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Effect of Piliation on Interactions of *Haemophilus influenzae* Type b with Human Polymorphonuclear Leukocytes

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Piliated, adherent (P+) and nonpiliated, nonadherent (P−) strains of *Haemophilus influenzae* type b (Hib) were compared with respect to their ability to induce polymorphonuclear leukocyte (PMN) chemiluminescence (CL) and superoxide (O_2^-) generation and their susceptibility to phagocytosis by PMNs. P+ strains opsonized in normal human serum (NHS) induced significantly greater CL than did P− strains ($500 \times 10^5 \pm 112 \times 10^5$ versus $242 \times 10^5 \pm 65 \times 10^5$ total counts per 60 min; $P < 0.001$) when reacted with normal PMNs. Contributions of immunoglobulin and complement to CL activity in these mixtures were shown by findings of lower overall levels of CL when hypogammaglobulinemic serum or heat-inactivated NHS was used to opsonize either P+ or P− organisms. Results obtained with mixtures of hypogammaglobulinemic plus adsorbed heat-inactivated NHS (with P+ or P− organisms) suggested a role for an antipilus antibody in the enhancement of CL by these strains. NHS-opsonized P+ strains also induced significantly greater ($P < 0.002$) O_2^- generation than did P− strains (2.83 ± 0.08 versus 1.94 ± 0.14 nmol of ferricytochrome c reduced per 10 min/10^6 PMN). Comparable ingestion of P+ or P− strains opsonized in NHS by PMNs was demonstrated by a radiolabeled uptake technique and transmission electron microscopy, and primary granule release (β-glucuronidase) was comparable during ingestion of P+ or P− strains. The basis for the observed enhanced capacity of P+ Hib to stimulate PMN oxidative metabolism as compared with P− organisms is uncertain. Possible clinical implications of these findings deserve further study.

Several species of pathogenic bacteria express surface pili which may contribute to the virulence properties of these organisms. In previous studies, piliation has been shown to be a determinant of microbial adherence to host epithelial surfaces (18, 19), susceptibility to phagocytosis (8, 20), stimulation of phagocyte oxidative metabolism (3, 4, 8, 13), or a combination of these. Recent interest has developed with respect to the pilus of *Haemophilus influenzae* type b (Hib) as a potential mediator of colonization or invasion, or both, through the nasopharynx (6, 10, 16). Interestingly, studies have shown that when piliated, adherent (P+) strains of Hib are inoculated into the nasopharynx of infant rats, organisms recovered from the blood or cerebrospinal fluid (CSF), or both, of rats which develop systemic disease are uniformly nonpiliated and nonadherent (P−) (10). P− may therefore be an advantageous state of Hib in this context. The possibility exists that P+ strains may interact with host defense mechanisms such that they are preferentially eliminated, thus allowing subpopulations of P− organisms to proliferate. Therefore, these studies were designed to determine possible effects of piliation on interactions between Hib and human polymorphonuclear leukocytes (PMNs).

MATERIALS AND METHODS

Bacterial strains. Of more than 50 children admitted to Texas Children's Hospital with meningitis due to Hib and from whom the organism was isolated from the nasopharynx, only 3 harbored strains that were found to be adherent to human epithelial cells (E. O. Mason, Jr., S. L. Kaplan, E. P. Norrod, W. A. Stenback, and R. D. Feigin, Program Abstr. 22nd Intersci. Conf. Antimicrob. Agents Chemother., abstr. no. 648, 1982). Each of these strains has been shown to be piliated. Table 1 lists the strains used in our experiments which were obtained from these children at the time of admission: P+ nasopharyngeal strains 884, 1264, and 1009, along with their corresponding P− CSF fluid isolates no. 880, 1228, and 1007, respectively. Another P− strain, 1250, was isolated from infant rat CSF after nasopharyngeal inoculation with strain 1009. For most experiments reported here, strains 1009 and 1007 were compared. Chemiluminescence (CL) experiments also were performed with each of the other three possible pairs, including comparisons between strains 1009 and 1250 (the rat CSF isolate) to ensure that our observations were not strain specific.

Preparation of bacterial strains for experiments. Strains of Hib used in these experiments were stored at −70°C in glycerolated Trypticase soy broth (BBL Microbiology Systems, Cockeysville, Md.). Organisms were grown overnight in either Trypticase soy broth or Todd-Hewitt broth (Difco Laboratories, Detroit, Mich.) supplemented with 10 μg each of hemin and NAD (Sigma Chemical Co., St. Louis, Mo.) per ml. Organisms were washed twice in Dulbecco phosphate-buffered saline (DPBS); pH 7.3; GIBCO Laboratories, Grand Island, N.Y.). Suspensions were adjusted to an optical density of 0.62 to 0.66 at 540 nm. Colony counts of these suspensions consistently showed approximately 4×10^9 CFU/ml. Organisms in radiolabel uptake experiments were processed in a similar manner except that [^{3}H]thymidine (Sigma Chemical Co.) was added to the broth medium. Piliation (or lack thereof) was confirmed for each strain used

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TABLE 1. Clinical strains of Hib from children admitted with Hib meningitis

Patient	Strains	
	P+[a]	P−[b]
X	884	880
Y	1264	1228
Z	1009	1007, 1250[c]

[a] Nasopharyngeal isolates.
[b] CSF isolates.
[c] Isolated from rat CSF after nasopharyngeal innoculation with strain 1009.

by transmission electron microscopy on numerous occasions throughout and after our studies.

Isolation of PMNs. Venous blood samples were collected from a single healthy adult donor in a syringe containing 10 U of sodium heparin per ml of blood. After sedimentation of erythrocytes in dextran, leukocyte-rich plasma was layered over a Ficoll-Hypaque solution consisting of 10 parts of 33.9% Hypaque (Winthrop Laboratories, New York, N.Y.) and 24 parts of 9% Ficoll (Sigma Chemical Co.) and centrifuged at 400 × *g* for 30 min at 4°C.For most experiments, erythrocytes were eliminated by hypotonic lysis. Purified cell suspensions were adjusted to a final concentration of 10^7 PMNs per ml of DPBS (1).

Preparation of sera. Whole blood samples obtained by phlebotomy were allowed to clot for 30 min at 21°C, after which serum was separated by centrifugation and frozen at −70°C to preserve complement activity. The sources of sera for these experiments included a pool of six healthy adult laboratory workers (NHS) and an adult patient with common variable immune deficiency disease with hypogammaglobulinemia (immunoglobulin G, 125 mg/dl; immunoglobulin M, 12 mg/dl; immunoglobulin A, 7 mg/dl). Confirmation of normal hemolytic complement activity in these sera was accomplished as previously described (14). Levels of antibody against Hib capsular polysaccharide (PRP) were determined with an enzyme-linked immunosorbent assay (21). NHS contained an anti-PRP titer of 1:512. Hypogammaglobulinemic serum and all sera adsorbed with Hib organisms contained undetectable (<1:8) levels of anti-PRP.

Opsonization of bacteria. Mixtures of serum (0.5 ml) and bacterial suspensions (0.5 ml) were incubated at 37°C for 45 min in a shaking water bath. Mixtures were centrifuged at 6,000 × *g*, and the cell pellets were washed in DPBS. DPBS was used in place of serum as a control in some experiments. For reconstitution experiments in which various sources of antibody were added to hypogammaglobulinemic serum, equal numbers of bacteria were pelleted and used in the assay to maintain the concentration of all the reactants (21).

Luminol-enhanced CL assay. Bacterial pellets from opsonic mixtures were washed and resuspended in 0.5 ml of DPBS. Reaction mixtures containing opsonized or unopsonized bacterial suspensions (0.4 ml), 10^{-6} M luminol (5-amino-2,3-dyhydr-1,4-phthalazinedione; Sigma Chemical Co.) (0.1 ml), DPBS (0.2 ml; 1% human serum albumin in DPBS [0.05 ml]), and PMNs (10^7/ml; 0.25 ml) were prepared in 1.5-ml conical polyethylene microvials (bacteria-to-PMN ratio, 800:1). Quantitation of CL was performed in a liquid scintillation counter (model C2425 Tricarb; Packard Instruments Co., Inc., Downers Grove, Ill.) in the out-of-coincidence mode, and CL values were expressed as the integral or area under the curve (AUC) in total counts per 60 min (11).

Measurement of superoxide (O_2^-) generation (ferricytochrome c reduction). A modification of a method previously described was used, with opsonized bacteria as a stimulus to measure O_2^- generation by PMNs (2). PMNs (2.5×10^6) were incubated at 37°C for 5 min in the presence or absence of superoxide dismutase (100 μg/ml; Sigma Chemical Co.). Horse heart ferricytochrome c (final concentration, 0.08 mM; Sigma Chemical Co.) and 2×10^9 CFU of P+ or P− Hib were added to produce a final volume of 1.5 ml, which was gently tumbled at 37°C for 10 min. Controls were used, with DPBS instead of bacteria. Reactions were terminated by placing tubes in an ice-water bath. Cells and bacteria were pelleted, and supernatants were analyzed by spectrophotometry at 550 nm. Results were expressed as nanomoles of ferricytochrome c reduced per 10 min per 10^6 PMNs.

PMN uptake (attachment or ingestion) of radiolabeled Hib. Organisms grown in medium containing [^{3}H]thymidine (50 μCi/10 ml of broth) were prepared as above, washed, and resuspended in Hanks balanced salt solution (GIBCO Laboratories) containing 0.1% gelatin (Hanks balanced salt solution-gel). From this bacterial suspension 2×10^8 CFU were added to 2.5×10^6 PMNs suspended in Hanks balanced salt solution-gel (total volume, 0.35 ml; bacteria-to-PMN ratio, 80:1). The mixtures were incubated at 37°C for 30 min and then subjected to slow centrifugation (160 × *g*) for 5 min, three times with washing, to separate PMNs from the free bacteria. Cell pellets were recovered, and counts per minute of tritium were determined in a liquid scintillation

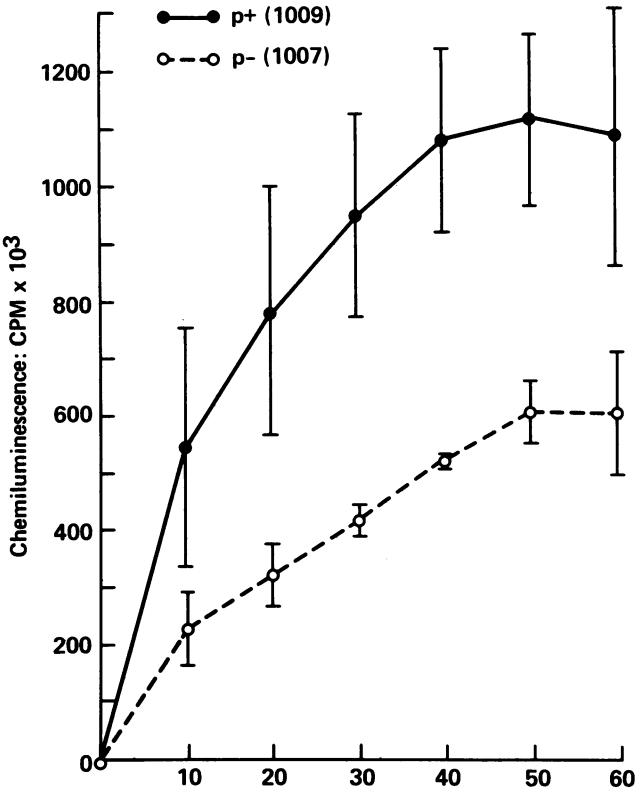

FIG. 1. Composite tracing of results of five separate experiments comparing PMN CL induced by 2×10^9 CFU of Hib strains 1009 and 1007 in a luminol-enhanced CL assay with 2.5×10^6 PMNs showing a marked increase in CL induced by strain 1009 (P+) versus strain 1007 (P−) ($P < 0.001$). Organisms were opsonized in NHS before the CL assay. Means ± 1 standard deviation for five separate experiments are shown.

spectrometer. Percent cell-associated counts were calculated by dividing counts per minute in the cell pellet by the total counts detected in vials containing 2×10^8 opsonized Hib alone.

Transmission electron microscopy. Unlabeled opsonized Hib and purified PMNs were incubated as in ^{3}H-labeled Hib uptake experiments for 15 min. After incubation, intact PMN pellets were fixed under 3% glutaraldehyde (Sigma Chemical Co.), postfixed in OsO_4, dehydrated with ethanol, and embedded into Araldite 502. Thin sections were prepared and stained with uranyl acetate and lead citrate. Photomicrographs of PMNs from six separate experiments were evaluated independently by three observers under blinded conditions. Based on the counts of each observer, a mean phagocytic index was computed and expressed as the percentage of PMNs containing ingested bacteria.

PMN degranulation. By using modifications of methods previously described, 5×10^6 PMNs were incubated with approximately 10^9 CFU of opsonized bacteria or DPBS (5, 15). PMNs were warmed to 37°C for 5 min in a shaking water bath, and bacteria were then added. The mixture was incubated for an additional 5 min at 37°C (total reaction mixture volume, 0.5 ml). Supernatants of these mixtures and pellets disrupted in 2% Triton X-100 (Sigma Chemical Co.) were assayed for β-glucuronidase, and results were expressed as percent release of total cell content (12).

Statistical methods. All statistical comparisons between results for P+ versus P− strains were performed by using the paired or unpaired Student *t* test.

TABLE 2. PMN CL induced by P+[a] versus P−[b] strains of Hib[c] in paired determinations[d]

Strain	CL[e]
P+	
884	445 ± 102
1264	468 ± 95
1009	500 ± 112
P−	
880	257 ± 80
1228	260 ± 52
1007	242 ± 65
1250	198 ± 70

[a] Nasopharyngeal isolates.
[b] Corresponding CSF isolates obtained from the same patient or rat (strain 1250).
[c] Opsonized in pooled NHS.
[d] Paired determinations: 884/880, $n = 2$; 1264/1228, $n = 3$; 1009/1007, $n = 5$; 1009/1250, $n = 3$.
[e] AUC or total counts per 60 min (mean ± standard deviation).

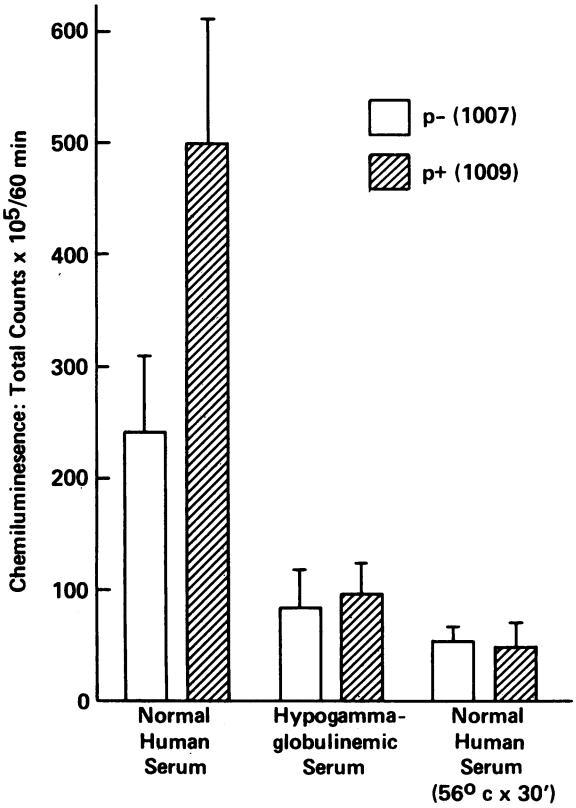

FIG. 2. Comparison of PMN CL induced by 2×10^9 CFU of Hib strain 1009 versus strain 1007 after opsonization in NHS, hypogammaglobulinemic serum, or heat-inactivated NHS. Results of CL after opsonization in NHS were higher ($P < 0.001$) for strain 1009 (P+) than for strain 1007 (P−). Opsonizing with hypogammaglobulinemic serum or heat-inactivated NHS produced lower overall CL values and showed no differences between P+ and P− strains. Results are for five separate experiments (mean ± 1 standard deviation).

RESULTS

CL induced by P+ versus P− strains of Hib. A composite tracing representing the mean of five separate experiments

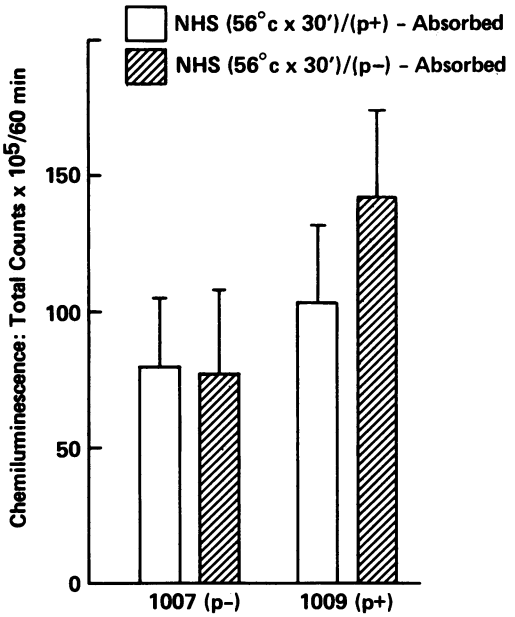

FIG. 3. Effect of pilus-directed antibody on CL induced by P+ versus P− strains of Hib is demonstrated. Heat-inactivated NHS absorbed with 5×10^{10} CFU/ml of Hib strain 1009 (P+) or 1007 (P−) was used to supplement equal volumes of hypogammaglobulinemic serum. These mixtures were used to opsonize P+ or P− strains for CL experiments. For P+ strains, CL was significantly higher ($P < 0.005$) when (P−)-absorbed NHS was used to supplement hypogammaglobulinemic serum than when (P+)-absorbed NHS was used. No such difference was observed for P− strains. Results are for four separate experiments (mean ± 1 standard deviation).

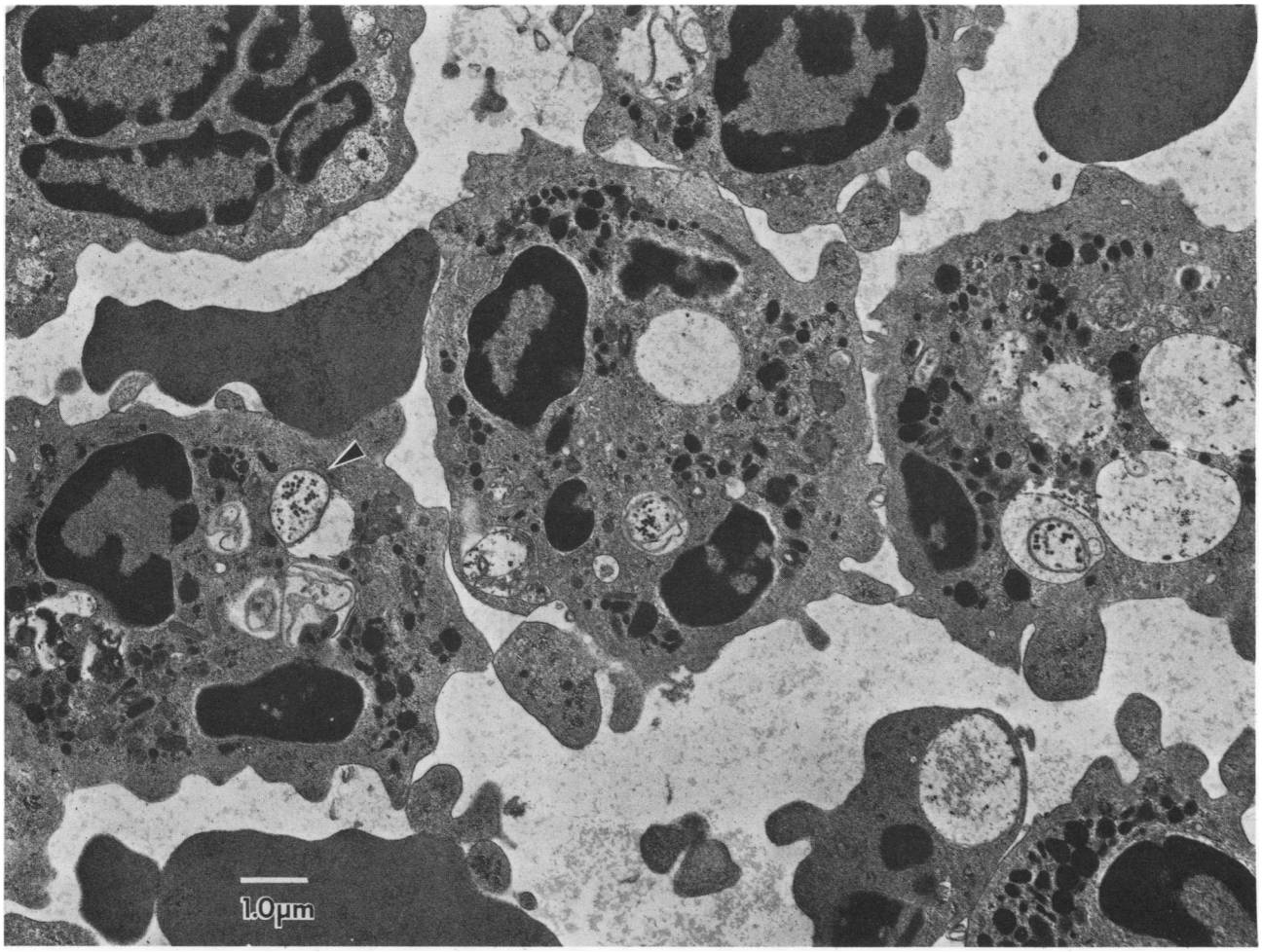

FIG. 4. Transmission electron micrograph of PMNs containing ingested Hib organisms (arrow). These are examples of cells which were counted as positive in determining a phagocytic index by examination of photomicrographs of random thin sections through fixed cell pellets after incubation with opsonized P+ or P− strains of Hib.

comparing PMN CL induced by a P+ strain (1009) as compared with a P− strain (1007) opsonized in NHS is shown in Fig. 1. The same data also are shown in a bar graph (Fig. 2) expressed as the integral (AUC) of CL evolution over a 60-min interval. As shown, CL generated by P+ organisms was significantly ($P < 0.001$) greater than that induced by P− organisms opsonized in NHS. In contrast, CL generated was comparable for P+ as compared with that for P− strains (and overall values were much lower) when organisms were opsonized in either hypogammaglobulinemic serum or heat-inactivated NHS (56°C for 30 min). No CL activity was detectable for P+ or P− strains incubated in DPBS (data not shown). Similar relationships between other P+ and P− pairs were observed as shown in Table 2.

Contribution of anti-pilus antibody. Figure 3 demonstrates CL activity generated by P+ or P− strains opsonized in the presence of hypogammaglobulinemic serum (source of exogenous complement) plus heat-inactivated serum adsorbed with either a P+ strain (1009) or a P− strain (1007). As shown, CL generated by P+ was significantly ($P < 0.005$) greater when opsonized in the presence of (P−)-adsorbed serum (AUC, $142 \times 10^5 \pm 32 \times 10^5$) as compared with (P+)-adsorbed serum (AUC, $103 \times 10^5 \pm 28 \times 10^5$). No such differences were observed for similarly opsonized P− strains.

PMN superoxide generation. In agreement with observations in CL assays, generation of O_2^- by PMNs was stimulated to a greater extent by P+ strains opsonized in NHS as compared with P− strains of Hib (2.83 ± 0.08 versus 1.94 ± 0.14 nmol of ferricytochrome c reduced per 10^6 PMNs, respectively; $n = 4$; $P < 0.002$).

Phagocytosis experiments using NHS-opsonized P+ or P− strains of Hib. In contrast to findings in CL and O_2^- assays, no significant differences were observed between radiolabeled P+ and P− Hib strains with respect to their attachment or ingestion, or both, by PMNs (11.6 ± 3.3 versus $11.7 \pm 3.6\%$ PMN-associated counts per min; $n = 6$). Phagocytic indices determined by transmission electron microscopy (Fig. 4) also demonstrated comparable ingestion (17.8 ± 0.7 versus $19.1 \pm 0.2\%$ PMNs containing ingested microorganisms; $n = 6$). In further experiments, phagocytosis-associated release of β-glucuronidase was comparable for opsonized P+ ($5.03 \pm 0.32\%$) versus P− ($4.75 \pm 0.11\%$) strains ($n = 3$).

DISCUSSION

Evolution of CL by human PMNs reflects oxidative metabolic activity associated with phagocytosis (with fusion of phagosomes and myeloperioxidase-containing lysosomal

granules) or as a result of microbial PMN membrane pertubations or interaction with soluble stimuli (7–9, 17). Findings in these studies that P+ Hib generate significantly enhanced CL activity and O_2^- generation or release, or both, as compared with P− strains emphasize that piliation represents an important determinant of the ability of Hib to stimulate PMN oxidative metabolism. These findings were evident only when Hib were opsonized with both complement and antibody from NHS. Further, experiments with heat-inactivated serum adsorbed differentially with either P+ or P− strains of Hib suggest that differences in CL activity observed are, in part, related to antibody directed against the pilus.

Our inability to detect significant differences between opsonized P+ and P− strains of Hib with respect to their ingestion by or association with PMNs, coupled with findings of increased O_2^- or CL generated by P+ as compared with P− strains, suggests that opsonized P+ Hib may interact with the PMN surface membrane in a manner distinct from P− strains. The precise nature of this interaction and subsequent activation of cellular oxidative activity is unclear and deserves more intensive study.

Our finding for NHS-opsonized Hib, demonstrating equivalent ingestion by PMNs of P+ and P− strains but greater stimulation of PMN oxidative metabolism by P+ versus P− organisms, contrasts with findings from previous studies with *Escherichia coli* (3, 4, 13), which did show increased CL levels induced by piliated (or fimbriated) *E. coli* compared with their nonfimbriated counterparts but which also clearly demonstrated enhanced phagocytosis of fimbriated strains. Results of similar studies with *Neisseria gonorrheae* (8, 20) in the presence of serum showed that P− strains were actually ingested by PMNs to a greater extent and generated higher levels of oxidative metabolism in the form of myeloperoxidase activity than did P+ strains. Interestingly, in the absence of serum, P+ *N. gonorrheae* stimulated much greater O_2 consumption than did P− *N. gonorrheae* (8). These comparisons with our findings for Hib serve to emphasize that the presence of pili or fimbriae may not have the same functional significance among different bacterial species. Hib pili are mannose resistant, in contrast with most *E. coli* pili, which also suggests functional heterogeneity among pili (10).

Our studies were designed to explore the effect of piliation on Hib-PMN interactions in the hope of shedding light on the nature of the apparent advantage of the P− state of Hib in the context of systemic infections (10). Our results demonstrating enhanced stimulation by P+ Hib of PMN oxidative microbicidal mechanisms, an index of host defense mobilization, would appear at least consistent with, and might play some role in mediating, this apparent advantage of P− strains. Other possible explanations for the isolation of P− strains from blood and CSF in systemic disease despite isolation from or inoculation (in rats) of the nasopharynx with P+ strains also should be entertained.

Studies to further explore the fundamental nature of Hib pilus-PMN surface interactions which might account for our observations, as well as additional immunochemical studies of the pilus of Hib, seem warranted.

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