Multiple Mechanisms in Serum Factor-Induced Resistance of
Haemophilus influenzae Type b to Antibody

MANO KURATANA,† ERIC J. HANSEN, and PORTER ANDERSON

Department of Microbiology, University of Rochester, Rochester, New York 14642, and Department of Microbiology, The University of Texas Southwestern Medical Center, Dallas, Texas 75235

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Incubation of Haemophilus influenzae type b at \( \leq 10^7 \) CFU/ml with serum ultrafiltrate induces a phenotypic conversion in which complement-mediated bactericidal activity by somatic antibodies decreases while killing by capsular antibody is unchanged. Conversion had been shown to occur in a capsule-deficient (b−) mutant of strain Eag (thus appearing independent of capsulation), to include an increase in lipopolysaccharide content, and to be inhibited by chloramphenicol or puromycin. In the present study, in several strains not previously examined, conversion was not inhibited by the drugs and the corresponding b− mutants did not convert. Incubation in ultrafiltrate was also found to increase capsulation, as detected by radioassay, only 1.6-fold in Eag but 4.5-fold in DL26, the strain with the largest increase in resistance; moreover, complement-mediated opsonization by capsular antibody was greatly decreased. Thus, multiple mechanisms, capsule dependent as well as independent, appear to contribute to the serum factor-induced resistance of H. influenzae type b to antibody.

Haemophilus influenzae type b (Hib), like other gram-negative bacteria, can be killed by bactericidal (BC) antibody and complement in vitro. Hib from the blood of infected rats was more resistant to BC activity than the same strain grown in conventional broth. By incubation at 37°C and \( \leq 10^7 \) CFU/ml with a 500-molecular-weight filtrate of normal serum, broth-grown Hib was converted within 30 min to a resistant phenotype resembling that of in vivo organisms (23). Some degree of this phenotypic conversion has been found in all of nine tested Hib strains (3, 13, 15). The structural basis of the difference between broth-grown (Sen) and resistant (Res) bacteria is difficult to study because of the limited yields of Res bacteria. The resistance affects killing by antibodies to lipopolysaccharide (LPS) and some other outer membrane proteins (OMPs) (16), while Res and Sen cells are equally susceptible to the BC activity of capsular antibody (3, 16, 23). In six of six studied strains, cells with the Res phenotype appeared to contain two- to fourfold more LPS of the same electrophoretic mobility as that of the LPS from cells with the Sen phenotype. In the strain (Eag) studied in most detail, chloramphenicol or puromycin, which blocked the increase in resistance, also blocked the apparent increase in LPS content. Sen and Res cells of strain Eag produced roughly equal amounts of the polyribosyl-ribitol-phosphate capsular antigen (PRP) per CFU (13, 23). Moreover, a capsule-deficient (b−) mutant, strain Eag b− S2, although much more sensitive to LPS antibody, responded to serum filtrate with a relative increase in resistance similar to the parent strain. Therefore, it appeared that the phenotypic conversion was independent of capsule expression (13, 23).

In the present study, when additional strains were examined, several were found in which conversion was not blocked by chloramphenicol or puromycin and was not expressed in the b− mutants. This observation led to reexamination of capsule expression, and it was found that Res cells expressed more PRP per unit of cell protein than Sen cells. Additional evidence for an increase in functional capsulation was found in the effect of the shift on uptake by leukocytes in vitro. Res cells were more resistant to opsonization not only by antibodies to somatic antigens but also by antibodies to PRP. Overall the data indicate that at least two mechanisms are involved in the acquisition of resistance in response to low-molecular-weight components of serum.

MATERIALS AND METHODS

Bacterial strains. Hib strains Eag, Rab, and Mad (3), DL26 (9), and DL42, DL63, and OA104 (7) have been described. Hib strains Eag b− S2 (2) and DL26 b− #1 (9) are spontaneous capsule-deficient (b−) mutants that appear as colonies smaller and less mucoid than those of their parents; these mutants were shown to elaborate <1% of the parental amount of PRP but to express the same OMP (9, 18) and LPS (9, 11) electrophoretic profiles. The additional b− mutants were isolated in the same way and were assayed for PRP but not for OMP or LPS profiles.

Bacterial culture. The bacteria were kept at \( \sim \)70°C in skim milk. A subculture plate was made by partially defrosting the skim milk suspension, streaking a loopful onto agar made with brain heart infusion (Difco Laboratories, Detroit, Mich.) containing horse red blood cell lysate (1:1,000 [vol/vol]) and 1 µg of NAD per ml (BHI-XV), and incubating at 37°C for about 18 h. The bacteria were then grown in BHI-XV broth at 37°C in shaker, baffled flasks to an optical density known to correspond to about \( 10^8 \) CFU/ml (late exponential phase). For opsonization studies, a sample of the bacteria was diluted 1/10 in prewarmed BHI-XV containing [methyl-3H]thymidine (2 Ci/mmol; Dupont, NEN Research Products, Boston, Mass.) at 8 µCi/ml and further incubated to \( 10^8 \) CFU/ml; these bacteria were washed once in sodium phosphate-buffered saline (pH 7.4) containing 0.15 mM CaCl₂, 0.5 mM MgCl₂, and 0.1% bovine serum albumin (PCMA) to remove unincorporated 3H.

Phenotypic conversion. To make Sen bacteria, bacteria were diluted in PCMA to \( 5 \times 10^6 \) CFU/ml and assayed...
immediately or after further incubation in PCMA for 30 min at 37°C; both preparations are equally susceptible to BC activity (13). To make Res bacteria, bacteria at 5 × 10⁶ CFU/ml in PCMA were mixed 1:1 with serum filtrate and incubated for 30 min at 37°C. Adjustments were made for the somewhat larger increase in CFU in the serum filtrate. The effect of chloramphenicol (0.01 to 0.06 mM) or puromycin (0.02 mM) on conversion was tested by adding the drugs at the onset of the 30-min incubation; the concentration of antibiotics used was capable of inhibiting the growth of each strain in control studies. Assays to compare phenotypes were done after either washing or diluting 300-fold in PCMA so the test environments were equal.

**Biological reagents.** The precolostral calf serum used as the complement source and the filtrate of normal human serum (PM10 filter, 10,000-molecular-weight cutoff; Amicon Corp., Danvers, Mass.) were described previously (13). Preparation of rabbit antiserum to purified LPS of strain Eag (RT214) and to live Eag b⁻² S2 in the Res phenotype (RT212) was also described previously (13); Western blot (immunoblot) analysis later showed a majority of antibodies reactive with the characteristic LPS band but also antibodies reactive with OMPs or possibly traces of LPS bond to these proteins (16). RT212 killed all strains in the study except Mad and OA104, and RT214 killed all but Mad. Rabbit antiserum R17 was raised against whole cells of strain Mad b⁻²; it killed Mad as well as Mad b⁻². In the antisera described above at the dilutions used, the concentrations of capsular (PRP) antibodies were <0.04 μg/ml in the assay described below. Affinity column-purified rabbit antiserum against Eag LPS was the gift of Marilyn Loeb, and immunoglobulin G (IgG) from human donors vaccinated with various bacterial polysaccharides including that of Hib (BPIG) was the gift of George Siber (1). Monoclonal antibodies (MAbs) to Hib LPS (6A2 and 5G8, murine IgG) (8) and to PRP (16M3C8, human IgG) (6) were previously described. MAbs 6A2 and 5G8, which were produced in tissue culture, were dialyzed twice at 4°C overnight with 200 volumes of PCMA (similar to PCMA but without albumin) to avoid the possible BC effect of antibodies present in the culture supernatant. MAB 16M3C8, from mouse ascites, was partially purified by ammonium sulfate fractionation and dialysis.

**BC assay.** Duplicate mixtures of the bacteria (4 × 10⁴ CFU/ml), the complement source (see below for dilutions), and serial dilutions of antiserum were brought to total volumes of 30 μl with PCMA in 96-well microtiter plates. The mixtures were incubated for 30 min at 37°C and chilled to stop the BC reaction. Samples (10 μl) were spread on BHI-XV agar, and the number of colonies after 24 h of incubation at 37°C was counted to determine the percent survival [100 × (CFU after incubation with antiserum/CFU after incubation without antiserum)]. The mean percent survival values were graphed to estimate the dilution of antibody giving 50% survival (D₅₀), and the shift ratio was computed as D₅₀ of Res bacteria/D₅₀ of Sen bacteria. Every strain was preliminarily tested in a checkerboard titration in which both antibody source and complement were independently diluted; thus, a plateau range of complement concentrations was defined that caused no killing per se but supported antibody-dependent killing. The capsumlated strains were not killed by complement at the highest concentration tested (33% [vol/vol]); 10% (vol/vol) complement fell on the plateau and was used in the BC assay for these strains. The b⁻² mutants were killed to some degree by complement at higher concentrations, but 2.5% (vol/vol) could be used as a plateau concentration for these strains.

**Radioantigen binding inhibition assay for PRP content.** Bacteria of the Sen and Res phenotypes were centrifuged at 12,000 × g for 15 min, washed once with PCMA, and resuspended in a known volume of PCMA at an estimated 10⁸ CFU/ml. Small samples of the bacterial suspensions were diluted in PCMA and plated onto BHI-XV agar to determine the actual CFU. The bulk of the bacteria were then centrifuged, the supernatants were discarded, and the bacterial pellets were kept at −70°C. After the number of CFU was determined (the following day), the bacterial pellets were suspended with distilled water to an equivalent of ~1.8 × 10⁹ CFU/ml and samples of the suspensions were assayed for protein by the version of the method of Lowry et al. intended for insoluble proteins (19). All the suspensions were then adjusted to the same protein concentration by adding appropriate amounts of distilled water; 25 μl of each bacterial suspension (containing ~15.5 ng of protein) was mixed with 25 μl of ³H-labeled PRP (1 nCi, 0.25 ng) (2), and 25 μl of a PRP antibody (U.S. Food and Drug Administration standard human antiserum) was then added at a concentration that resulted in about 50% binding of [³H]PRP when no inhibitor was used. After incubation overnight at 4°C, the percentage of [³H]PRP bound by ammonium sulfate-precipitable antibody was determined as described previously (2), and the amount of nonradioactive PRP active as an inhibitor in the bacterial suspensions was determined from a standard inhibition curve made with twofold dilutions of purified PRP (4) at 400 to 3.125 ng/ml in place of bacterial cells.

**Quantitation of LPS by electrophoresis.** The apparent content of LPS in the bacteria was examined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (17) and periodate-silver staining (28). The bacteria were collected, washed, resuspended, and adjusted to the same protein concentration as described above. Twenty-five microliters of each bacterial suspension in distilled water (containing about 3.9 μg of protein) was mixed with 25 μl of 2× cracking buffer, and the mixture was heated for 5 min at 100°C. The mixture was then combined with 10 μl of proteinase K (2.5 mg/ml in water) and incubated at 60°C for 1 h to digest the cell protein as described previously (27). Samples (40 μl) containing 2.6 μg of protein were electrophoresed in a 14% separating gel containing 3 M urea by using the discontinuous system of Laemmli (17) and stained by the periodate-silver method (28). If LPS from Sen and Res bacteria is assumed to stain equally, bands of equal density by eye would represent equal quantities of LPS. Thus, to better estimate the apparent quantity of LPS in bacteria of different phenotypes, 25-μl portions of diluted bacterial suspensions containing one-half and one-quarter of the amount of protein mentioned above were also included as samples of each phenotype.

**In vitro uptake of bacteria by human PMN.** Human polymorphonuclear leukocytes (PMN) were isolated by dextran and Ficoll-Paque sedimentation as described previously (5). Just before use, the cells were suspended at 10⁶/ml with Hanks balanced salt solution containing 0.1% gelatin (HBG). ³H-labeled, washed bacteria were suspended at 10⁶ CFU/ml with PCMA; half were kept as the Sen phenotype and half were converted to the Res phenotype by a 30-min incubation with PCMA or PCMA-serum filtrate (1:1). Bacteria of both phenotypes were then washed and suspended at 3 × 10⁸ CFU/ml in HBG. Small samples of the suspensions were diluted in PCMA and plated on BHI-XV agar to determine the
TABLE 1. Shifts in resistance of several strains and their capsule-deficient (b−) mutants induced by serum filtrate without and with chloramphenicol

<table>
<thead>
<tr>
<th>Strain</th>
<th>Antibody source</th>
<th>Shift ratio with:</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td>No drug</td>
</tr>
<tr>
<td>Eag</td>
<td>RT212</td>
<td>6.0</td>
</tr>
<tr>
<td>Eag b−</td>
<td>RT212</td>
<td>4.0</td>
</tr>
<tr>
<td>Mad</td>
<td>R17</td>
<td>&gt;2.0∗</td>
</tr>
<tr>
<td>Mad b−</td>
<td>R17</td>
<td>4.0</td>
</tr>
<tr>
<td>DL26</td>
<td>RT214</td>
<td>60</td>
</tr>
<tr>
<td>DL26 b− #1</td>
<td>RT214</td>
<td>1.0</td>
</tr>
<tr>
<td>DL26 b− #2</td>
<td>RT214</td>
<td>1.0</td>
</tr>
<tr>
<td>OA104</td>
<td>RT214</td>
<td>40</td>
</tr>
<tr>
<td>Rab</td>
<td>RT212</td>
<td>20</td>
</tr>
<tr>
<td>Rab b−</td>
<td>RT212</td>
<td>1.0</td>
</tr>
<tr>
<td>DL42</td>
<td>MAb 5G8</td>
<td>4.0</td>
</tr>
<tr>
<td>DL42 b−</td>
<td>MAb 5G8</td>
<td>1.0</td>
</tr>
</tbody>
</table>

* Concentrations used (0.01 to 0.06 mM) were the minimum shown in separate experiments to completely inhibit A50 increase of the particular strain.

The lowest antiserum dilution used was not concentrated enough to reach the D50 for bacteria with the Res phenotype.

Table 1 also shows variations among strains in the inhibition of conversion by chloramphenicol at minimally bacteriostatic concentrations and in the conversion of b− mutants. The strains seemed to fall into two groups. With Eag and Mad, the b− mutants and the parents exhibited shifts and the shifts were blocked by chloramphenicol completely in the mutants and substantially in one of the parents (Eag) (similar results were found with puromycin and were not tabulated). The blocking in strain Mad was not apparent; however, the lowest antiserum dilution used was not concentrated enough to reach the D50 for bacteria of the Res phenotype. In contrast, in DL26, OA104, Rab, and DL42 there was no blocking of the shift by the antibiotics and no shift in the b− mutants (Table 1). PRP expression by the cells was measured in a radioantigen binding inhibition assay (Table 2). Eag and DL26 were chosen for study from the two tentative strain groupings. Both versions of the Sen phenotype (direct assay and buffer incubated) were examined; in both DL26 and Eag the PRP/cell protein values were identical for the two versions of Sen. In the Sen phenotype, Eag had a threefold higher PRP content than DL26. Relative to Sen, DL26 with the Res phenotype had a 4.5-fold increase in response to filtrate while Eag had only a 1.6-fold increase.

Bacteria of both phenotypes were further examined for uptake by PMN in vitro. In this system Hib cells were biosynthetically labeled with [3H]thymidine, half were converted to the Res phenotype, cells of both phenotypes were exposed briefly to various opsonic combinations, PMN were added, and the uptake of radioactivity was measured. Strain DL26 (Fig. 3) showed modest uptake without opsonization, and there was greater uptake of bacteria of the Sen (10%) than Res (6%) phenotype (P < 0.05). Uptake was not increased by exposure to complement alone. There was clear opsonization by the LPS MAb 6A2 plus complement, and uptake of bacteria of the Sen phenotype was increased substantially more than that of Res bacteria (P < 0.01). The strongest observed opsonization was by a PRP MAb plus complement; notably, Sen bacteria were more effectively opsonized than Res bacteria. In strain Eag (Fig. 4) there was little uptake without opsonization and no increase with complement alone. With PRP antibody plus complement there was an additional increase in uptake of Sen bacteria but less so for Res bacteria. As with DL26, the Res bacteria

RESULTS

When examined with sources of polyclonal somatic antibody such as RT212 and RT214, strain DL26 showed a much greater increase in resistance than strains previously described (15; Table 1); therefore, the specificity of its phenotypic conversion was further examined. The effect of serum filtrate on killing of strain DL26 in the BC assay is shown in Fig. 1. Conversion to resistance to a particular antibody source is given as the shift ratio: the fold increase in antibody required for 50% killing with a constant dilution of complement. For strain DL26 the shift ratio was 6.0 with a LPS-directed MAb but 1.0 with a PRP-directed MAb. Thus, as with previously studied strains, the difference between Sen and Res phenotypes was "recognized" by somatic but not capsular antibodies in the BC assay. Likewise, DL26 showed an apparent increase in LPS; Sen bacteria assayed directly from broth culture and Sen bacteria incubated in buffer had bands of equal density in SDS-PAGE, while Res bacteria showed bands about 1.5-fold denser in samples equalized for bacterial protein (Fig. 2A). Very similar results were found under these conditions with strain Eag (Fig. 2B), for which earlier studies had correlated periodate-silver-stained band density with LPS concentration in an immunoassay (13). Additional Hib strains not previously examined (Mad, OA104, and DL42) were tested with serum filtrate, and all showed an increase in resistance to somatic but not PRP-directed antibodies. In general the extent of increase with a particular strain differed with the source of somatic antibody. The antibody source giving the highest shift ratio observed with the strains in this study is shown in Table 1. The maximal shift differed among strains and could be seen even with a common antibody source, e.g., strains Eag and Rab with RT212. Table 1 also shows variations among strains in the inhibition of conversion by chloramphenicol at minimally bacteriostatic concentrations and in the conversion of b− mutants. The strains seemed to fall into two groups. With Eag and Mad, the b− mutants and the parents exhibited shifts and the shifts were blocked by chloramphenicol completely in the mutants and substantially in one of the parents (Eag) (similar results were found with puromycin and were not tabulated). The blocking in strain Mad was not apparent; however, the lowest antiserum dilution used was not concentrated enough to reach the D50 for bacteria of the Res phenotype. In contrast, in DL26, OA104, Rab, and DL42 there was no blocking of the shift by the antibiotics and no shift in the b− mutants (Table 1). PRP expression by the cells was measured in a radioantigen binding inhibition assay (Table 2). Eag and DL26 were chosen for study from the two tentative strain groupings. Both versions of the Sen phenotype (direct assay and buffer incubated) were examined; in both DL26 and Eag the PRP/cell protein values were identical for the two versions of Sen. In the Sen phenotype, Eag had a threefold higher PRP content than DL26. Relative to Sen, DL26 with the Res phenotype had a 4.5-fold increase in response to filtrate while Eag had only a 1.6-fold increase.

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were less effectively opsonized by capsular, as well as LPS, antibody. Thus, in contrast to BC activity, the conversion from the Sen to the Res phenotype diminished the effect of capsular antibody when opsonic activity was compared.

**DISCUSSION**

Incubation with low-molecular-weight serum components decreases the susceptibility of all tested Hib strains to killing by somatic antibodies, but the present study indicates that a single mechanism is unlikely to account for all the resistance in all strains. As a working hypothesis we envision at least two mechanisms: (i) one mechanism is inhibited by chloramphenicol or puromycin, can occur in b− mutants, gives at most a modest increase in resistance, and is accompanied by an apparent increase in LPS content; (ii) the other mechanism is insensitive to the inhibitors, does not occur in b− mutants, can give large increases in resistance, and is accompanied by an increase in PRP that may be its structural basis. The resistance of particular strains to bacteriolysis can come from various combinations of mechanisms i and ii. For example, the capsule-deficient mutant Eag b− S2 acquires resistance entirely by mechanism i, while its parent may employ mechanism i but get a small increment of resistance also from mechanism ii. Strain DL26 gets a large increase in resistance by mechanism ii; since its b− mutants did not convert, it is possible that mechanism i contributes no resistance to the mutants or parent.

**TABLE 2.** Capsular polysaccharide (PRP) expression by Sen and Res bacteria of strains DL26 and Eag as detected by radioantigen binding inhibition assay

<table>
<thead>
<tr>
<th>Treatment (phenotype)</th>
<th>ng of PRP/ng of cell protein</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Strain DL26</td>
</tr>
<tr>
<td>No further incubation (Sen)</td>
<td>0.052 ± 0.002</td>
</tr>
<tr>
<td>Incubated with PCMA (Sen)</td>
<td>0.053 ± 0.004</td>
</tr>
<tr>
<td>Incubated with serum filtrate</td>
<td>0.23 ± 0.008</td>
</tr>
</tbody>
</table>

FIG. 1. Dilution-killing curves for Sen and Res bacteria of strain DL26 by MAbs. Sen (○) and Res (■) bacteria were tested with capsular polysaccharide (PRP) MAb 16M3C8; Sen (△) and Res (▲) bacteria were also tested with LPS MAb 6A2. The vertical bars denote the standard error of replicate determinations.

FIG. 2. Electrophoretic band density of LPS in proteinase K-digested cells of the Sen and Res phenotypes. (A) Strain DL26; (B) strain Eag. Lanes 1 to 3, Broth-grown bacteria collected without further incubation; lanes 4 to 6, bacteria incubated with PCMA; lanes 7 to 9, bacteria incubated with PCMA-serum filtrate (1:1). Lanes 1, 4, and 7 contained 2.6 μg of cell protein; lanes 2, 5, and 8 contained 1.3 μg of protein; and lanes 3, 6, and 9 contained 0.65 μg of protein.
The serum component(s) responsible for conversion of Hib remains to be identified. The sialyl donor, cytidine 5'-monophospho-N-acetylneuraminic acid, present in blood and other host tissues, was found to induce a superficially similar phenotypic conversion in gonococci (20). However, this compound has recently been shown not to increase the resistance of Hib strains Eaq b^- S2 and DL26 (13); therefore, it appears not to be involved in either mechanism i or ii.

The structural basis of mechanism i is poorly understood. Although conversion was inhibitable by chloramphenicol or puromycin, no new membrane proteins could be detected by one-dimensional SDS-PAGE in the conversion of strain Eaq or Eaq b^- S2. No Res-specific antigens were revealed in Eaq b^- S2 by adsorption of Res antiserum on Sen cells (13). Since the only noted structural correlate was an increased content of LPS of identical size (i.e., same mobility pattern in SDS-PAGE), Inzana postulated an increase in an enzyme involved in LPS biosynthesis and an increased multiplicity of LPS chains per unit area of outer membrane that would sterically interfere with antibody binding to proximal epitopes in the LPS (13). If correct, this model might also account for the subsequently observed resistance to antibodies against certain outer membrane proteins (16). However, strains DL26 (Fig. 2A) and Rab (13) show the LPS increase while appearing to gain no resistance by mechanism i (Table 1); thus, additional LPS does not necessarily give greater resistance. Additional work is needed on the composition and steric relationships of membrane components in response to serum filtrate. The present finding of strains that appear not to gain resistance by mechanism i will assist future analysis. Such research would better be done with b^- mutants since the present study also shows (Table 2) that incubation of capsulated Hib in serum filtrate can superpose a capsule increase upon hypothetical mechanism i.

It seems likely that the observed increase in capsule is the structural basis for mechanism ii. In gram-negative bacteria generally (26) and Hib in particular (12, 13, 29, 30), it is known that genetic or phenotypic increases in capsulation can reduce the BC activity of somatic antibodies. The increased in PRP content brought about by serum filtrate was missed in two earlier studies (13, 23). These studies compared Sen and Res bacteria on the basis of CFU rather than cell protein and also examined only strain Eaq, which the present study showed to have a small, although consistent, increase. Eaq is relatively highly capsulated even in Sen cells, so its 1.6-fold increase in conversion to Res may account for the very modest chloramphenicol-insensitive component of resistance to BC activity. At the other extreme, DL26 has low capsulation in Sen cells but had a large relative increase in response to serum filtrate, which may account for its large shift ratio.

For both strains, additional evidence that the increase in PRP (as determined by radioassay) increases capsulation in a functional sense was seen in the opsonization assay, in which the activity of capsular, as well as somatic, antibodies was diminished. It is an interesting but instructive paradox that the shift decreased the complement-dependent opsonization by PRP antibody but not the complement-dependent killing. In complement-mediated killing of gram-negative bacteria, it is thought that antibody must initiate the formation of the C5b-9 complex near the outer membrane (14). Thus, in Hib, access of antibody to PRP epitopes proximal to the outer membrane evidently is not affected by the serum factor-induced increase in PRP content. Why the PRP increase diminishes the functional access of LPS and other somatic antibodies remains to be explained. The difference may be related to the observation that the BC activity of somatic antibodies against Hib requires classical pathway components (24) while killing by PRP antibody can utilize either classical or alternative pathways of complement activation (24, 25). For best opsonization, the antibody-bound C3 must be activated in a site accessible to phagocytic cell receptors and protected from degradation by serum factors H and I (14). Evidently in Res Hib, although more PRP antibody is bound than in Sen Hib, the site or mode of the subsequent complement activation is less effective.

Although serum BC activity in vitro is an accepted correlate of immunity to Hib (for a review, see reference 1), there

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**FIG. 3.** Effects of several opsonizing combinations on uptake of ^3^H-labeled Sen and Res bacteria of strain DL26 by human PMN. Unopsonized bacteria incubated without PMN and with PMN were also tested as controls for radioactive background and nonspecific uptake by PMN, respectively. C, Complement; LPS MAb, MAb 6A2 to LPS; PRP MAb, MAb 16M3C8 to capsular polysaccharide.

**FIG. 4.** Effects of several opsonizing combinations on uptake of ^3^H-labeled Sen and Res bacteria of strain Eaq by human PMN. Unopsonized bacteria incubated without PMN and with PMN were also tested as controls for radioactive background and nonspecific uptake by PMN, respectively. C, Complement; LPS Ab, affinity-purified rabbit antibody to strain Eaq LPS; PRP Ab, human IgG fraction containing a high level of antibody to capsular polysaccharide (1).
is evidence that immune clearance in vivo operates through opsonization (21, 30). Thus, the Sen/Res phenomenon would appear to affect the protective potential not only of planned Hib vaccines based on somatic antigens but also the existing, capsule-directed vaccines. It should be noted that, with any specificity studied thus far, the resistance is quantitative: an antibody killing Sen cells can also kill Res cells if the concentration is raised. For example, the LPS MAb 6A2 (although subject to a shift in strain DL26) can clear an established DL26 bacteremia in infant rats (10), in which presumably a Res-like phenotype pertains. Future animal challenge studies of antibody protectiveness might appropriately use Hib preconverted to the Res phenotype rather than the usual broth cultures grown to high density.

It appears that low-molecular-weight factor(s) able to produce the Res phenotype are present in the nasopharynx (15, 22), the usual habitat of Hib. Thus, although the present study examined mechanisms of phenotypic conversion by these host factor(s), their actual role may be the maintenance of a Res-like phenotype in a bacterium that normally resides only in the human host.

ACKNOWLEDGMENT

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LITERATURE CITED