Antibodies to *Haemophilus influenzae* Type b Polysaccharide Affect Bacterial Adherence and Multiplication

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Since immunization of infants with conjugated *Haemophilus influenzae* type b (Hib) capsular polysaccharide (PS) vaccines results in a reduction of colonization, we determined the inhibitory effect of anti-Hib PS on two steps in the colonization, i.e., adherence of *H. influenzae* to nasopharyngeal epithelium and bacterial growth. Monoclonal antibody (MAb) E117-5 specific for Hib PS inhibited at a concentration of at least 80 µg/ml in vitro the adherence of Hib strain 770235f⁺b⁺ to oropharyngeal epithelial cells by 50% ($P < 0.02$), but this MAb and sera from children immunized with Hib PS conjugate vaccine ($n = 10$) were not inhibitory in final dilutions containing up to 20 µg of anti-Hib PS per ml. The growth of Hib strain 770235f⁺b⁺ did completely stop in the presence of 5 µg of anti-Hib PS MAb E117-5 per ml and human sera with an anti-Hib PS concentration of 2 µg/ml or more, in contrast to the growth of the nonencapsular variant strain 770235f⁺b⁰.

The nasopharynx of healthy human beings is the natural habitat for *Haemophilus influenzae* type b (Hib). In childhood Hib is the leading cause of invasive disease, including meningitis, epiglottitis, and cellulitis (18). Immunization with conjugated vaccines consisting of the capsular polysaccharide (PS) of Hib covalently coupled to a protein carrier molecule results in prevention of these invasive Hib diseases by eliciting sufficient levels of antibodies to the type b PS (12). Takala et al. demonstrated that immunization of infants with the Hib PS-diphtheria toxoid conjugate vaccine in Finland resulted in a reduction of Hib disease and carriage (16). In a prospective study by Murphy et al. a reduction of carriage of Hib was demonstrated in day-care centers among vaccinated children, although these children had been exposed to Hib (10), and in a prospective study by Takala et al. the carrier rate was found to be lower among vaccinated than nonvaccinated American Indian children (17).

Colonization by *H. influenzae* requires bacterial adherence to epithelial cells of the nasopharynx and in situ bacterial multiplication. Adherence of *H. influenzae* to epithelial cells is promoted by fimbriae (24) and nonfimbrial adhesins, including in nonencapsulated strains the high-molecular-mass proteins of 105 to 110 kDa (2). Four types of fimbriae have been described, of which the LKP fimbriae have been characterized most extensively. The gene cluster encoding expression of LKP-4 fimbriae in Hib has been sequenced and analyzed (25, 26), and it has become clear that the fimbriae consist of polymers of the major subunit protein HifA of 23 kDa and two minor proteins, HifD and HifE (26). The major subunit mediates adherence to nasopharyngeal epithelial cells containing a mono-sialoganglioside 2 (GM2) structure (20). The contribution of fimbriae in the colonization of Hib has been affirmed in a monkey infection model, since an *H. influenzae* variant unable to express fimbriae showed decreased colonization ability (28). Moreover, in organ culture models, adherence of encapsulated *H. influenzae* in contrast to nonencapsulated vari-

ants was facilitated by the expression of fimbriae (9). Fimbriae may therefore promote adherence of encapsulated *H. influenzae* by exposing the required bacterial adhesin at the outside of the shielding and negative charge of the PS capsule.

Binding of antibodies to PS capsules results in the formation of a gel-like structure around the bacteria known as the Quellung reaction, the capsule swelling of the bacteria. The antibody-PS complexes around the bacteria may influence sterically and in a physical-chemical way the interaction of the bacteria with epithelial cells of the nasopharynx and the outgrowth of the bacteria. The infant rat model for nasopharyngeal colonization by Hib was used by Kauppi et al. (6) to study the effect of anti-Hib PS antibodies on nasopharyngeal colonization. Human secretory immunoglobulin A (IgA) and IgG and murine monoclonal IgG1 anti-Hib PS administered intranasally or intraperitoneally reduced colonization significantly (8). Since Fab₂ fragments of these antibodies also inhibit colonization (7), a direct blocking effect of anti-PS on bacterial colonization seems likely.

Therefore, we determined whether binding of anti-Hib PS in contrast to antibodies to other surface components results in blockage of fimbria-mediated adherence of Hib to oropharyngeal epithelial cells and the growth of Hib on epithelial cell layers in culture.

MATERIALS AND METHODS

Bacteria and culture conditions. Hib strains 760705 and 770235 were isolated from cerebrospinal fluid. These strains do not express fimbriae (23). Fimbriated bacteria of strain 770235 (i.e., strain 770235f⁺b⁺) were obtained after enrichment on erythrocytes as described before (23). Nonfimbriated *H. influenzae* mutant 770235f⁰b⁺ was obtained by insertion of a kanamycin cassette in the fimbrial subunit *hifA* gene (26). Nonencapsulated variants of these strains (770235f⁺b⁰ and 770235f⁰b⁰) were selected on antiserum agar plates as described elsewhere (22). The absence of the type b capsule was analyzed by coagglutination (3) and a whole-cell enzyme-linked immunosorbent assay (ELISA) (19) with monoclonal antibody (MAb) E117-5, specific for the type b PS (11). *H. influenzae* strains were routinely cultured on chocolate agar plates overnight at 37°C in a humid atmosphere enriched with 5% CO₂.

Bacterial growth on cell layers. Monolayers of cell line NCI-H292 (ATCC CRL 1448) derived from a human pulmonary mucocypidermoid carcinoma (1) maintained in RPMI containing 10% heat-inactivated fetal calf serum were subcultured twice a week. The H292 cells grown to confluency in 24-well plates on glass slides were inoculated with *H. influenzae* at a density of 2×10^5 bacteria per ml in RPMI. Bacterial growth was monitored by subculturing aliquots of the

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medium at various time intervals on chocolate agar plates and, after incubation, counting of the number of CFU.

Assessment of adherence of *H. influenzae* to epithelial cells. Adherence of *H. influenzae* to human oropharyngeal epithelial cells was performed as follows. Oropharyngeal epithelial cells were obtained from a healthy adult volunteer (not smoking, not using antibiotics) by scraping the posterior of the pharyngeal cavity. The cells suspended in phosphate-buffered saline (PBS; 15 mM sodium phosphate, 0.1 M NaCl, pH 7.4) were spun down by centrifugation for 10 min at $250 \times g$, washed with PBS, and resuspended in PBS at a density of 5×10^5 cells per ml as assessed by microscopy with a counting chamber. Fifty microliters of this suspension was transferred to a round glass slide. The cells were dried on air and fixed with paraformaldehyde (1%, wt/vol) for 30 min at room temperature. *H. influenzae* was suspended in Dulbecco modified Eagle medium (DMEM; GIBCO Laboratories) including 15% fetal calf serum (FCS) to a density of 10^{10} bacteria per ml. Ten microliters of the bacterial suspension was diluted with 50 μ l of DMEM–15% FCS and transferred to the epithelial cells on the glass slides, which were incubated at 37°C in a humid atmosphere for 1.5 h. Then glass slides were rinsed five times with PBS containing 0.05% Tween 20, treated with methanol for 10 min to fix adherent bacteria, and subsequently stained overnight with a 1:30-diluted (in water) standard crystal violet solution. The number of attached bacteria per cell was determined by counting bacteria on 20 cells with a light microscope. Since up to 20 bacteria per cell were found to adhere independently of the *H. influenzae* variant exposed, only cells with at least 20 adherent bacteria were used to assess the number of attached bacteria. The proportion of epithelial cells without attached bacteria was usually low (0 to 16%).

To determine the effect of antibodies on adherence, 6 μ l of human serum samples, milk samples, or murine ascitic fluid containing MAbs was added to 44 μ l of DMEM containing 15% FCS. From a bacterial suspension of 10^{10} bacteria per ml in DMEM containing 15% FCS, 10 μ l was added to the mixture of DMEM and serum or murine ascitic fluid and then incubated at 37°C for 30 min. Then 10 μ l of the suspension was transferred to the epithelial cells on glass slides, and bacterial adherence was determined as described above.

Antibodies. Burro antibody 132 (anti-Hib PS; 14.1 mg/ml) (15), elicited by immunizing a burro with Hib PS, was a generous gift of John Robbins (Food and Drug Administration, Bethesda, Md.), and MAb E117-5, an IgG1 (κ) murine MAb containing about 800 μ g of anti-Hib PS per ml, was kindly provided by Dace Madore (Praxis Biologics) (11). MAbs specific for lipopolysaccharide serotypes 1 and 2 (LPS1 and LPS2) (MAb 3DA11), outer membrane protein (OMP) P1 (MAb 5HA5), OMP P5 (4BF8), and OMP P6 (MAb 8BD9) were used as controls. By immunoelectron microscopy these antibodies were shown to bind to intact bacteria (19, 23). MAb 6HE8 was specific for the fimbriae of strain 770235. These MAbs have been described before (21, 23). Fluorescein isothiocyanate-labeled anti-mouse immunoglobulins as the secondary antibody in flow cytometry were obtained from the Central Laboratory for Blood Transfusion Services, and horseradish peroxidase-labeled anti-immunoglobulins for ELISA were obtained from Dakopatts.

Human pre- and postvaccination serum samples were obtained from 10 children boosted with Hib PS conjugate vaccine at 14 months (6). Also a pool of pre- and postvaccination serum samples from similar children was used. Breast milk samples were obtained from a mother before and after vaccination with Hib conjugate vaccine (8).

Antibody determinations. The titers of the murine MAbs specific for *H. influenzae* were determined with the whole-cell ELISA as described before (19). The total amount of anti-Hib PS of the MAb and in the human serum and milk samples was determined with the radioimmunoassay. IgG anti-Hib PS antibodies were determined with an ELISA as described before (6).

Flow cytometry. Flow cytometry was performed as described by Vogel et al. (27).

Scanning electron microscopy. *H. influenzae* attached to polylysine-coated coverslips was fixed in McDowell fixative, dehydrated in graded ethanol, and finally dried in hexamethyldisilazane (Sigma). After drying, the coverslips were sputter coated with 12-nm-diameter gold particles and studied in a scanning electron microscope (ISLSSyo, Tokyo, Japan) at a magnification of $\times 3,000$.

Statistics. All experiments were performed at least in triplicate, and the values were expressed as the mean $\pm$ standard deviation. The significance of inhibition of adherence was determined with the Student *t* test.

RESULTS

Fimbria-dependent adherence of *H. influenzae* to human oropharyngeal epithelial cells. Adherence of *H. influenzae* 770235f⁺b⁺ to oropharyngeal epithelial cells resulted in attachment of 115 ± 69 bacteria per cell (69 positive cells) (Fig. 1A). The nonencapsulated variant *H. influenzae* 770235f⁺b⁰ adhered equally well (136 ± 69 bacteria per cell, 51 positive cells) (Fig. 1B). This result is in agreement with data described previously (23, 26). The fimbria-specific adherence was inhibited by preincubating the bacteria with ascitic fluid containing the fimbria-specific MAb 6HE8, diluted 200-fold in DMEM

FIG. 1. Effects of various concentrations of anti-Hib PS MAb E117-5 on fimbria-dependent adherence of *H. influenzae* 770235 variants to human oropharyngeal epithelial cells. (A) *H. influenzae* 770235f⁺b⁺; (B) 770235f⁺b⁰. Bars 1, control without antibodies; bars 2 to 4, with 80, 40, and 20 μ g of anti-Hib PS MAb 117-5 per ml, respectively; bars 5, with 1.4 mg of anti-Hib PS (burro) per ml; bars 6, with MAb 6HE8 (antifimbriae). Adhering bacteria on cells containing at least 20 bacteria per cell were counted. Values are the means $\pm$ standard deviations.

plus FCS to reach a titer of 5×10^3 (Table 1). The number of bacteria of the fimbrialess variant attached to the epithelial cells was at the background level for all 20 cells analyzed. Preincubation of the nonfimbriated variants with MAb 6HE8 did not change the adherence of the bacteria to the cells (data not shown).

The effect of incubation of bacteria with antibodies specific for the Hib PS on adherence was analyzed in inhibition experiments with polyclonal and monoclonal anti-Hib PS antibodies. Preincubation of *H. influenzae* 770235f⁺b⁺ with polyclonal antiserum raised in a burro to Hib PS (final concentration of anti-Hib PS, 1.41 mg/ml) resulted in a number of oropharyngeal epithelial cells without bacteria attached which was significantly less than that in the control ($P < 0.002$). Antiserum raised in rabbits against Hib strain 760705 had the same inhibitory effect (data not shown). The experiments were repeated with the anti-Hib PS MAb E117-5 in various concentrations ranging from 2.5 to 80 μ g/ml. A dose-dependent reduction in adherence was observed (Fig. 1A). No effect was seen in the

TABLE 1. Titers of antibodies to Hib PS, OMPs, LPS, and fimbriae

Antibody	Specificity	Titer of antibody to <i>H. influenzae</i> strain ^a	
		770235f ⁺ b ⁺	770235f ⁺ b ⁰
Burro 132 ^b	Hib PS	ND	ND
MAb E117-5 ^c	Hib PS	1.6×10^5	4.0×10^3
MAb 5HA5	OMP P1	2.0×10^5	ND
MAb 4BF8	OMP P5	1.3×10^6	ND
MAb 8BD9	OMP P6	6.3×10^5	4.0×10^5
MAb 6HE8	Fimbriae	1.0×10^6	1.0×10^3
MAb 3DA11	LPS1 and LPS2	6.3×10^5	ND
MAb D1D1	Negative control	3.2×10^3	1.2×10^3

^a The titer of the antibodies was determined by whole-cell ELISA. ND, not determined.

^b Burro serum contained 14.1 mg of anti-Hib PS per ml (15).

^c This titer corresponded with 800 µg of anti-Hib PS per ml as determined with the radioimmunoassay (11).

presence of 20 µg of antibodies per ml, in the presence of 40 µg/ml some decrease in the number of bacteria per cell was noted, and in the presence of 80 µg of anti-Hib PS MAb per ml a significant reduction in adherence occurred ($P < 0.02$). The inhibition of adherence in the presence of burro anti-Hib PS and murine anti-Hib PS MAb was specific for the *H. influenzae* strain with the type b capsule, since adherence of the nonencapsulated variant *H. influenzae* 770235f⁺b⁰ was not affected by 80 µg of anti-Hib PS MAb per ml (Fig. 1B). The nonfimbriated variants strain 770235f⁺b⁺ and strain 770235f⁺b⁰ did not adhere in the absence or presence of anti-Hib PS.

To analyze the specificity of the inhibition of adherence for anti-Hib PS, the effect of antibodies reacting with other surface components on adherence was determined. MAb 3DA11 (anti-LPS1 and anti-LPS2), MAb 5HA5 (anti-OMP P1), MAb 4BF8 (anti-OMP P5), and MAb 8BD9 (anti-OMP P6) were chosen since by immunoelectron microscopy these antibodies were shown to bind to intact bacteria (19, 23). Ascitic fluid (diluted 10 times) containing the anti-OMP and the anti-LPS antibodies did not inhibit adherence significantly (Fig. 2). This concentration resulted in antibody titers which were similar to the antibody titers of anti-Hib PS MAb E117-5 causing inhibition of adherence (Table 1; Fig. 1A). These results showed that inhibition of adherence was specific for antibodies to Hib PS.

The inhibitory effect of antibodies elicited in humans by vaccination with Hib conjugate vaccines on the adherence of fimbriated Hib was determined with serum samples from 10 individual children, pooled serum samples, and a breast milk sample taken before and after maternal vaccination. The anti-Hib PS concentrations in the prevaccination serum samples were ≤ 4.0 µg/ml (geometric mean concentration, 0.59 µg/ml). In the postvaccination serum samples antibody concentrations ranged from 1.7 to 202.2 µg/ml (geometric mean concentration, 70.0 µg/ml). The total anti-Hib PS concentration in the pooled serum samples obtained from children before and after vaccination rose from <0.1 to 160 µg/ml (IgG, 150 µg/ml; IgM, 7.1 µg/ml; and IgA, 4.7 µg/ml). A breast milk sample from a vaccinated mother had increased anti-Hib PS IgA compared to a milk sample of the mother before vaccination (prevaccination, 0.025 µg/ml; postvaccination, 4.6 µg/ml) and no detectable IgG or IgM (<0.01 µg/ml). Adherence of Hib strain 770235f⁺b⁺ was not inhibited significantly in the presence of 10-fold dilutions (final concentrations) of any of these serum samples. After preincubation of *H. influenzae* 770235f⁺b⁺ with a milk sample obtained from a nonvaccinated mother, the

number of adhering bacteria was 137 ± 49 per cell; for strain 770235f⁺b⁰ the number was 225 ± 68 per cell. After preincubation with the milk sample (1:10 diluted) from the vaccinated mother, 193 ± 91 bacteria of *H. influenzae* 770235f⁺b⁺ adhered per cell and 230 ± 79 bacteria of strain 770235f⁺b⁰ adhered per cell. These results indicate that the human samples did not show an inhibitory effect on adherence, although they contained up to 20 µg of anti-Hib PS per ml (final concentration).

Effect of anti-Hib PS on the growth of *H. influenzae*. Since bacterial multiplication on the mucosa is an essential step in colonization, the effect of anti-Hib PS on the growth of *H. influenzae* on epithelial cells was investigated. Hib strain 770235f⁺b⁺ and its acapsular variant multiplied equally well on H292 epithelial cell monolayers (Fig. 3A and B) despite differences in adherence to these cells: after 4 to 6 h Hib was not seen in association with the cells and the nonencapsulated variant was found in patches, as observed with light microscopy after staining with diluted crystal violet solution (not shown).

FIG. 2. Effects of MAbs to various outer membrane components on fimbria-dependent adherence of *H. influenzae* 770235f⁺ variants to human oropharyngeal epithelial cells. (A) *H. influenzae* 770235f⁺b⁺; (B) 770235f⁺b⁰. Bars 1, control; bars 2, anti-Hib PS MAb (80 µg/ml); bars 3, MAb 3DA11 (anti-LPS); bars 4, MAb 5HA5 (anti-OMP P1); bars 5, MAb 4BF8 (anti-OMP P5); bars 6, MAb 8BD9 (anti-OMP P6). Adhering bacteria on cells containing at least 20 bacteria per cell were counted. Values are the means $\pm$ standard deviations.

FIG. 3. Effects of anti-Hib PS MAb (E117-5) (A and B) and anti-OMP P6 or anti-LPS MAb (C) on the growth of *H. influenzae* variants inoculated on monolayers of lung epithelial cell line H292. (A and C) *H. influenzae* 770235f⁺b⁺; (B) strain 770235f⁺b⁰.

In the presence of 1 µg of anti-Hib PS MAb E117-5 per ml the growth rate of *H. influenzae* 770235f⁺b⁺ was reduced, and the growth stopped completely in the presence of 5 µg of anti-Hib PS per ml (Fig. 3A). No effect of 5 µg of anti-Hib PS per ml on the growth rate of the nonencapsulated variant *H. influenzae* 770235f⁺b⁰ was seen (Fig. 3B). To analyze whether inhibition of growth is a general phenomenon for *H. influenzae* with antibodies attached, growth curves of *H. influenzae* were determined in the presence of MAbs specific for OMP P6 and LPS. Diluted MAbs with antibody titers similar to or higher than the titer of the growth-inhibiting amount of anti-Hib PS MAb (5 µg/ml) did not affect the growth rate of *H. influenzae* 770235f⁺b⁺ (Fig. 3C) and the nonencapsulated variant *H. in-*

fluenzae 770235f⁺b⁰. Similar results were obtained with antibodies specific for OMPs P1 and P5 (not shown). These results indicate that the presence of antibodies on the surface of *H. influenzae* per se is not sufficient to inhibit the growth of either type b or nonencapsulated *H. influenzae* and that growth inhibition was specific for Hib in the presence of anti-Hib PS antibodies.

The effect of human serum antibodies elicited by vaccination with conjugate Hib PS vaccine on bacterial growth is shown in Fig. 4. Human sera inhibited the growth of *H. influenzae* 770235f⁺b⁺ in an anti-Hib PS dose-dependent way. In general, the sera from the children after vaccination had higher antibody levels and a stronger inhibitory effect than sera obtained from these children before vaccination. The fourfold increase in the amount of anti-Hib PS in the serum of infant 3 did hardly result in increased inhibition of growth. Since this may be explained by low affinity of the antibodies raised by vaccination, we did not analyze this further. Although vaccination of children resulted in increased amounts of anti-Hib PS antibodies, the growth of the nonencapsulated variant *H. influenzae* 770235f⁺b⁰ was not inhibited in the presence of the pre- and postvaccination sera (data not shown).

Bacterial agglutination. We had to exclude the possibility that the effects of anti-Hib PS on bacterial adherence and growth were caused by bacterial agglutination. Flow cytometry was used to determine bacterial agglutination since bacterial agglutination results in increased values of the forward scatter due to an increase in particle size and in a decrease of the number of particles (an agglutinate of bacteria gives only one signal). The forward scatter-fluorescence diagrams of *H. influenzae* 770235f⁺b⁺ and 770235f⁺b⁰ were identical (Fig. 5A, B, and E). The size of the *H. influenzae* particles was about 0.1% of that of the internal standard *Candida albicans*. The forward scatter of the nonencapsulated variant strain 770235f⁺b⁰ did not change in the presence of anti-Hib PS (Fig. 5E and F). In contrast, the forward scatter of *H. influenzae* 770235f⁺b⁺ increased about 100-fold during a 5-h incubation with 16 µg of anti-Hib PS per ml (final concentration) (Fig. 5B, C, and D), corresponding with an increase in size of *H. influenzae* 770235f⁺b⁺ from 0.1 to 1% of the size of *C. albicans* (Fig. 5C and D). The ratio of the number of *H. influenzae* bacteria over the number of yeast particles did not change over 5 h of incubation with anti-Hib PS at room temperature (0.84 for *H. influenzae* 770235f⁺b⁺ and 0.80 for *H. influenzae*

FIG. 4. Effects of various concentrations of anti-Hib PS as they occur in pre- and postvaccination sera from human individuals 1 to 3 on the increase of *H. influenzae* 770235f⁺b⁺ in 2.5 h of growth on H292 cell layers. The starting inoculum was 2.6×10^3 CFU/ml.

FIG. 5. Effect of anti-Hib PS (aPS) incubation on the size of the bacteria as determined from the fluorescence (FL1)-versus-forward scatter (FSC) plot of FACS analyses. *H. influenzae* bacteria were labeled with fluorescein isothiocyanate. (A) FL1-versus-side scatter (SSC) to show uniformity of the population of *H. influenzae* f⁺b⁺. (B to D) Fluorescein isothiocyanate-labeled *C. albicans* was added to each sample as an internal control. *H. influenzae* f⁺b⁺ incubated for 0 min (B), 2 h (C), and 5 h (D) with MAb E117-5 (5 μ g/ml); (E and F) *H. influenzae* f⁺b⁰ incubated for 0 min (E) and 5 h (F) with MAb E117-5 (5 μ g/ml).

770235f⁺b⁰; the time zero value was arbitrarily taken as 1). Since the bacteria did not multiply or die during the incubation period as determined by colony counting, these data show that incubation of Hib with anti-Hib PS resulted in a size extension of the bacteria and not in agglutination. The increase in the bacterial size was confirmed with scanning electron microscopy. Single bacteria were observed. The diameter of *H. influenzae* 770235f⁺b⁺ cells in the absence of anti-Hib PS was $0.82 \pm 0.01 \mu\text{m}$ (mean of 117 cells $\pm$ standard deviation) and with 16 μ g of anti-Hib PS per ml was $0.96 \pm 0.02 \mu\text{m}$ (mean of 122 cells $\pm$ standard deviation). Such an increase in diameter results in an increase in volume of the bacteria by a factor of 1.7. It should be noted that increases observed with flow cytometry and scanning electron microscopy cannot be compared, since in flow cytometry light scattering is determined

and with electron microscopy sizes of bacteria are visualized after fixation and staining. The diameter of the nonencapsulated *H. influenzae* 770235f⁺b⁰ cells in the presence of 80 μ g of anti-Hib PS per ml remained $0.80 \pm 0.01 \mu\text{m}$ (mean of 107 cells $\pm$ standard deviation). As a positive control for agglutination, *H. influenzae* 770235f⁺b⁺ was incubated with 1 mg of burro anti-Hib PS per ml for 1 h. This resulted in visual agglutination and the formation of large clumps of bacteria as seen by light and scanning electron microscopy. These suspensions could not be analyzed by flow cytometry, since they caused obstruction of the measuring cell. Summarizing, the fluorescence-activated cell sorter (FACS) and scanning electron microscopy results exclude bacterial agglutination during incubation with the antisera under the conditions used in the inhibition experiments.

DISCUSSION

Adherence of *H. influenzae* to oropharyngeal epithelial cells is generally assumed to prevent colonizing bacteria from being expelled by mucociliary clearance. This first step in the colonization of *H. influenzae* is probably the same for the carrier state, respiratory tract infections, and systemic disease. Subsequently, the route followed by *H. influenzae* during infection differs, especially between encapsulated type b strains and nonencapsulated strains (24). Nonencapsulated strains remain localized in the airway epithelium, and *H. influenzae* effectively penetrates the mucosa and spreads systemically in the circulation and even the cerebrospinal fluid.

Blockage of bacterial adherence has been shown to be an effective way of preventing colonization (14). In the case of Hib antibodies specific for the type b capsular PS were shown to inhibit adherence to oropharyngeal epithelial cells (Fig. 1). The inhibition of adherence of encapsulated *H. influenzae* strains was not observed in the presence of antibodies specific for OMPs and LPS, indicating that inhibition of adherence by anti-Hib PS is not just the consequence of binding of antibodies to the surface of *H. influenzae* but that anti-Hib PS and antifimbriae inhibited adherence specifically. These data correlate well with observations in humans. Hib colonization was inhibited after vaccination with Hib PS conjugate vaccines (10, 16, 17), but nonencapsulated *H. influenzae* was shown to colonize the nasopharynx despite the presence of strain-specific antibodies to OMPs (5). However, the concentration of anti-Hib PS antibodies required to inhibit the fimbria-mediated adherence in vitro was quite high (more than 20 µg/ml) (Fig. 1). These are concentrations in serum, and their relevance on mucosal surfaces can be questioned. Generally, the concentrations of anti-Hib PS in human sera and in breast milk after vaccination were insufficient for inhibition of adherence. Since they were high enough to prevent colonization in the infant rat model (6), the primary effect of anti-Hib PS is neither blocking of adherence nor infidelity of the in vitro assay condition for measuring inhibition of adherence. In vivo the number of bacteria colonizing the throat is much lower than in the in vitro assay, but it has not been possible to perform this in vitro assay or similar assays with more relevant numbers of bacteria. Robbins et al. recently argued that prevention of colonization of the mucosa by low numbers of bacteria probably requires much lower concentrations of antibodies than does prevention of infection (13).

The inhibitory effect of anti-Hib PS on bacterial multiplication of Hib was observed at concentrations (1 µg/ml) about 50-fold lower than were required for inhibition of adherence (Fig. 3 and 4). These concentrations are reached in sera and saliva after vaccination with conjugate vaccines (8).

Inhibition was not due to bacterial agglutination as analyzed by flow cytometry (Fig. 5) and electron microscopy (data not shown). The anti-Hib PS antibodies were shown to bind to the capsule so that the size of the bacteria increased (Fig. 5). This phenomenon is known as the Quellung reaction (capsule swelling reaction). Most likely, the antibody-PS complexes form a negatively charged layer around the bacterium which may act as a diffusion barrier for nutrients into the bacteria. Antibodies to OMPs and LPS did not inhibit growth, and these do not cause an increase in the size of *H. influenzae*.

We cannot exclude the possibility that other antibody-dependent mechanisms also contribute to the prevention of colonization by Hib after vaccination with conjugated Hib PS. This mechanism was proposed by Robbins et al. for many bacteria (13). A critical level of vaccine-induced serum IgG to surface antigens of the microorganism could inactivate the

inoculum on mucosal surfaces because of the exudation of IgG and complement onto mucosal surfaces and the subsequent attraction of leukocytes and killing of the bacteria. Moreover, during colonization *H. influenzae* was shown to enter the mucosa (4), where IgG, complement, and leukocytes are more prominent than on the mucosa. IgA antibodies may not be very important for the prevention of colonization, since anti-Hib PS is not present in the rat model after passive immunization with colonization-blocking IgG antibodies (6). Although the murine MAb E117 activates rat complement, yielding bacterial lysis (11), it is not known whether the local concentrations of complement factors in humans are high enough to promote anti-Hib PS-dependent lysis or phagocytosis of Hib and thereby prevention of carriage. A direct effect of the anti-Hib PS antibodies on the bacteria is more likely, since Fab fragments of MAb E117-5 also prevented colonization in the rat model (7). In addition, it is hard to understand why antibody-dependent prevention of colonization was observed only in the presence of anti-Hib PS antibodies and not with antibodies to OMPs that also promote complement-mediated killing and phagocytosis. The capsule swelling caused by antibodies to PS capsules may easily act as a diffusion barrier for nutrients and cause steric hindrance.

Summarizing, rather high concentrations of antibodies specific for Hib PS were shown to block adherence of Hib to epithelial cells, but concentrations easily reached after vaccination inhibited multiplication of these encapsulated strains. These mechanisms may be operative for the prevention of colonization of other encapsulated bacteria, such as pneumococci, meningococci, and *Escherichia coli*. Antibodies to surface proteins do not seem to be effective in preventing colonization and local infections of *H. influenzae*.

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