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Effect of Amplification of the Cap b Locus on Complement-Mediated Bacteriolysis and Opsonization of Type b *Haemophilus influenzae*

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Amplification of the Cap b locus of *Haemophilus influenzae* occurs frequently in clinical isolates and has been proposed to be a mechanism by which this organism evades host defense. To determine if amplification of this locus affected complement fixation, in vitro studies to determine complement-mediated bacteriolysis and complement-mediated opsonization of an isogenic set of organisms containing 2, 3, and 4 copies of the Cap b locus were performed. Organisms containing 4 copies of the Cap b locus were significantly more resistant to antibody-dependent, classical complement pathway-directed bacteriolysis than were organisms containing 2 copies. Organisms containing 3 copies of this locus exhibited intermediate susceptibility to lysis. Complement-mediated opsonization of these organisms was assessed by determining the degree of binding of bacteria to murine or human macrophages or to nonphagocytic cells transfected with the genes for human Mac-1, the complement receptor type 3. In all three assay systems, organisms containing 4 copies of the Cap b locus bound less well than did organisms containing 2 copies of this locus. Consistent with their decreased susceptibility to lysis and opsonization, organisms with 4 copies of the Cap b locus fixed less C3 than did organisms containing 2 copies. These data demonstrate that amplification of the Cap b locus is associated with decreased susceptibility to complement-mediated lysis and decreased complement-mediated opsonization and suggest that amplification is used by these pathogens to increase their resistance to complement-dependent host defense mecanisms.

Haemophilus influenzae type b is an encapsulated bacterial pathogen whose virulence is, in part, mediated by the capsule. The importance of the capsule as a virulence factor is supported by two observations. First, nearly all invasive *H. influenzae* disease is caused by encapsulated isolates (19, 24). Second, unencapsulated mutants of type b organisms cause little or no disease upon intranasal or intraperitoneal challenge while encapsulated *H. influenzae* causes disseminated disease in infant animals (14, 22, 26). Despite the clinical and laboratory data supporting the role of the capsule as a major mediator of virulence, the precise mechanism of this virulence is not known.

The genes responsible for capsule expression by *H. influenzae* have been identified (6, 8). In most isolates 2 copies of an ~18-kb DNA segment of chromosome (the Cap b locus) are required for detectable capsule expression (6). Recent work, however, has shown that isolates of type b *H. influenzae* obtained from patients with invasive disease frequently have 3 or more copies of the Cap b locus (3). Because in vitro passage of isolates in this amplified state results in a shift to a 2-copy state, it has been proposed that amplification poses additional metabolic requirements that would not be spent unless some advantage was realized by the organism (3). Given the importance of bacteremia in the pathogenesis of invasive *H. influenzae* disease, improving bacterial survival in the bloodstream was considered to be an advantage that amplification might give to the organism.

Complement plays an important role in host defense against *H. influenzae*. Its role in mediating clearance of *H. influenzae* from the bloodstream has been described (4, 17). Previous work has shown that susceptibility to complement-mediated lysis and opsonization distinguishes unencapsulated *H. influenzae* that is promptly cleared from blood from virulent type b-encapsulated organisms that cause persistent bacteremia (16). Furthermore, increased expression of capsule has been proposed to impair complement-mediated host defense (11, 12). These observations served as the basis for considering that amplification of the Cap b locus may increase the organism's potential for surviving in the host by affecting the interaction with serum complement.

To determine the effect of amplification of the Cap b locus on the interaction of serum complement with encapsulated *H. influenzae*, an isogenic set of organisms containing 2, 3, and 4 copies of the Cap b locus was studied. Two criteria for complement activation were used. The first involved determining the level of complement-mediated bacteriolysis in human serum, and the second involved assessing complement-dependent opsonization for bacterial adherence to cells expressing complement receptors. Differences in susceptibility among these organisms to complement-mediated lysis and differences in the degree of complement-mediated opsonization were measured to test the hypothesis that amplification of the Cap b locus is associated with impaired complement-mediated host defense against type b *H. influenzae.*

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MATERIALS AND METHODS

Bacteria. Isogenic strains of type b *H. influenzae*, derived from isolate PC1005, were obtained from Paul Corn and Susan Hoiseth (3). Isolate PC1005 is a clinical isolate that has been shown to contain 5 copies of the Cap b locus. Organisms containing 4, 3, and 2 copies of this locus were derived from PC1005 as described previously (3). The Cap b locus copy number was determined by Southern analysis of DNA digested with *Kpn*I-*Sma*I by using the 2.1-kb *Pvu*II fragment as a probe (3). Hybridization and detection of the probe were done with the Bluegene nonradioactive nucleic acid detection system (Life Technologies, Grand Island, N.Y.) according to the manufacturer's directions. Organisms were stored in skim milk broth (Difco, Detroit, Mich.) at −70°C. The amount of polyribosyl ribitol phosphate (PRP) associated with organisms was measured by enzyme-linked immunosorbent assay (ELISA). A modification of a previously described sandwich ELISA (8) was used to measure PRP. A murine monoclonal antibody to PRP (E117-5, provided by Lederle-Praxis Biologics, West Henrietta, N.Y.) was used at a 1:200 dilution as the secondary antibody followed by peroxidase-conjugated goat anti-mouse antibody (Sigma, St. Louis, Mo.) used at a 1:250 dilution. Soluble and cell-associated PRP was measured according to the methods described by Buckmire (2).

Outer membrane components of organisms were isolated and characterized by previously described methods (5). Lipooligosaccharide was prepared and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) in 16.6% polyacrylamide, as described previously (25).

Shavings of frozen broth containing organisms were incubated on chocolate agar (BBL, Cockeysville, Md.) overnight. Colonies from this agar were then incubated in brain heart infusion (BHI) broth (Difco, Detroit, Mich.) supplemented with NAD and hemin (for bactericidal and immunostaining binding assays) or in Mueller-Hinton broth supplemented with NAD and hemin containing 0.01 mCi of [^{3}H]thymidine per ml (for radiobinding assays) for 4 h at 37°C in a shaking water bath as previously described (18). Organisms were washed three times in Hanks' balanced salt solution (HBSS; Gibco Laboratories, Grand Island, N.Y.) containing 0.2% glucose and 0.02% gelatin (PB) before being used in assays.

Serum and monoclonal antibodies. Normal human serum was obtained from four healthy adults and pooled. Nonimmune serum containing ≤160 ng of antibody to type b capsule per ml was obtained from an 11-month-old male who had transient hypogamma globulinemia. C8-deficient serum containing 718 ng of antibody to type b capsule per ml was obtained from a patient with a congenital deficiency (13). C4-deficient serum was obtained from a patient with serum C4 level of 3.3 mg/dl (normal range, 15 to 45 mg/dl). This serum had normal alternative complement pathway activity as assessed by its ability to lyse rabbit erythrocytes. Murine serum was obtained from mice congenitally deficient in C5 (strain B10D2./oSn; Jackson Laboratories, Bar Harbor, Maine) as previously described (17). This serum had no detectable antibody to type b organisms as previously described (17). C4-deficient murine serum was obtained from mice in which a C4 deficiency was created by gene targeting (27). For selected assays, serum was incubated at 56°C for 1 h to deplete complement activity. Serum was stored at −70°C and thawed immediately before use.

Monoclonal antibody M1/70 directed against CD11b (the alpha chain of Mac-1) was purified from a tissue culture supernatant of hybridoma ATCC TIB128 with the use of a protein G-Sepharose column (15). Tissue culture supernatant containing monoclonal antibody M51H9, an immunoglobulin M (IgM) antibody that recognizes type b capsule, was provided by Lederle-Praxis Biologicals.

Bactericidal assays. Complement-mediated bactericidal activity was assessed by methods similar to those previously described (20). Approximately 5×10^4 organisms were incubated in phosphate-buffered saline (PBS; Gibco) containing between 0 and 50% serum in a total volume of 200 μl. The number of CFU per 10 μl of each sample taken immediately after the addition of the organisms and after 1 h of incubation was used to calculate the percent loss in CFU per microliter as described previously (20).

Bacterium-binding assays. The binding of bacteria to phagocytes and transfected cells was determined by direct visualization of immunostained organisms and by radiobinding assays as previously described (18). Binding of organisms was determined after a 1-h incubation of bacteria with cells adherent to glass or plastic surfaces.

Resident peritoneal macrophages were obtained from CDF-1 mice (Charles River Breeding Laboratories, Wilmington, Mass.) as described previously (18). Human macrophages were cultured from peripheral monocytes obtained from healthy adult donors as previously described (15). Mac-1-transfected cells were prepared and maintained as described previously (7).

Complement fixation and antibody deposition assays. C3 fixation and antibody deposition on organisms were determined by flow cytometry. Approximately 5×10^7 organisms were incubated for 30 min at 37°C in HBSS containing either 20% human C8-deficient or murine serum, in serum which had been heat inactivated for 30 min at 56°C, or in buffer alone. Organisms were then washed extensively in HBSS to remove unbound opsonins. Immunostaining for C3 deposition was performed by incubating samples on ice for 45 min. in a 1:100 dilution of fluorescein isothiocyanate (FITC)-conjugated goat anti-human complement fragment C3c (Jackson ImmunoResearch, West Grove, Pa.) or FITC-conjugated goat anti-mouse complement C3 antibody (Cappel, Durham, N.C.). Staining for antibody deposition was performed by incubating samples in 1:100 dilutions of either FITC-conjugated goat anti-human IgG and IgM (heavy and light chains) or FITC-conjugated goat anti-mouse IgG and IgM (heavy and light chains) (Jackson ImmunoResearch). After being stained, the organisms were washed extensively and fixed in 2.5% paraformaldehyde. Flow cytometric analysis was performed with an Epics Elite flow cytometer (Coulter Diagnostics, Hialeah, Fla.).

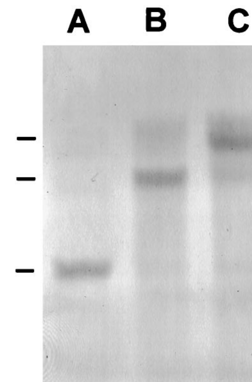

FIG. 1. Results of Southern analysis of organisms containing 2 (lane A), 3 (lane B), and 4 copies (lane C) of the Cap b locus. Bands (from bottom to top) at approximately 45, 63, and 81 kb correspond to 2, 3, and 4 copies of this locus, respectively. Chromosomal DNA was digested with *Kpn*I and *Sma*I and probed with a *Pvu*II fragment that recognizes a 2.1-kb segment within the Cap b locus.

RESULTS

Characterization of organisms. The colony morphologies on chocolate agar and BHI agar supplemented with 1.5% Fildes enrichment (Difco) were indistinguishable for all three isogenic strains of *H. influenzae* containing 2, 3, and 4 copies of the Cap b locus. Stereo dissecting microscopic views of colonies on BHI agar showed that the majority of colonies of all three strains had the opaque phenotype, described by Weiser (25). By SDS-PAGE analysis, all three isolates exhibited outer membrane and lipooligosaccharide profiles that were indistinguishable from each other (data not shown).

The number of copies of the Cap b locus of each of the isolates was confirmed by Southern analysis (Fig. 1). Organisms with 2, 3, and 4 copies of the locus demonstrated prominent bands at approximately 45, 63, and 81 kb, respectively. Faint bands in this analysis suggested that the populations of bacteria contained some contamination with organisms containing different numbers of the Cap b locus. On the basis of parallel analyses with mixed populations of organisms containing known ratios of each isolate, it was estimated that fewer than 5% of organisms within each of these populations were organisms containing numbers of the Cap b locus that differed from the number demonstrated by the predominant band. Previous work had demonstrated the relationship between the number of Cap b loci and the production of PRP (3). Our analysis confirmed this finding. The total amount of soluble and cell-associated PRP from organisms expressing predominantly 2 copies of the Cap b locus was 15.6 ng/10^6 CFU. Organisms containing predominantly 3 copies expressed 31.8 ng/10^6 CFU, and organisms containing predominantly 4 copies of the Cap b locus expressed 40.9 ng/10^6 CFU. Thus, by these criteria, the three strains used in this work were judged to be isogenic, differing only with respect to the number of copies of the Cap b locus and the amount of PRP expressed (3).

Serum bacteriolysis. The bacteriolysis of type b encapsulated *H. influenzae* containing 2, 3, and 4 copies of the Cap b locus was determined by incubating them in increasing con-

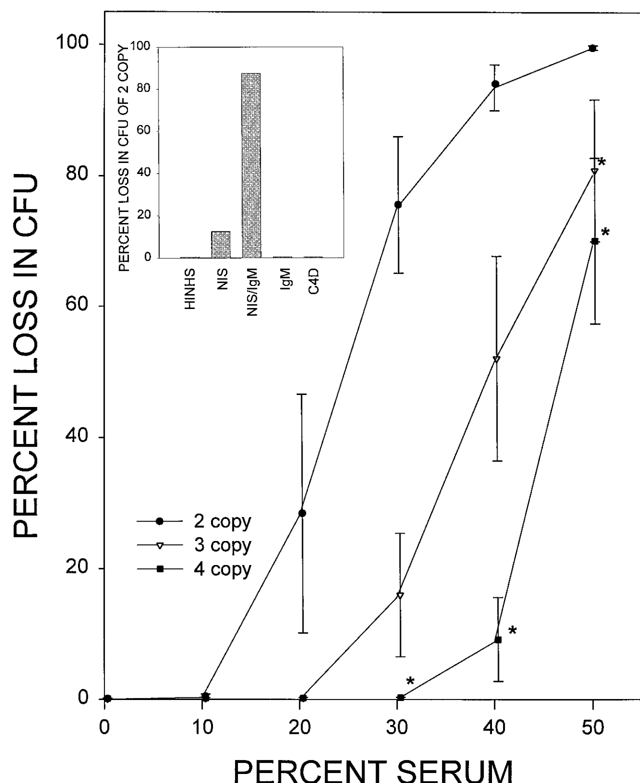

FIG. 2. Susceptibility of type b *H. influenzae* containing 2, 3, or 4 copies of the Cap b locus to lysis in increasing concentrations of healthy human serum. Organisms were incubated for 1 h in PBS containing 0 to 50% serum. Each point represents the mean of six experiments; error bars indicate standard errors of the means. Asterisks indicate significant differences ($P < 0.05$ by rank sum) from the results for organisms containing 2 copies. Inset, percent killing of organisms containing 2 copies in 50% heat-inactivated serum (HINHS), in 50% nonimmune serum (NIS), in 50% nonimmune serum containing IgM monoclonal antibody 5M1H9 (NIS/IgM), in IgM monoclonal antibody 5M1H9 alone, and in 50% C4-deficient human serum (C4D).

centrations of normal adult human serum (Fig. 2). Organisms with 2 copies of the Cap b locus were significantly more susceptible to bacteriolysis than were organisms with 4 copies when they were incubated in serum concentrations of 20% or greater. Bacteriolysis did not occur in serum incubated at 56°C for 60 min, nor did it occur in human serum containing undetectable levels (≤0.16 μg/ml) of antibody to type b capsule. The addition of murine IgM monoclonal antibody (final concentration, 0.5 μg/ml) to serum lacking antibody to *H. influenzae* resulted in a restoration of bacteriolysis (Fig. 2, inset), suggesting a role for the classical complement pathway in this process. Furthermore, bacteriolysis of the organisms with 2 copies did not occur in human serum deficient in the fourth component of complement, C4 (Fig. 2, inset), indicating that an intact classical complement pathway was necessary for bacteriolysis. Bacteria containing 3 copies of the Cap b locus exhibited intermediate levels of serum sensitivity.

Complement opsonization. Opsonization was determined in three assay systems that have been previously characterized. Two of these systems used mononuclear phagocytes, cells whose interaction with type b *H. influenzae* have been shown to depend of C3 opsonization (16, 18). The third system utilized Chinese hamster ovary (CHO) cells transfected with the cDNAs for human Mac-1, the complement receptor type 3 (CR3).

As previously shown (18), binding of type b organisms to murine macrophages depended on complement opsonization. Binding occurred in the presence of fresh murine serum but not heat-treated serum and was completely inhibited by preincubation of monolayers with the monoclonal antibody to Mac-1 (M1/70). Bacterial binding to murine macrophages was determined under various experimental conditions, which included varying the inoculum size and the concentration of serum in the assay. Bacterial binding to macrophages increased with inoculum size and plateaued above 150 organisms per macrophage (Fig. 3). A significantly greater number of type b *H. influenzae* organisms with 2 copies of the Cap b locus bound to macrophages compared with the number of organisms containing 4 copies of this locus (Fig. 3). The degree of binding of organisms containing 3 copies of the Cap b locus was intermediate between that of the 2- and 4-copy-containing organisms. Binding of organisms containing 2 copies of this locus to macrophages was consistently greater than binding of organisms containing 4 copies of this locus when measured in serum concentrations at or above 5% (1.7 ± 0.3 versus 0.3 ± 0.1 at 5% serum; P of <0.05 by t test). Differences in binding were not significant when measured under conditions with 1% or less serum (0.15 ± 0.03 versus 0.10 ± 0.05). The addition of 0.5 μg of IgM monoclonal antibody per ml had no effect on the levels of complement-dependent binding (1.2 ± 0.2 versus 1.2 ± 0.2 for organisms with 4 copies).

Experiments using human macrophages derived from peripheral blood monocytes also demonstrated differences between the number of 2- and 4-copy-containing organisms bound per cell when the assays were done with human serum (C8D). In the presence of 8.3% nonlytic C8D serum, macrophages bound an average of 1.9 (± 0.6) organisms with 2 copies of the Cap b locus, whereas parallel monolayers incubated under the same conditions bound an average of 0.9 (± 0.5) of the 4-copy-containing organism per macrophage.

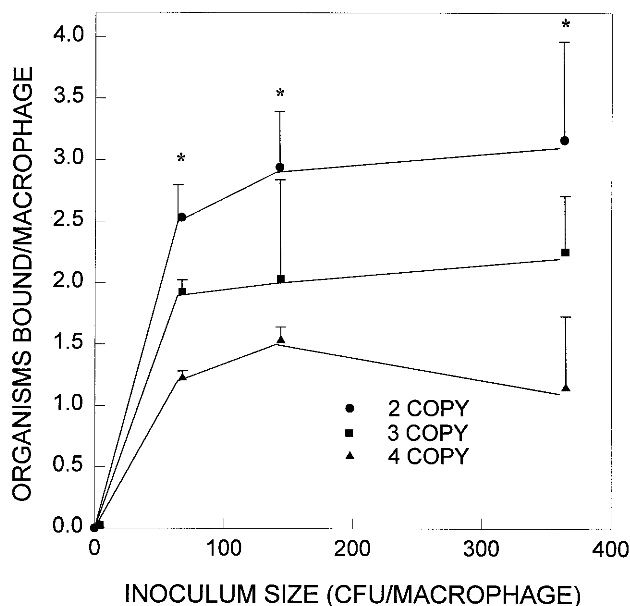

FIG. 3. Effect of inoculum size on binding of type b *H. influenzae* containing 2, 3, or 4 copies of the Cap b locus to murine resident peritoneal macrophages. Binding was measured by radiobinding assay after 1-h of incubation with macrophages in the presence of 8.3% C5-deficient serum. Points represent the means for at least three experiments; error bars indicate standard errors of the means. Differences in mean binding between and organisms with 2 and 4 copies are significant ($P < 0.05$ by t test) for inocula above 50 organisms per macrophage.

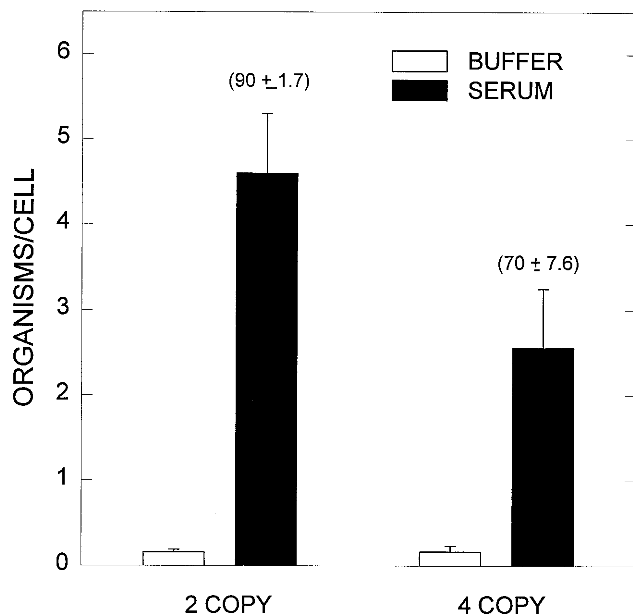

FIG. 4. Serum-dependent binding of type b *H. influenzae* to CHO cells transfected with genes encoding human Mac-1 (the receptor for iC3b). Binding was measured by counting immunofluorescently stained bacteria, and bars represent the means for three determinations, with error bars indicating the standard deviations. Mean binding is significantly different ($P < 0.05$ by t test). The numbers in parentheses indicate the percentage of cells binding at least one organism.

The addition of IgM monoclonal antibody to the type b capsule had no effect on this binding (1.9 ± 0.8 and 0.8 ± 0.3 for organisms with 2 and 4 copies, respectively). Although the total level of bacterial binding to human cells in the presence of human serum was considerably less than that observed in the murine system, the reduced binding to macrophages by organisms with 4 copies relative to that for organisms with 2 copies remained a consistent observation ($P < 0.05$ by t test).

To assess complement-dependent binding of these organisms to the human CR3, bacterial binding to transfected CHO cells expressing Mac-1 on their surfaces was determined. Similar to our observation with human macrophages, there was little or no serum-independent interaction of type b *H. influenzae* with these cells. Mac-1-transfected CHO cells bound only 0.2 (± 0.1) bacterium per cell in the absence of serum (Fig. 4), a level that was not different from that of bacterial binding to control-transfected CHO cells (data not shown). The addition of 10% murine serum to the assay increased bacterial binding to these cells dramatically. Similar to our observation with macrophages, the number of organisms with 4 copies that bound to CHO Mac-1 cells was significantly ($P < 0.05$ by t test) lower than the number of organisms with 2 copies bound to these cells (Fig. 4).

Measurement of C3 and antibody deposition on *H. influenzae*. Antibody and C3 complement deposition on bacteria incubated in serum was assessed by flow cytometry (Table 1). To measure antibody deposition, organisms were incubated in C8-deficient human serum and stained with an antibody to human immunoglobulin (Fig. 5). Both 2- and 4-copy-containing organisms exhibited comparable levels of staining, which was greater than the amount of background staining for organisms which were incubated in buffer alone. This indicated this human serum, as expected, contained antibody to type b *H. influenzae*. The intensity of staining was not different for 2- and 4-copy-containing organisms, indicating that comparable amounts of antibody were fixed by both these organisms. Antibody fixation following an incubation in murine serum was also examined. Consistent with previous published results (17), these methods demonstrated that murine antibody was not deposited on bacteria following an incubation in murine serum. The mean levels of intensity of fluorescence for 2- and 4-copy-containing organisms stained for murine immunoglobulin was 0.26 and 0.28, respectively, which was not different from those for bacteria incubated in buffer alone (0.26 and 0.29).

Bacteria were also stained for C3 complement fixation following incubation in serum (Fig. 6). The staining intensity of complement-opsonized bacteria was well above that for bacteria incubated in buffer alone (Table 1). Differences in C3 fixation between 2- and 4-copy-containing organisms were observed with both nonbactericidal human and murine serum (Table 1). Complement fixation did not depend on the presence of C4. The mean intensity of fluorescence of 2- and 4-copy-containing organisms was similar to that observed with murine C5-deficient serum (Table 1). This is consistent with previous work indicating that these organisms efficiently fix C3 via the alternate complement pathway (17, 21). Thus, in both human and murine sera, bacteria containing 2 copies of the Cap b locus fixed more C3 than bacteria containing 4 copies of this locus.

In vitro selection of organisms with amplified Cap b. Mixed populations of organisms were incubated in 50% serum to determine whether serum could select for organisms containing an amplified Cap b locus. Southern analysis of DNA from a mixed population (90% 2 copy and 10% 4 copy) of organisms after only 1 h of incubation in serum followed by an overnight incubation in BHI demonstrated an increased intensity of a band corresponding to approximately 81 kb (Fig. 7). This increase in intensity occurred along with a concomitant decrease in the intensity of the band at the 2-copy position (45 kb). Organisms incubated in parallel in buffer alone continued to exhibit the predominant band at the 2-copy position.

DISCUSSION

Amplification of the Cap b locus has been proposed to be a mechanism by which type b *H. influenzae* can increase its vir-

TABLE 1. Effect of amplification of Cap B locus of *H. influenzae* on C3 and serum antibody deposition assessed by fluorescence of bacteria immunostained for C3 complement or IgG, or IgM

Opsonin source	Mean log intensity of fluorescence in organisms containing the indicated no. of copies of Cap b locus stained for[a]:			
	C3 fixation		Antibody deposition	
	2	4	2	4
Human				
Buffer alone	0.46	0.57	0.46	0.53
Serum			3.50	2.73
C8 deficient	2.85	1.41		
Heated	0.46	0.58		
Murine				
Buffer alone	0.27	0.35	0.26	0.27
Serum			0.26	0.28
C5 deficient	23.5	8.2		
C4 deficient	38.0	13.7		
Heated	0.37	0.41		

[a] Results are the means for a single representative experiment as determined by flow cytometry.

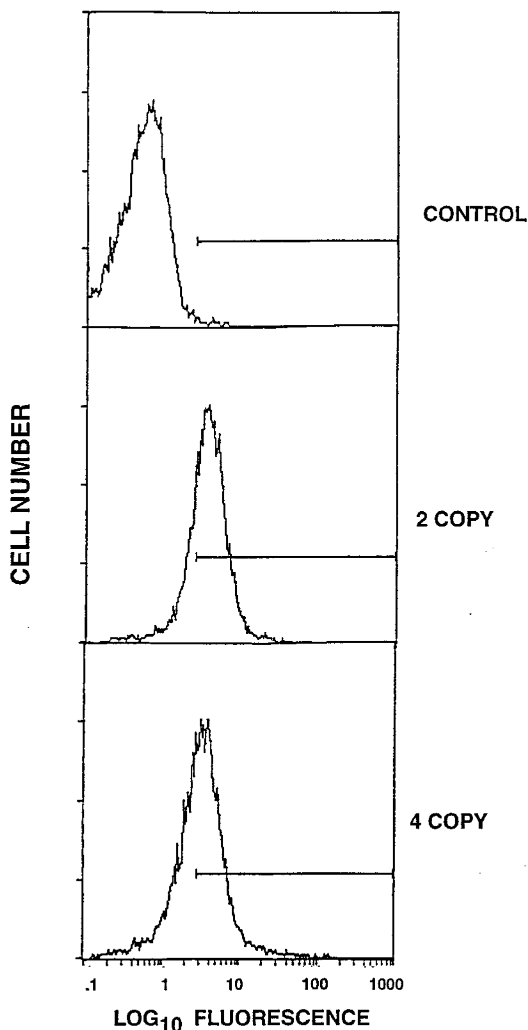

FIG. 5. Flow cytometric analysis of type b *H. influenzae* containing 2 or 4 copies of the Cap b locus immunostained for deposition of human IgG or IgM. The upper panel (control) represents fluorescence of bacteria incubated in buffer alone; the middle and lower panels represent organisms incubated in 20% C8-deficient human serum.

ulence (3, 10). Recent work has shown that 35% (23 of 66) of clinical isolates contain 3 or more copies of the Cap b locus (3). The results presented here demonstrate that this amplification is associated with decreased susceptibility to the complement's antibacterial activities. Organisms containing 2 copies of the Cap b locus were more susceptible than isogenic organisms containing 4 copies of this locus to complement-mediated bacteriolysis and to complement opsonization. This result thus supports the hypothesis that amplification of the Cap b locus imparts virulence by impairing complement-mediated host defense against type b *H. influenzae*.

The organisms used in this study were derived from a parent isolate that was shown to have 5 copies of the Cap b locus (3). These organisms have been characterized, and a gene dosage effect of the Cap b locus on production of the type b capsule of *H. influenzae* has been demonstrated (3) and confirmed in our laboratory. Southern analysis results presented here demonstrate that the organisms used in this study were composed of predominantly 2, 3, or 4 copies of the Cap b locus. Measurement of PRP by ELISA confirmed that organisms containing 3 and 4 copies of the Cap b locus exhibit progressive increases in the production of PRP compared with organisms containing 2 copies of this locus. In contrast to differences in capsule production among these organisms, there was no difference among organisms in colony morphology or by SDS-PAGE analysis of outer membrane components. On the basis of these results, we propose that differences noted among the organisms tested in the work presented here are largely, if not exclusively, due to the increased expression of the type b capsule by the organisms.

Consistent with previous work (1, 23), serum-dependent lysis of *H. influenzae* that expresses the type b capsule occurred in normal adult human serum and depended on the presence of antibody and an intact complement pathway. Lysis did not occur in heated serum or in serum with undetectable levels of antibody to *H. influenzae*. Bacteria containing only 2 copies of the Cap b locus were much more susceptible to complement-mediated lysis than were organisms containing 4 copies. At a serum concentration of 30%, for example, greater than 75% of

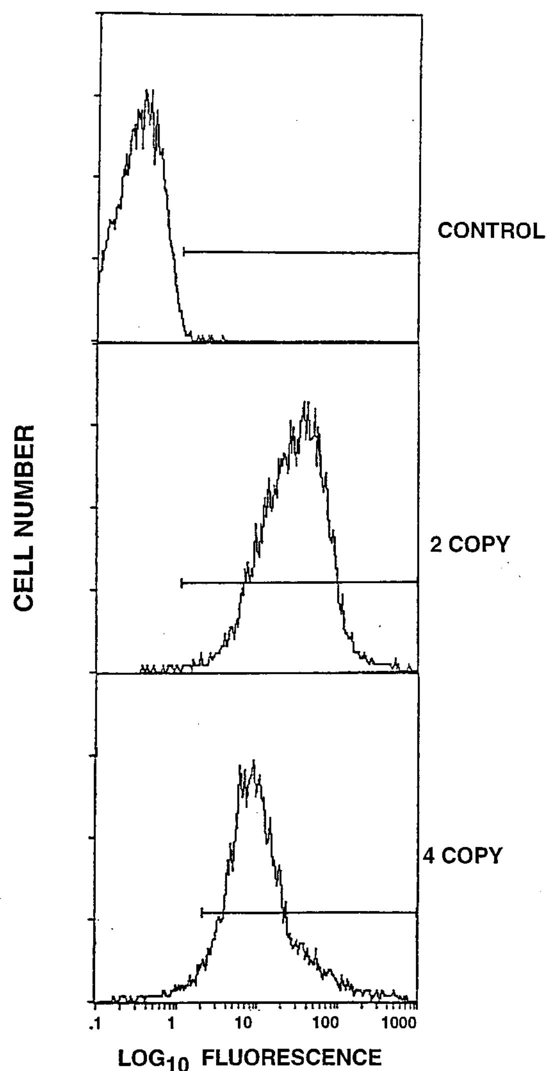

FIG. 6. Flow cytometric analysis of type b *H. influenzae* containing 2 or 4 copies of the Cap b locus immunostained for deposition of murine C3 complement. The upper panel represents the fluorescence of organisms incubated in buffer alone; the middle and lower panels represent fluorescence of organisms incubated in 10% murine serum.

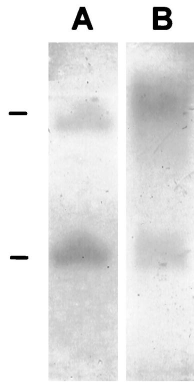

FIG. 7. Southern analysis of mixed populations of amplified organisms and organisms with 2 copies of the Cap b locus. Bands at approximately 45 kb (lowest band) and 81 kb (highest band) correspond to 2 and 4 copies of the Cap b locus, respectively. Chromosomal DNA was digested with *Kpn*I and *Sma*I and probed with a *Pvu*II fragment which recognizes a 2.1-kb segment within the Cap b locus. A mixed population of organisms containing 2 (90%) and 4 (10%) copies were incubated in buffer alone (lane A) or in 50% healthy human serum (lane B) for 1 h and then incubated in supplemented BHI broth overnight before analysis.

the 2-copy-containing organisms were lysed whereas bacteria with 4 copies of the Cap b locus remained 100% viable. This difference in susceptibility to lysis was not due to differences in antibody fixation, because by immunofluorescence, 2- and 4-copy-containing strains fixed comparable amounts of antibody from adult human serum.

Complement-mediated opsonization is considered to be an important host defense against type b *H. influenzae* (1, 17, 18). Previous work has demonstrated that complement, and specifically iC3b, is an important opsonin for type b *H. influenzae* (18). In the present studies, functional complement opsonization was determined with murine and human macrophages as well as nonphagocytic cells transfected with the genes encoding human Mac-1, the receptor for iC3b. Results in both the murine and the human systems indicate that organisms containing 4 copies of the Cap b locus bound less well to complement receptors than did organisms with 2 copies of this locus. This difference was observed over a broad range of bacterial inocula and serum concentrations. In these in vitro systems, increased binding could not be demonstrated with the addition of an IgM monoclonal antibody that was shown to direct bacteriolysis of type b organisms in the presence of nonimmune serum. This suggests that an antibody capable of directing complement fixation is incapable of increasing the levels of complement-mediated binding of organisms with 4 copies to macrophages.

The amount of C3 deposition on bacteria was determined by flow cytometry. This approach also indicated that amplification of the Cap b locus is associated with decreased deposition of C3. These differences were demonstrated not only in human serum that contained antibody but also in murine serum lacking antibody. Surprisingly, despite lacking the antibody to *H. influenzae*, murine serum was fully capable of opsonizing bacteria with complement. Moreover, murine serum deficient in C4 also fixed amounts of C3 comparable to that fixed in murine serum with an intact classical pathway. This demonstrates that the classical pathway is not required for efficient C3 deposition on type b *H. influenzae* incubated in murine serum. By two criteria, the intensity of fluorescence as measured by flow cytometry and the number of bacteria adherent to CR-3-expressing cells, bacteria exposed to low concentrations of murine serum efficiently fixed complement. It was only in relatively high concentrations of human serum that bacteria fixed sufficient complement to allow opsonization and lysis. Furthermore, bacteriolysis experiments using C4-deficient human serum indicate that complement-mediated lysis did not occur even in serum concentrations as high as 50%. These results suggest that the mechanisms of complement activation may differ between the murine and human systems. Nevertheless, observations in both systems indicate that organisms with 4 copies of the Cap b locus consistently fix less complement than do organisms with 2 copies of this locus.

Previous work has shown that unencapsulated strains of *H. influenzae* are much more susceptible to serum bacteriolysis and to complement-mediated opsonization than are type b-encapsulated organisms (14, 22, 26). It has also been shown that type b isolates incubated under conditions that enhance type b capsule production and/or the lipooligosaccharide phenotype are more resistant to bacteriolysis and complement opsonization (11, 25). The resistance to complement due to amplification of the Cap b locus that we describe is most likely a mechanism that is distinct from those previously attributed to growth conditions or phase variation. This is proposed for two reasons. First, the basis for these previously noted differences, although not defined, has been proposed to be related to impaired antibody recognition of resistant phenotypes. The results with C8-deficient human serum presented here indicate that amplification of the Cap b locus does not affect antibody recognition of these organisms. Furthermore, antibody plays little or no role in iC3b opsonization in the murine system that clearly demonstrated differences in the amount and activity of the C3 that was fixed. Second, previous work demonstrating differences in susceptibility to complement lysis and opsonization showed that certain growth conditions influenced resistant phenotypes (11, 12). The studies reported here were conducted with organisms incubated under identical culture conditions.

The results presented here provide additional evidence that the type b capsule plays an important role in resistance to the biologic activities of the complement. The intriguing possibility suggested by the work presented here and the prevalence of amplification among clinical isolates is that type b *H. influenzae* may use amplification of the Cap b locus as a mechanism to overcome complement-mediated host defenses. In the in vitro experiments presented here, it was shown that human serum which mediates bacteriolysis of organisms containing 2 copies when tested at a 50% concentration can serve to select organisms with 3 or more copies of the Cap b locus from mixed populations of organisms that contained predominately 2 copies of this locus. These results suggest that amplification of the Cap b locus could be used to evade complement-dependent host defense. We speculate that such selective pressure may also occur in vivo and may be further enhanced by the selective pressure of C3 opsonization and clearance of bacteria by the reticuloendothelial system that is likely to occur in nonimmune and immune patients. This might explain clinical observations made by Corn and colleagues that organisms with 3 or more copies of that Cap b locus can be isolated frequently from patients (3). Ongoing work is aimed at demonstrating this selection in vivo and examining the effect of amplification on clearance of these bacteria from the bloodstream of immune and nonimmune animals.

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