytosis of nonencapsulated *H. influenzae* was compared with that of *Staphylococcus aureus* (Wood 46) (19) and *Candida albicans* (18). *C. albicans* also served as an internal standard (18) for quantitative analysis of opsonophagocytosis of *H. influenzae* and *S. aureus*, and *H. influenzae* served as a standard for the measurement of opsonophagocytosis of *C. albicans*. Bacteria and *C. albicans* were grown to the late exponential phase while being shaken (120 rpm) in brain heart infusion broth (Difco Laboratories, Detroit, Mich.) supplemented with NAD^+ and hemin (10 mg/liter each). The bacterial suspensions were centrifuged at 3,000 × *g* for 10 min and washed with 20 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; pH 7.4) containing 132 mM NaCl, 1 mM $MgCl_2$, 5 mM $CaCl_2$, and 0.1% (wt/vol) glucose. The pellets were resuspended in the

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same buffer to an optical density of 1.0 at 530 nm, corresponding to 10^9 CFU/ml as determined by colony counting.

Monoclonal antibodies and sera. The outer membrane protein P6-specific monoclonal antibody (MAb) 8BD9 and the outer membrane protein P2-specific MAbs 19DA11 and 21BG2 have been described elsewhere (28, 29). As a source of polyclonal antibodies, the serum of a healthy adult (serum M) and human pooled sera (HPS) prepared from sera collected from five healthy adults were used. Human sera contain antibodies to a variety of *H. influenzae* strains (12). Agammaglobulinemic serum (AGS), used as a source of complement, was obtained from a patient with agammaglobulinemia. This serum, eluted through a protein G column (2) to remove residual immunoglobulin G, had a complement activity of at least 96% of the original activity as determined by a bactericidal assay (28). C7-deficient serum was from a patient with a complement component C7 deficiency. No bacterial killing by the terminal complement C5-C9 complex formation occurred in this serum during incubation.

Measurement of antibody titers against *H. influenzae*. Antibody titers in HPS, AGS, C7-deficient serum, and serum M and those of MAbs 19DA11, 21BG2, and 8BD9 were measured by a whole-cell enzyme-linked immunosorbent assay (ELISA) with killed *H. influenzae* d1 and d3 as antigen (1). Twofold dilutions were used to determine the titer, which is the inverse of the dilution, corresponding to an optical density at 495 nm of 0.2 (12). Antibody titers to the polysaccharide of *H. influenzae* type b were determined by radioimmunoassay as described by Robbins et al. (22).

Fluorescent labeling of microorganisms. Bacteria or yeast cells were incubated with FITC (0.015 mg/ml) while being shaken for 15 min (37°C). Subsequently, microorganisms were washed three times with HEPES buffer (pH 7.4) containing NaCl, $MgCl_2$, $CaCl_2$, and glucose. The bacterial populations were homogeneously labeled with FITC as analyzed by flow cytometry.

Isolation of PMNs. The PMNs were isolated as described by Roos and de Boer (24). In short, mononuclear cells and platelets were removed from heparinized blood by centrifugation over isotonic Percoll (Pharmacia, Uppsala, Sweden) with a specific gravity of 1.076 g/ml. Erythrocytes were lysed by incubating the pellet fraction with ice-cold isotonic NH_4Cl solution (155 mM NH_4Cl, 10 mM $KHCO_3$, 0.1 mM EDTA [pH 7.4]). PMNs were washed twice in ice-cold HEPES buffer (pH 7.4) containing NaCl, $MgCl_2$, glucose, 0.1% (wt/vol) gelatin, and 0.1% (wt/vol) human serum albumin (HSA; Central Laboratory of the Red Cross Blood Transfusion Service, Amsterdam, The Netherlands) and resuspended in the same buffer with the addition of 5 mM $CaCl_2$. The density of the cell suspension was adjusted to 10^7 PMNs per ml. The viability of the PMNs was more than 90%, as determined by lactate dehydrogenase activity.

Opsonophagocytosis assay. FITC-labeled *H. influenzae* bacteria were suspended in HEPES buffer (pH 7.4) containing NaCl, $MgCl_2$, $CaCl_2$, glucose, gelatin, and HSA to a final concentration of 1.0×10^7 bacteria per ml. In opsonophagocytosis experiments in which a bacterium/PMN ratio of 10:1 was used, the bacteria were suspended in a final concentration of 5.0×10^7 bacteria per ml. For opsonization, heat-inactivated HPS or MAb and complement were added to final concentrations of 15 and 1%, respectively. In some experiments, C7-deficient serum served as opsonin. Subsequently, PMNs were added to the incubation mixture in a final concentration of 0.5 $\times 10^7$ cells per ml. The bacterium-PMN mixture was incubated (37°C) for 30 min in a shaking water bath. The phagocytosis was stopped by a fivefold dilution of 100-μl samples of the incubation mixture in ice-cold HEPES buffer (pH 7.4) containing NaCl, $MgCl_2$, glucose, gelatin, and HSA. A standard number of FITC-labeled *C. albicans* cells (1.0×10^6) was added to each sample as an internal standard to quantitate the decrease of free bacteria from the mixture. The reaction mixture was fixed by adding paraformaldehyde (final concentration, 1%). Flow cytometric analysis was not affected by paraformaldehyde fixation.

Controls were always run in parallel with the opsonophagocytosis assay. In these controls, antibodies or complement or both were omitted from the incubation mixture to determine the opsonic effect of antibodies and complement separately. In other controls, PMNs were omitted from the incubation mixture to determine bacterial killing or agglutination.

Flow cytometric analysis of opsonophagocytosis. Analysis was done with a FACScan flow cytometer (Becton Dickinson, Heidelberg, Germany) with computer-assisted evaluation of data (FACScan software). Data were acquired by means of an instrument setting with a logarithmic data mode for forward and side scatter and a logarithmic mode for fluorescence (FL1 and FL3). FITC fluorescence was measured in the FL1 channel (515 to 545 nm), and PI fluorescence was measured in the FL3 channel (≥650 nm). A live gate was set around the bacterial, *C. albicans*, and PMN populations. Individual bacterial, yeast, and PMN populations were used for standardization. Routinely, 4,000 events were counted. Phagocytosis was quantitated as the percent reduction of the number of free bacteria from the incubation mixture after 30 min of incubation in the presence of PMNs and calculated as follows: number of phagocytized bacteria (%) = 1 − [(number of bacteria/number of *C. albicans* cells), where t is 30 min × (number of *C. albicans* cells/number of bacteria), where $t = 0$ min] × 100%. The concentrations of *C. albicans* at times (t) of 0 and 30 min are equal since a standard number of FITC-labeled *C. albicans* cells was added to each sample. After gating for the PMN population, the number of PMNs participating in the phagocytosis process could be determined. The upper limit of the autofluorescence of the PMNs was measured in a control sample containing PMNs only. A marker was positioned just above the upper FL1 value of the autofluorescence, and the portion (in percent) of the PMNs in the histogram above the marker was determined.

Microscopic examination of phagocytosis. The association of bacteria with PMNs was visualized by fluorescence microscopy. Internalization of bacteria was confirmed by electron microscopy of thin sections of samples obtained after phagocytosis. Ruthenium red (0.4% [wt/vol]) was added as a marker for extracellular surfaces before the samples were sectioned.

To discriminate between bacteria internalized by PMNs and bacteria adhering to PMNs, ethidium bromide (50 μg/ml) was added to the incubation mixture after phagocytosis, the fluorescence of extracellular bacteria was quenched, and the color shifted to orange. Since ethidium bromide does not penetrate rapidly into intact PMNs, intracellular bacteria remain fluorescent green (10). The number of fluorescent bacteria associated with 50 PMNs was counted. Quenching of nonphagocytized fluorescent bacteria served as a control.

Staining of killed *H. influenzae*. To differentiate between opsonophagocytosis of viable and dead *H. influenzae*, PI staining was used. PI penetrates only into dead bacteria and binds to DNA (6). The percentage of killed extracellular bacteria was determined by flow cytometry after PI (final concentration, 0.1 mg/ml) was added to the unfixed samples obtained from opsonophagocytosis experiments. Viable counting was performed for comparison.

Viability of intracellular bacteria. To determine the number of viable intracellular bacteria after 30 min of incubation, the

TABLE 1. Reciprocal dilutions of antibody titers against unlabeled and FITC-labeled *H. influenzae* d1 and d3 measured by whole-cell ELISA

Antibody source	Reciprocal dilution of antibody titer against:			
	Strain d1		Strain d3	
	Unlabeled	Labeled	Unlabeled	Labeled
HPS	1.7×10^3	1.7×10^3	1.6×10^3	2.5×10^3
Serum M	3.0×10^3	3.5×10^3	4.9×10^3	7.0×10^3
C7 def[a]	9.2×10^3	ND[b]	7.5×10^3	ND
AGS	$\leq 10^2$	ND	$\leq 10^2$	ND
MAb 19DA11	2.2×10^5	2.0×10^5	ND*[c]	ND*
MAb 21BG2	5.7×10^4	4.9×10^4	2.0×10^4	5.7×10^4
MAb 8BD9	1.0×10^5	1.0×10^5	1.0×10^4	3.0×10^4

[a] C7 def, serum from a patient with C7 deficiency.
[b] ND, not determined.
[c] ND*, not determined since the MAb is specific for strain d1.

PMN fraction was spun down by centrifugation at 250 × *g* for 10 min and resuspended in HEPES buffer (pH 7.4) containing glucose, gelatin, and HSA and then incubated with gentamicin (100 μg/ml) for 1 h to kill extracellular bacteria. PMNs were washed with HEPES buffer and lysed in 1% saponin (10 min at room temperature). This treatment did not affect the viability of *H. influenzae*. Aliquots of the suspension were plated for colony counting. The remaining bacterial suspension was stained with PI to quantitate the killed bacteria by flow cytometry.

Statistics. Statistical analysis was done with the two-tailed Student *t* test. *P* values of <0.05 were considered to be significant. Results were expressed as means ± standard deviations.

RESULTS

The effect of bacterial labeling with FITC on antigenic properties of outer membrane proteins and viability of *H. influenzae*. The titers of serum antibodies and of MAbs specific for outer membrane proteins P2 and P6 were determined by whole-cell ELISA before and after FITC labeling of *H. influenzae* d1 and d3. The FITC labeling did not affect the antibody titers of HPS, serum M, and the two MAbs (Table 1).

After FITC labeling, the number of CFU of *H. influenzae* d1 per milliliter was 83% ± 33% (*n* = 5) of the number of CFU per milliliter before labeling, indicating that the viability of the bacteria was not affected by the labeling. Similar results were obtained for *H. influenzae* A920044 (147%), A920045 (100%), and A920047 (116%).

Flow cytometric analysis of phagocytosis of *H. influenzae*. The FITC labeling of bacteria and *C. albicans* resulted in homogeneous populations as shown in the plots of side scatter-FL1 obtained after flow cytometry (Fig. 1A and B). The bacteria were uniform in granularity and FITC labeling. The fluorescence signal of the population of labeled bacteria showed no overlap with that of the population of unlabeled bacteria in the histogram of counts versus FITC fluorescence (Fig. 1C). This indicates that the whole population of *H. influenzae* was labeled.

Fig. 2A and B depicts the side scatter, indicating the granularity of cells, and fluorescence signals of killed labeled *H. influenzae* d3, the internal marker *C. albicans*, and nonfluorescent and fluorescent PMNs after opsonophagocytosis in the presence of HPS and AGS. PMNs became fluorescent because of the association with FITC-labeled bacteria. There was no overlap between the *H. influenzae*, *C. albicans*, and PMN populations. In experiments performed with a bacterium/PMN ratio of 2:1, a gradual decrease in the number of free bacteria was observed over time (Fig. 2A and B). The number of fluorescent PMNs increased accordingly (Fig. 2C to F), and 2 ± 1 bacteria (*n* = 3) were associated per PMN during phagocytosis as was observed with fluorescence microscopy. When experiments were performed with a bacterium/PMN ratio of 10:1, more than 90% of the PMNs were fluorescent. Under these conditions also, 2 ± 1 bacteria (*n* = 3) were associated per PMN. Therefore, the observed reduction of 16% ± 10% in the number of free bacteria correlated with the reduction of 18 to 20% calculated from the number of bacteria associated per cell and the number of positive PMNs. These results indicate that the number of bacteria per cell was low irrespective of the bacterium/PMN ratio and that the great majority of the PMNs were able to phagocytize *H. influenzae*.

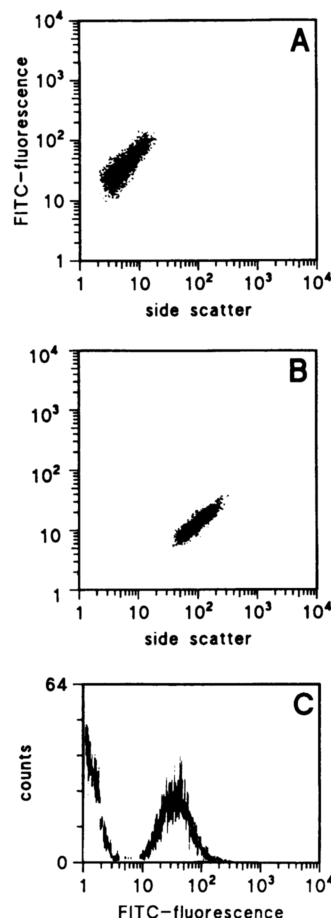

FIG. 1. Side scatter-FL1 plots of FITC-labeled *H. influenzae* (A) and FITC-labeled *C. albicans* (B) and FL1 histogram of unlabeled (left peak) and FITC-labeled (right peak) *H. influenzae* (C).

Opsonophagocytosis of nonencapsulated *H. influenzae*, *S. aureus*, and *C. albicans*. The opsonophagocytoses of nonencapsulated *H. influenzae*, *S. aureus*, and *C. albicans* in the presence of HPS and AGS were compared. The opsonophagocytosis of *S. aureus* and *C. albicans* was significantly better (*P* < 0.05) than that of nonencapsulated *H. influenzae* (Fig. 3), although the antibody titers in HPS against *S. aureus*, *C. albicans*, and *H. influenzae* were similar as determined by whole-cell ELISA (1.2×10^3, 2.7×10^2, and 1.7×10^3, respectively).

The opsonophagocytosis of *H. influenzae* d1 in the presence

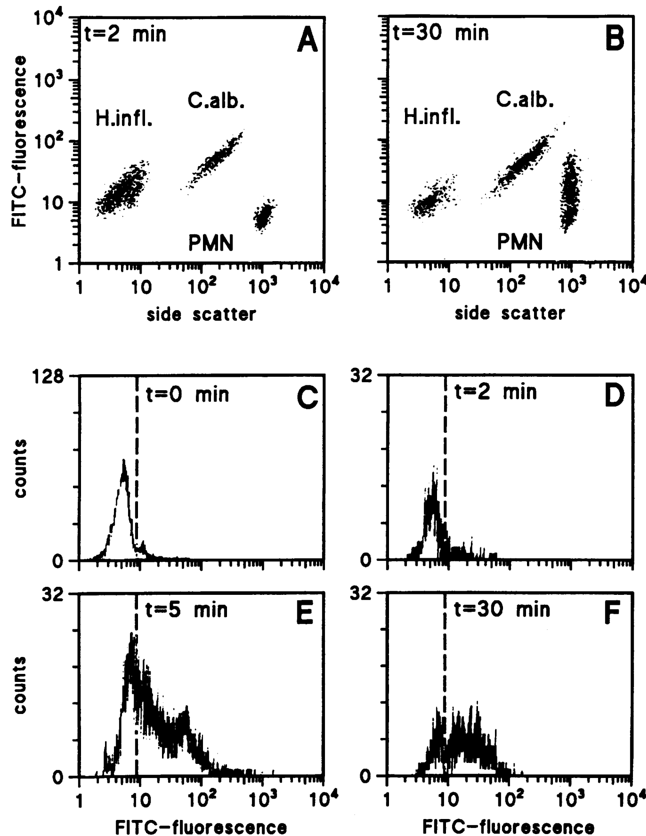

FIG. 2. (A and B) Side scatter-FL1 plots of samples containing killed labeled *H. influenzae* d3, internal marker *C. albicans*, nonfluorescent PMNs, and fluorescent PMNs, after two time intervals in the opsonophagocytosis process, i.e., *t* of 2 (A) and *t* of 30 (B) min; (C to F) histograms of the fluorescence of the PMNs at *t* of 0, 2, 5, and 30 min in the opsonophagocytosis process of killed FITC-labeled nonencapsulated *H. influenzae* d3. Bacteria were opsonized with 15% HPS and 1% AGS. A bacterium/PMN ratio of 10:1 was used.

of HPS and AGS was 17% ± 29% (n = 11). The percentages of phagocytized bacteria in the presence of antibodies and complement were similar to those found in the absence of opsonins or in the presence of antibodies or complement alone. To determine if the poor opsonophagocytosis of *H. influenzae* was strain dependent, the additional *H. influenzae* strains d3, A920044, A920045, A920047, G946, and G947 were tested. The viability of these strains was more than 95%. The phagocytosis of these strains in the presence of HPS and AGS was as low as it was for strain d1, and no significant differences between these strains were observed (Table 2), irrespective of the source of the bacterial strains.

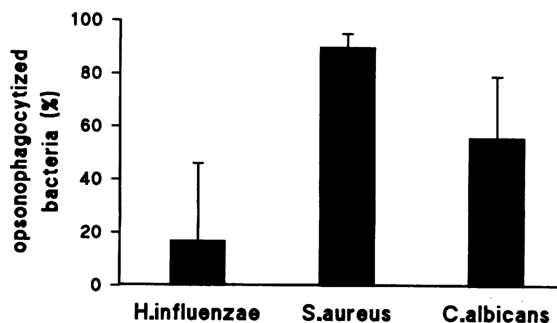

FIG. 3. Opsonophagocytosis of nonencapsulated *H. influenzae* d1 (n = 11) in comparison with that of *S. aureus* (n = 4) and *C. albicans* (n = 3). Heat-inactivated HPS (15%) was used as an antibody source, and AGS (1%) was used as a complement source. A bacterium/PMN ratio of 2:1 was used.

TABLE 2. Percentages of phagocytized bacteria of several nonencapsulated *H. influenzae* strains in the presence of HPS and AGS

Strain	Source	Phagocytized bacteria (% ± SD)	n
d1	Chronic bronchitis sputum	17 ± 29	11
d3	Chronic bronchitis sputum	30 ± 36	7
A920044	Chronic bronchitis sputum	7 ± 27	3
A920045	Chronic bronchitis sputum	25 ± 42	3
A920047	Chronic bronchitis sputum	16 ± 35	3
G946	Healthy throat	0	2
G947	Healthy throat	0	2

Since *H. influenzae* shows strong antigenic differences (20), specific opsonic antibodies against the tested strains may be absent in HPS. Therefore, the opsonophagocytosis of *H. influenzae* in the presence of MAbs was determined. The titer of these MAbs was approximately 100 times higher than that of HPS (Table 1). The number of phagocytized *H. influenzae* d1 bacteria in the presence of MAb 19DA11 was low (2% ± 32%). The opsonophagocytosis of *H. influenzae* d3 in the presence of the complement-activating MAb 30DA5 (28) and C7-deficient serum was significant (64% ± 54%). However, 68% of the bacteria were killed during opsonization as determined by colony counting.

Since *H. influenzae* type b was reported to be phagocytized in the presence of anti-polyribitol ribosyl phosphate antibodies (4, 27), the opsonophagocytosis of *H. influenzae* type b was studied with our assay. Serum samples from a healthy adult collected before and after vaccination with the HbOC vaccine (Praxis Biologics, Rochester, N.Y.) were used as opsonin. The antibody levels in the pre- and postvaccination serum samples were 1.1 and 9.1 μg/ml, respectively. During opsonophagocytosis of *H. influenzae* type b in the presence of prevaccination serum and AGS, the number of free bacteria increased 26% ± 28% (n = 3), indicating no opsonophagocytosis. In contrast, the number of free bacteria decreased 55% ± 6% (n = 3) during opsonophagocytosis in the presence of postvaccination serum. This difference was significant ($P < 0.05$).

Association of *H. influenzae* with PMNs. Ethidium bromide was used to discriminate between bacteria adhering to PMNs and bacteria internalized by PMNs. The fluorescence signal of free bacteria was quenched completely in the presence of ethidium bromide, as was observed with flow cytometry and fluorescence microscopy. With flow cytometry, it is, however, not possible to determine the number of bacteria which are internalized, since any internalized bacterium makes the PMN fluorescent, irrespective of the number of adhering bacteria with a quenched fluorescence signal. After opsonophagocytosis of *H. influenzae* d1 in the presence of HPS and complement in an incubation mixture with a bacterium/PMN ratio of 20:1, 10% of the bacteria were associated with PMNs. Of these PMN-associated bacteria, 44% (range, 27 to 59; n = 3) were fluorescent green as observed with the fluorescence microscope in the presence of ethidium bromide. This percentage did not increase after prolonged opsonophagocytosis, indicat-

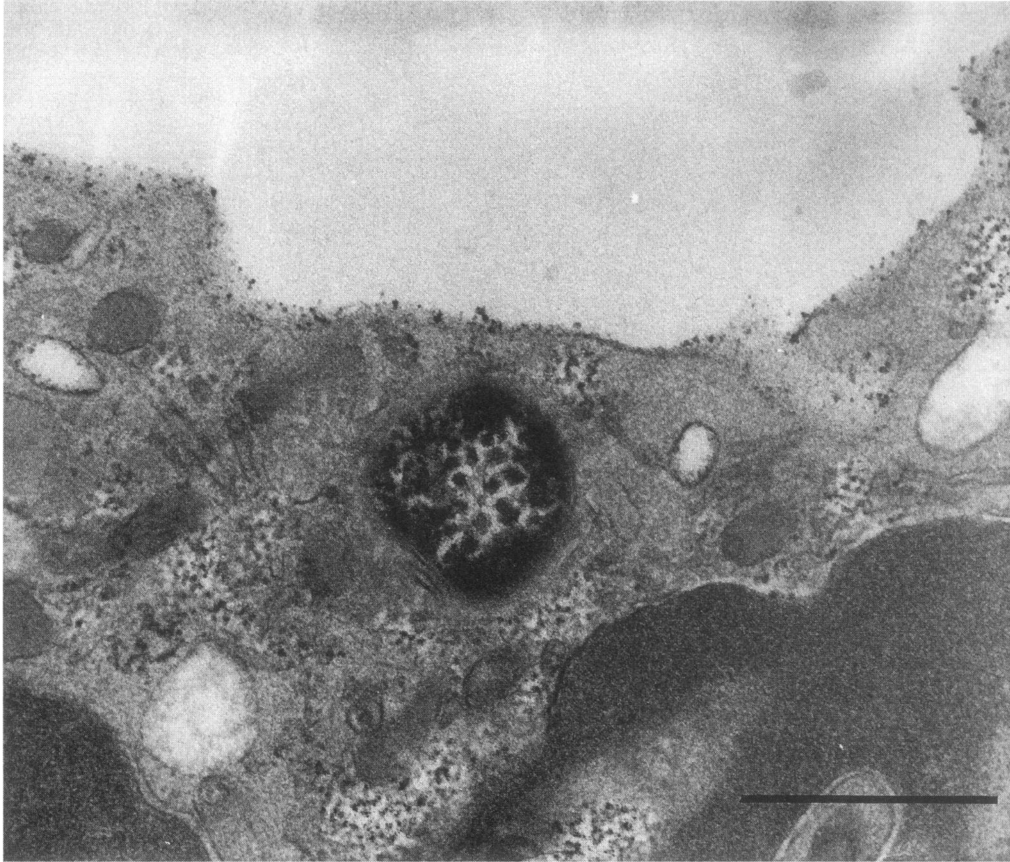

FIG. 4. Electron micrograph of a PMN with internalized *H. influenzae* d3. Ruthenium red was used as a marker for extracellular surfaces. Bar, 1 μm.

ing that external bacteria were not internalized later and that only a rather small number of bacteria were internalized. Electron microscopy showed bacteria internalized by PMNs since they were observed in vesicles not containing ruthenium red (Fig. 4). Opsonophagocytosis experiments performed at a temperature of 0°C to prevent internalization resulted in PMNs with adherent bacteria only since the fluorescence signal of all PMN-associated bacteria was quenched with ethidium bromide.

Intracellular killing of nonencapsulated *H. influenzae*. To investigate whether bacteria survived intracellularly after phagocytosis, the number of viable intracellular bacteria was determined by colony counting and PI staining (in triplicate). After gentamicin treatment, the bacteria not associated with PMNs were killed as determined by colony counting and PI staining (data not shown). Viable bacteria were also not recovered from the PMNs lysed with saponin, although the saponin treatment itself did not affect the viability of *H. influenzae* as determined by colony counting. The number of CFU of *H. influenzae* d1 after saponin treatment was 120% of the untreated bacteria. Flow cytometry showed that, after saponin treatment, PMNs disappeared from the side scatter-FL1 diagrams, indicating that lysis of PMNs was complete. Bacteria released from saponin-lysed PMNs stained with PI, confirming that the intracellular bacteria were killed.

Phagocytosis of killed *H. influenzae*. The low number of opsonophagocytized, viable, nonencapsulated *H. influenzae* bacteria in the presence of antibodies may be due to a poor exposure of epitopes on the surface of viable bacteria. We compared the opsonophagocytoses of heat-killed (30 min at 56°C) and viable nonencapsulated *H. influenzae* d1. The heat treatment resulted in a 4-log reduction in the number of CFUs in the suspension, and 95% of the bacteria were PI positive (Fig. 5). In the unheated bacterial suspension, only 4% of the bacteria stained with PI. After killed bacteria were mixed with viable bacteria in a ratio of 1:1, 45% of the bacteria were PI positive (Fig. 5). Opsonophagocytosis of killed bacteria was 41% ± 20%. This is significantly better than opsonophagocytosis of viable bacteria ($P < 0.05$) (Fig. 6).

DISCUSSION

With flow cytometry, opsonophagocytosis of nonencapsulated *H. influenzae* was measured. Fluorescent labeling of the microorganisms with FITC did not affect the reactivity of antibodies with labeled nonencapsulated *H. influenzae* (Table 1) nor the viability of nonencapsulated *H. influenzae*. Moreover, FITC labeling did not affect opsonophagocytosis as measured by colony counting (data not shown). The percentage of opsonophagocytized bacteria showed a great interassay variation. This is a general characteristic of opsonophagocytosis assays (3) and may be related to individual differences in Fc (9) and complement receptors on PMNs.

At a bacterium/PMN ratio of 10:1, almost all PMNs participated in the phagocytosis process because more than 90% of the PMNs became fluorescent by the opsonophagocytosis of *H. influenzae*. In experiments with lower bacterium/PMN ratios (2:1), the percentage of fluorescent PMNs was lower, indicat-

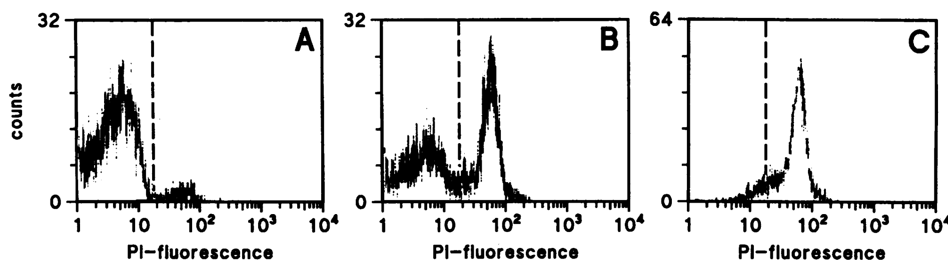

FIG. 5. The percentages of PI-positive, killed *H. influenzae* d1 bacteria determined by flow cytometry were as follows: 4% in a viable population (A); 45% in a 50% viable population prepared by mixing viable and heat-killed bacteria (B); and 95% in a population of killed bacteria (C). The percentage of killed bacteria correlated well with the percentage of PI-positive bacteria ($n = 3$).

ing a ratio-dependent association of *H. influenzae* with PMNs. At high ratios (10 to 20 bacteria per PMN), the reduction in the percentage of free bacteria was far less than the percentage of fluorescent PMNs. This indicates that the PMNs became fluorescent irrespective of the number of bacteria associated with the PMNs and that only a small number of bacteria became associated with the PMNs.

Quantitative flow cytometric analysis showed that nonencapsulated *H. influenzae* isolated from either the sputa of chronic bronchitis patients or the throats of healthy adults is poorly opsonophagocytized in the presence of high titers of specific antibodies and complement. In contrast, the opsonophagocytosis of *S. aureus* and *C. albicans* was efficient, as reported in other studies (6, 7, 18, 19, 30). Apparently, the poor opsonophagocytosis of *H. influenzae* is not due to inappropriate assay conditions. The percentage of phagocytized *H. influenzae* bacteria did not increase with incubation for longer than 30 min and higher concentrations of antibodies (data not shown). Binding of specific MAb 19DA11 did not result in a statistically significant increase of opsonophagocytosis of the homologous *H. influenzae* strain, despite binding of the MAb to the bacteria (Table 1) (26, 28). The significant opsonophagocytosis of strain d3 in the presence of MAb 30DA5 and C7-deficient serum was probably due to killing of the bacteria. In a separate report, we show that antibodies against some strain-specific epitopes of noncapsular antigens bind to nonencapsulated *H. influenzae*, activate complement, and promote some binding of the opsonized bacteria to PMNs (26). Anti-PRP antibodies elicited by HbOC vaccination promoted the opsonophagocytosis of *H. influenzae* type b, indicating that antibodies of the proper specificity can promote phagocytosis.

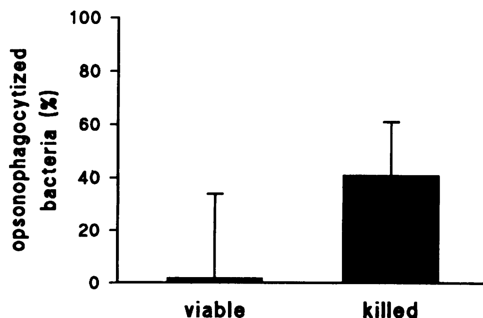

FIG. 6. Opsonophagocytosis of viable and heat-killed nonencapsulated *H. influenzae* d1 in the presence of MAb 19DA11 and AGS. A bacterium/PMN ratio of 2:1 was used. Killed bacteria ($n = 5$) were phagocytized significantly better ($P < 0.05$) than viable bacteria ($n = 12$).

In contrast to our findings, Garofalo et al. (11) and Hansen et al. (15) reported that almost 90 and 100%, respectively, of the number of nonencapsulated *H. influenzae* bacteria were phagocytized. Hansen et al. (15) measured phagocytosis by means of colony counting. Bacterial aggregation or bactericidal activity of the serum could have reduced the number of viable bacteria. Garofalo et al. (11), measuring the association of radioactive labeled bacteria with PMNs at a bacterium/PMN ratio of 10:1, did not include controls to assess the viability of the bacteria, which we found to be an important factor. Heat-killed bacteria were opsonophagocytized better than viable bacteria (Fig. 6). This finding suggests that the accessibility of epitopes at the surface of *H. influenzae*, to which opsonic antibodies may bind, is better on killed than on viable bacteria. Surface epitopes on viable *H. influenzae* other than the capsular polysaccharide may be shielded by structures, such as lipopolysaccharide structures (17). This is also suggested for some antigenic determinants of *H. influenzae* type b (23). Alternatively, the observation that only 2 ± 1 bacteria were associated per PMN irrespective of the bacterium/PMN ratio suggests that *H. influenzae* is detrimental for phagocytes.

Only a low number of nonencapsulated *H. influenzae* is internalized. These internalized bacteria are rapidly killed since no viable bacteria were recovered after gentamicin treatment of the extracellular bacteria and subsequent lysis of the PMNs. In addition, bacteria obtained after lysis of the PMNs stained with PI, confirming that they were killed. After prolonged incubation, internalization of the PMN-adherent bacteria did not occur, suggesting that *H. influenzae* blocks internalization by the PMNs. Since the PMNs did not stain with PI (0.01 mg/ml) after the opsonophagocytosis (data not shown), the poor internalization of the bacteria was not due to death of the PMNs.

In summary, flow cytometric analysis is appropriate for quantitative analysis of opsonophagocytosis of *H. influenzae*. Viable nonencapsulated *H. influenzae* is poorly opsonophagocytized by PMNs in the presence of strain-specific antibodies and complement.

Complement-mediated killing has been shown to be effective for the killing of nonencapsulated *H. influenzae* (26). It is likely that in the respiratory tract the host is more dependent on opsonophagocytosis than on complement-mediated killing because of the low concentration of complement. Since the association of bacteria with PMNs is dependent on complement, we hypothesize that internalization of opsonized *H. influenzae* via complement receptors is inefficient because of shielding of complement components on the surface of *H. influenzae* by the bacterial surface structure. During complement-mediated killing, complement components are inserted in the bacterial membrane. During phagocytosis, these com-

ponents should be exposed in such a way that interaction with receptors on the surface of the PMNs occurs. Opsonophagocytosis is likely to play a role in eradicating mainly dead bacteria.

Poor opsonophagocytosis of *H. influenzae* is not specific for patients with chronic bronchitis. It is likely that *H. influenzae* grows and survives better in these patients than in other individuals. Local factors present in the epithelial lining fluid and sputa of patients with chronic bronchitis may act as growth factors and shield the bacteria from harmful components of the nonspecific defense mechanisms of the host. Our findings that nonencapsulated *H. influenzae* is poorly opsonophagocytized in the presence of specific antibodies and complement offer an explanation for the persistence of this microorganism in the lower respiratory tracts of patients with chronic bronchitis.

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