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Immunochemical Characterization of Variable Epitopes of Outer Membrane Protein P2 of Nontypeable *Haemophilus influenzae*

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Monoclonal antibodies (MAbs) were elicited to the nontypeable *Haemophilus influenzae* variants d1 to d4, which differ in the outer membrane protein P2 to analyze the immunological properties of the variable parts of this protein. Five MAbs reacted in a whole-cell enzyme-linked immunosorbent assay (ELISA) only with the homologous strain and in some cases with its variants, but not with 69 unrelated nonencapsulated *H. influenzae* isolates; nine MAbs also reacted with some other *H. influenzae* isolates, and four MAbs showed broad cross-reactivity. All of the MAbs reacted with purified protein P2 in ELISAs and immunoblotting. The five MAbs which reacted with the homologous strain d3 and not with the variants d1, d2, and d4 promoted complement-dependent bactericidal activity against strain d3. These and four other MAbs reacted with the intact bacteria of strain d3 in immunogold electron microscopy, indicating that they were directed against surface-exposed epitopes of outer membrane protein P2. A mutant of strain d3 was isolated as a survivor from bacterial killing by complement and MAb 30DA5. This mutant had an altered P2 protein on sodium dodecyl sulfate-polyacrylamide gels and had lost its reactivity with all of the five *H. influenzae* d3-specific MAbs but not with the other MAbs. From these results, we conclude that the variable parts of outer membrane protein P2 of nonencapsulated *H. influenzae* from the sputum of patients with chronic obstructive pulmonary disease are immunogenic and mostly surface exposed. Only strain-specific MAbs promoted complement-dependent killing of the bacteria, which was abolished in a spontaneous mutant with an altered P2 protein.

Patients with chronic obstructive pulmonary disease (COPD) are persistently infected by nontypeable *Haemophilus influenzae* (12). Especially after exacerbations of the *H. influenzae* infections in these patients, changes were observed in the molecular weights of the major bacterial outer membrane proteins (OMP) P2 (b or c, the pore protein; molecular weight, 39,000 to 42,000) and/or P5 (d, a heat-modifiable protein; molecular weight, 37,000 to 39,000) as analyzed by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (5, 6). The changes in the electrophoretic mobility of the major OMP P2 appeared to be caused by minor differences in its amino acid composition (7).

Differences in OMP composition of *H. influenzae* have been shown to cause strong antigenic differences between the bacteria; polyclonal antisera raised to nontypeable *H. influenzae* bacteria reacted strongly with the strain which was used for the immunization and much more weakly with strains in which OMP were changed (7). Moreover, these antibodies promoted complement-dependent bactericidal activity which was strain specific (7). In an animal model for recurrent otitis media caused by *H. influenzae*, protection against reinfection was achieved if the animals were reinoculated with the same *H. influenzae* strain but not if they were reinoculated with a strain with a different major OMP composition (8).

To determine the specificity of the epitopes of OMP P2 of nontypeable *H. influenzae* from patients with COPD with an antigenic drift, we prepared monoclonal antibodies (MAbs) specific for variable and preserved epitopes on this OMP. The reactivity of these MAbs with various *H. influenzae* isolates was analyzed and the biological activity of these antibodies was determined in complement-dependent bactericidal tests. The surface exposure of the strain-specific and cross-reactive epitopes was assessed by immunogold electron microscopy. Finally, bacteria which survived in the bactericidal assay containing one of the various MAbs were characterized to analyze whether specific antibodies contribute to the selection of new variants with changed OMP P2.

MATERIALS AND METHODS

Isolation and characterization of *H. influenzae* strains. Nonencapsulated *H. influenzae* strains were isolated from the sputum of patients with COPD during a 3-year longitudinal study which has been documented elsewhere (5, 6). Strains d1 to d4, which are isolates from a single patient, are related because they had identical DNA fragment patterns on agarose gels after restriction endonuclease digestions and differences in mobility of the major OMP P2 and P5 on SDS-polyacrylamide gels (5). *H. influenzae* type b strains Eagan (OMP subtype 1L), 770235 (OMP subtype 2L), 859 (OMP subtype 2H), 760705 (OMP subtype 3L), and 1481 (OMP subtype 6U) were isolated from patients with invasive disease (2, 17). *Haemophilus parainfluenzae* isolated from the throats of healthy carriers and *Escherichia coli* were used as controls.

Production and specificity of MAbs. Murine MAbs were produced after immunization of BALB/c mice with Formalin-killed bacteria or with purified protein P2 as described elsewhere (7, 19). The isolation and purification of protein P2 has been described previously in detail (7). After fusion of spleen cells of immunized mice with cells of the myeloma cell line NS-1, clones producing antibodies which reacted with the OMP P2 of the homologous strain were selected. Selection was done by an enzyme-linked immunosorbent assay (ELISA) using heat-killed bacteria and purified protein P2 of the homologous and heterologous strains as the antigen

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TABLE 1. Properties of MAbs specific for OMP P2 of nonencapsulated *H. influenzae* from patients with COPD as analyzed by a whole-cell ELISA, bactericidal activity, and immunogold electron microscopy[a]

MAb	Homologous strain	ELISA titer[b]	Reactivity with variants[c]	No. of other strains reactive			IgG subclass	Bactericidal activity[f]	Immunogold labeling
				Other HiNT[d] (n = 69)	Hib[e] (n = 5)	*H. parainfluenzae* (n = 5)			
25DA5	d2	$1:10^3$	d2, d3, d4	0	0	0	IgG2a	−	−
28AC7	d4	$1:10^2$	d2, d3, d4	0	0	0	IgG1	−	−
28CF2	d4	$1:10^4$	d2, d3, d4	0	0	0	IgG2b	−	−
30DA5	d3	$1:10^3$	d3	0	0	0	IgG3	+	+
30DA10	d3	$1:2 \times 10^3$	d3	0	0	0	ND[g]	+	+
19DA11	d1	$1:10^3$	d1	8	0	0	IgG2b	−	+
25BE5	d2	$1:5 \times 10^3$	d2, d3, d4	3	0	0	IgG2a	−	+
25DE10	d2	$1:10^3$	d1, d2, d3, d4	5	0	0	IgG1	−	+
26AC12	d3	1:10	d1, d2, d3, d4	10	5	0	IgG1	−	+
27CC4	d1	$1:3 \times 10^3$	d1, d2, d3, d4	1	0	0	ND	−	ND
28DD3	d4	$1:8 \times 10^1$	d1, d2, d3, d4	3	0	0	IgG1	−	−
30BD1	d3	$1:2 \times 10^2$	d3	12	0	0	ND	+	+
30DA11	d3	$1:10^3$	d3	7	0	0	IgG3	+	+
30DD1	d3	$1:2 \times 10^3$	d3	4	0	0	IgG2a	+	+
21BG2	d1	$1:10^4$	d1, d2, d3, d4	69	5	5	IgG2b	−	−
23AA12	d1	$1:2 \times 10^3$	d1, d2, d3, d4	65	5	0	IgG2a	−	−
24BC9	d1	$1:5 \times 10^2$	d1, d2, d3, d4	65	5	0	IgG2a	−	−
28BC2	d4	$1:5 \times 10^1$	d1, d2, d3, d4	69	5	5	ND	−	±

[a] Antigen specificity of MAbs was assessed by immunoblotting.
[b] As measured with the whole-cell ELISA to cells of the homologous strain.
[c] Variants include the homologous strain and related strains.
[d] HiNT, Nontypeable *H. influenzae*.
[e] Hib, *H. influenzae* type b.
[f] Killing was strain specific.
[g] ND, Not determined.

(7). MAb 19DA11 has been described previously (7). The specificity of the MAbs was assessed by a whole-cell ELISA (1) and by immunoblotting (4).

The subclass of the antibodies was determined by immunoprecipitation (Ouchterlony method) with rabbit anti-mouse immunoglobulin G subclass-specific antibodies (Dakopatts, Copenhagen, Denmark).

Bactericidal assay and immunochemical characterization of surviving bacteria. The complement-dependent bactericidal killing assay (7) was performed with 4×10^4 bacteria of the related *H. influenzae* strains d1 to d3 per ml and MAbs specific for OMP P2 of these *H. influenzae* strains and human complement of an agammaglobulinemic patient.

Bacteria with changes in their OMP P2 were selected in a similar bactericidal assay as follows. Bacteria of strain d3 (10^8/ml) were exposed to complement and MAb 30DA5 for 2 h. This antibody to OMP P2 exerts a bactericidal effect on this strain. The reaction mixture was then plated in 10-fold dilutions. After incubation of the plates at 37°C in a CO_2 incubator overnight, plates containing 30 to 200 colonies were blotted onto sterile nitrocellulose filter paper. The filter was incubated with MAb 30DA5, and the bound antibodies were visualized with *Staphylococcus aureus* protein A coupled to horseradish peroxidase; incubation was then performed with tetramethylbenzidine as described previously (7). Those colonies negative on this immunoblot were selected, and their OMP patterns were analyzed (5).

Immunogold labeling of bacteria. The method described by Van Ham et al. (20) was used. In short, bacteria grown on chocolate agar plates for 16 h were suspended in phosphate-buffered saline, transferred to Formvar-coated copper grids and incubated on the grids with various MAbs, and then washed and incubated with protein-gold. When immunoglobulin G1 (IgG1) MAbs were analyzed in this assay, an additional incubation with polyclonal anti-mouse immunoglobulins was included before the reaction with protein A-gold. Negative staining of the bacteria was omitted to improve the resolution of gold spheres on the bacteria.

RESULTS

Characteristics of the isolated MAbs. Hybridoma cell lines were obtained from eight fusions between myeloma cell line NS-1 and spleen cells from mice immunized with cells of strains d1 through d4 or OMP P2 from these cells. Eighteen hybridomas produced MAbs which reacted in ELISAs with cell envelopes and purified protein P2 from the strain used for the immunization of a particular mouse. The titers of these culture supernatants are shown in Table 1. The specificities of the MAbs were further determined by immunoblotting using cell envelopes of the homologous and related strains (with changed OMP P2) as antigens. The results of these analyses are summarized in Table 1. All MAbs reacted with the protein P2 band of the homologous strain on the immunoblot. This reaction is illustrated for nine MAbs in Fig. 1A and C. Analysis of the cross-reactivity of the MAbs between the homologous and related strains by immunoblotting (Fig. 1B) and ELISA (Table 1) showed that MAb 19DA11 reacted with the homologous strain d1 and not with strains d2, d3, and d4; MAbs 30DA5, 30DD1, and 30DA10 were specific for the homologous strain d3; MAbs 25BE5, 25DA5, 28CF2, and 28AC7 cross-reacted with strains d2, d3, and d4; and the remaining MAbs cross-reacted with strains d1 to d4.

Strong differences were observed in the whole-cell ELISA when various MAbs were tested for their reactivity with 69

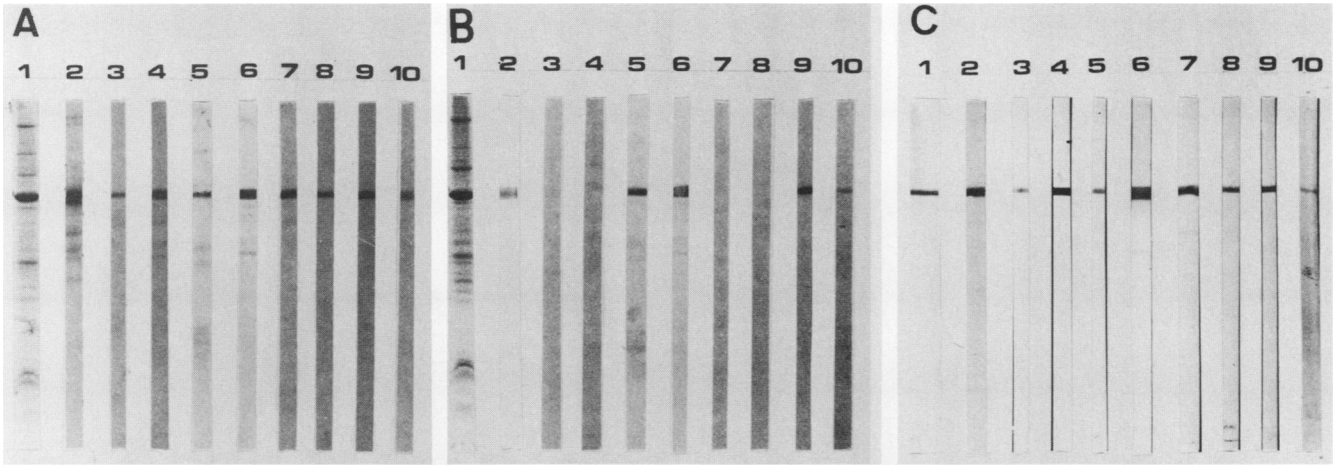

FIG. 1. Immunoblot analysis of monoclonal antibodies to OMP P2 of nonencapsulated *H. influenzae*. Cell envelopes of strain d3 (A), cell envelopes of variant strain d4 (B), and purified protein P2 from strain d3 (C) were used as antigens. Lanes 1, Protein staining of the gel; lanes 2 to 10, immunoblotting results after incubation with MAbs 28AC7 (lane 2), 30DA5 (lane 3), 30DA10 (lane 4), 25BE5 (lane 5), 26AC12 (lane 6), 30BD1 (lane 7), 30DA11 (lane 8), 21BG2 (lane 9), and 28BC2 (lane 10). Lanes 3, 4, and 7 to 9 were incubated with culture supernatants of the hybridomas diluted 1:10; in lanes 5, 6, and 10 a 1:25 dilution was used, and in lane 2 a 1:50 dilution was used. See Table 1 for the titers of these MAbs.

nonencapsulated *H. influenzae* sputum isolates which were shown to have different OMP composition and DNA fragment patterns on agarose gels after restriction endonuclease digestion (5, 6). Five of the 18 MAbs appeared specific for the homologous strain (MAbs 25DA5, 28AC7, 28CF2, 30DA5, and 30DA10). Nine MAbs showed a low degree of cross-reactivity; they reacted with up to nine strains (MAbs 19DA11, 25BE5, 25DE10, 26AC12, 27CC4, 28DD3, 30BD1, 30DA11, and 30DD1). On the basis of the reactivity pattern of these MAbs, it was not possible to indicate subgroups of strains which shared reactivity with some of these MAbs. The remaining four MAbs reacted with all or almost all strains (MAbs 21BG2, 23AA12, 24BC9, and 28BC2). These last MAbs also reacted with five *H. influenzae* type b strains differing in OMP composition, and two of them reacted with *H. parainfluenzae* (MAbs 28BC2 and 21BG2). None of the MAbs reacted with *E. coli*, which was used as a control. We did not find a relationship between the cross-reactivity of the MAbs and the kind of antigen (whole cells or purified protein P2) used for the immunization of the mice from which the spleen cells were obtained.

These results indicate that strain-specific MAbs were predominant in the series and that the majority of these showed limited cross-reactivity with the isolated variants of the same strain.

Bactericidal activity of MAbs. The MAbs were tested for their ability to kill the homologous and related strains of *H. influenzae* in the presence of complement. These results are summarized in Table 1. None of the broadly cross-reactive MAbs exerted a bactericidal effect. Five MAbs killed the homologous strain in the presence of human serum from an agammaglobulinemic patient which was used as the complement source (7). The slope of the killing curve was dependent on the concentration of the MAb in the incubation mixture. A culture supernatant diluted 1:10 (final concentration) was sufficient for killing within 30 min; a 1:100 dilution did not promote killing during the 90-min study period. Killing was not observed with heat-inactivated serum in the absence of these MAbs. The results of the bactericidal assays for one of these MAbs (MAb 30DA5) and a negative MAb (MAb 19DA11) are shown in Fig. 2. All of the bactericidal MAbs (30BD1, 30DA5, 30DA10, 30DA11, and 30DD1) were produced by hybridoma cell lines from one fusion, suggesting that they may be identical clones. However, the strain specificity of MAbs 30DA5 and 30DA10 was different from the specificity of MAbs 30BD1, 30DA11, and 30DD1 (Table 1), indicating that the bactericidal antibodies are at least directed to two (partly) different epitopes (see below).

The bactericidal antibodies killed the homologous strain d3 but not the related strains d1, d2, and d4. The lack of killing of these heterologous strains is in agreement with the lack of reactivity of these MAbs with these strains in ELISAs and immunoblotting. MAb 30BD1 did not promote complement killing of five other strains which reacted with this MAb in ELISA.

Immunogold electron microscopy with MAbs to OMP P2. To determine whether the various MAbs reacted with surface-exposed epitopes of OMP P2, freshly prepared bacterial

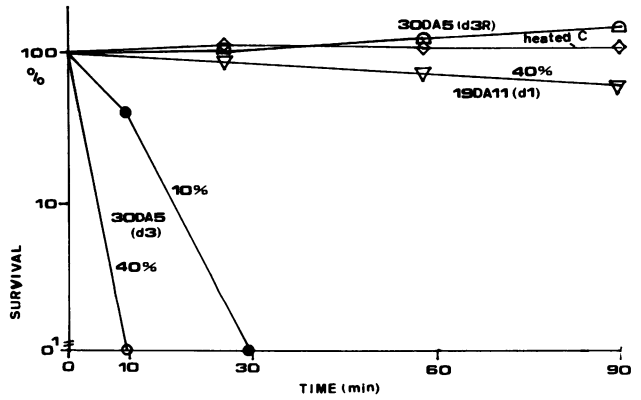

FIG. 2. Complement-dependent activity of MAb 30DA5 to strain d3 (○, ●, and ◇) and d3R (⌂) and of MAb 19DA11 to strain d1 (▽). Various concentrations of culture supernatants of the hybridomas producing MAb 30DA5 were used as indicated to show concentration-dependent killing. Killing after heat inactivation of complement is indicated (◇).

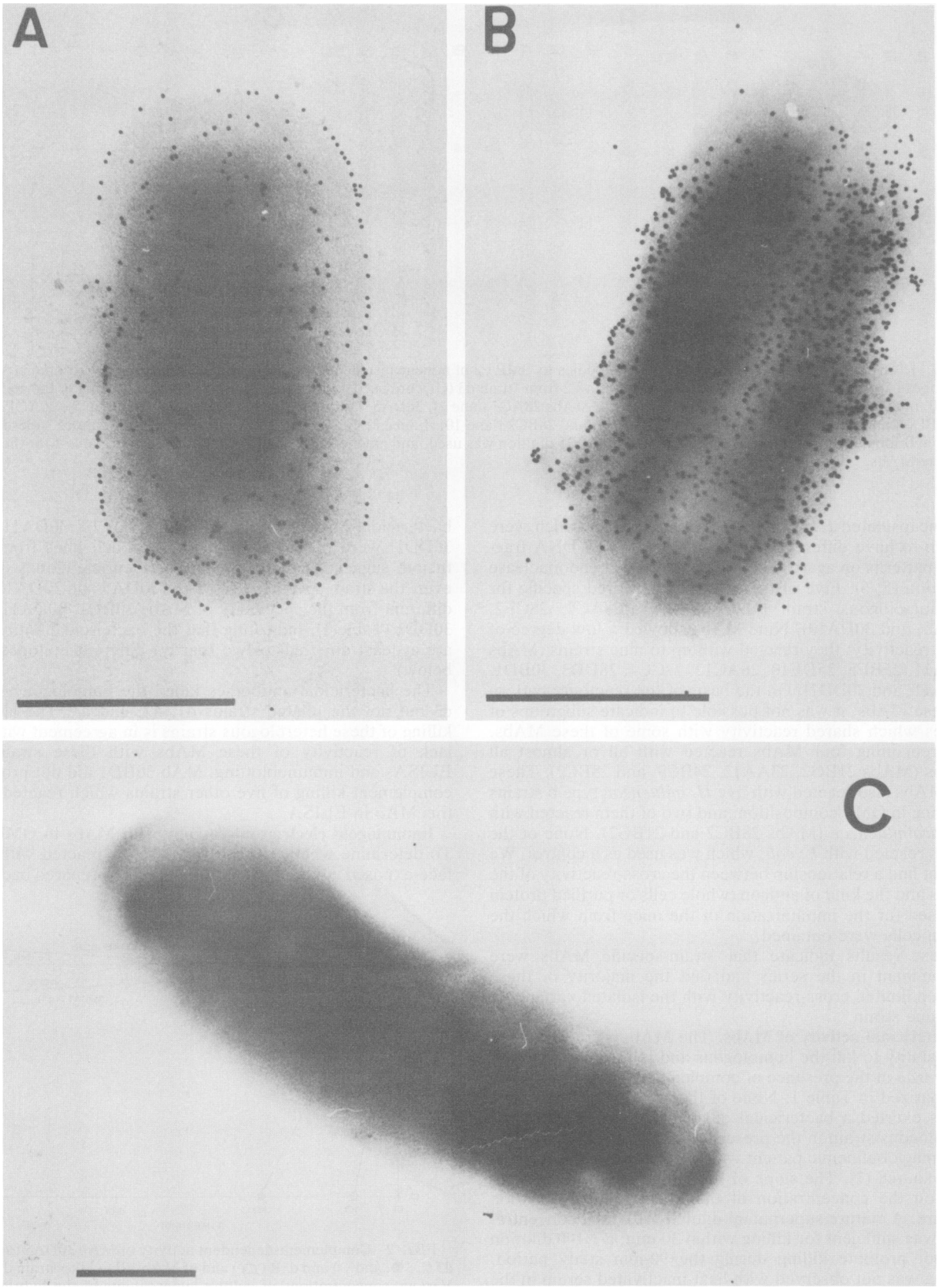

FIG. 3. Immunogold electron microscopy after incubation of strain d3 with MAb 30DA5 (A), strain d1 with MAb 19DA11 (B), and strain d3 with MAb 21BG2 (C). Bar, 0.5 μm. A culture supernatant diluted 1:5 with MAbs was used in all cases.

suspensions were analyzed by immunogold electron microscopy. The results of these experiments are summarized in Table 1, and electron micrographs are shown in Fig. 3. Gold spheres were seen on the surface of the homologous strain after incubation of the bacteria with the five bactericidal antibodies and four of eight other strain-specific MAbs. Bacteria were not labeled after incubation with the broadly cross-reactive MAbs. The results indicate that the majority of the strain-specific MAbs were directed against surface-exposed epitopes, in contrast to the cross-reactive MAbs.

OMP analysis and reactivity with MAbs of survivors from MAb-mediated killing. Bacteria of strain d3 were incubated with complement and MAb 30DA5 at 37°C for 2 h. An 8-log-unit decrease in the bacterial count was observed. The mixture was then plated, and the plates were incubated at 37°C overnight. Only one of the colonies obtained after three independent experiments did not react with MAb 30DA5 on a colony blot. The frequency of MAb 30DA5-negative mutants was calculated as approximately 1 out of 10^9 bacteria. The MAb 30DA5-negative mutant strain d3R was insensitive to killing by MAb 30DA5, in contrast to bacteria from three positive colonies. Mutant d3R appeared to be a variant of strain d3, since the DNA patterns of strains d3 and d3R on agarose gels after digestion with restriction endonucleases were identical.

Both strains d3R and d3 reacted in the whole-cell ELISA with MAbs 21BG2, 23AA12, 27CC4, and 28CF2, indicating that the changes in OMP P2 did not affect the strongly preserved epitopes and the epitopes which occur only on strains d2 through d4 and d1 through d4. These results also show that the d3-specific epitope which is recognized by MAb 30DA5 is different from the epitopes which are common to the variant strains d1 through d4. The mutant strain d3R did not react with MAb 19DA11, excluding a reversion to the d1-specific epitope. The mutant did not react with any of the other MAbs specific for strain d3 (MAbs 30BD1, 30DA10, 30DA11, and 30DD1).

The OMP analyses of strains d3R and strains d1 to d4 are shown in Fig. 4. The electrophoretic mobility of OMP P2 of strain d3R was different from those of strains d1 to d4. The lack of reactivity of strain d3R with MAb 30DA5 was apparently due to a change in protein P2 similar to the changes in OMP P2 observed in *H. influenzae* isolates from persistently infected patients with COPD.

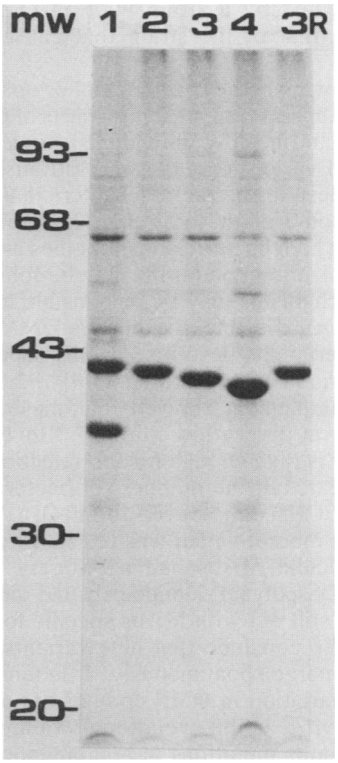

FIG. 4. SDS–polyacrylamide gel electrophoretic analysis of the OMP composition of *H. influenzae* variants d1 to d4 (lanes 1 to 4, respectively) and the mutant d3R (lane 3R), which is resistant to bactericidal killing mediated by MAb 30DA5. Molecular weight markers in thousands (mw) are indicated at the left.

DISCUSSION

The OMP composition of nonencapsulated *H. influenzae* is strongly heterogeneous (10, 14, 18). Persistence of *H. influenzae* in patients with COPD contributes to this heterogeneity, since new variants emerge frequently, most often during exacerbations of infection (5, 6). These changes in OMP composition were shown to result in strong antigenic differences between the variants (7). The MAbs which were elicited to the variable parts of OMP P2 appeared to react almost exclusively with the homologous strain and its isogenic variants, especially when they showed complement-dependent bactericidal activity (Table 1). The discrepancy between ELISA reactivity and killing of some strains may be due to slight differences in the amino acid composition and surface localization of these epitopes. These results indicate that the variable parts of OMP P2 are unique and that it is unlikely that patients have antibodies to these epitopes before a new variant has emerged. This was a surprising observation, since the amino acid compositions of OMP P2 of related *H. influenzae* isolates are almost identical (7).

The MAbs specific to strain d3 did not react with the mutant strain d3R. Although these MAbs were all from one fusion experiment, they are probably not identical; they showed different reaction patterns with various *H. influenzae* strains (Table 1). These mutants may recognize overlapping epitopes but could also be specific for epitopes whose conformations are changed because of a mutation elsewhere in the protein.

Very well-preserved epitopes of OMP P2 were recognized by a few MAbs. These reacted with all *H. influenzae* strains analyzed (Table 1). Murphy and Apicella found partially overlapping antigenic determinants in OMP P2 of nontypeable *H. influenzae* analyzed with a series of OMP P2-specific polyclonal antisera (13).

Most of the strain-specific parts of OMP P2 appeared surface exposed, in contrast to the well-preserved parts of this molecule (Table 1; Fig. 3). These preserved parts may be the sites of the protein which are essential for the pore function (9, 16) or for the embedding of the protein in the outer membrane (11). Since only strain-specific MAbs were bactericidal, the results indicate that the humoral defense mechanisms are probably largely strain specific. The results which we obtained previously (7) with polyclonal antisera are in agreement with the data obtained with MAbs. The animal protection studies described by Karasic et al. (8) also stress the importance of antigenic heterogeneity of OMP as a cause of the lack of cross-protection against recurrent otitis media by strains with different OMP compositions. In addition, Barenkamp et al. (3) and Murphy et al. (15) showed that infants with recurrent otitis media due to *H. influenzae*

become reinfected by a strain with an OMP composition different from that of the strain that caused the previous infection.

It is not known whether bactericidal or opsonic antibodies can be elicited against the preserved parts of protein P2. Sera from patients and healthy individuals showed strong cross-reactivity with various *H. influenzae* strains in immunoblot experiments despite the lack of bactericidal activity of the sera against some of these strains (3). Preserved parts of protein P2 to which bactericidal antibodies are elicited under special immunization conditions may be found.

Several mechanisms may be responsible for the antigenic drift. *H. influenzae* is able to exchange DNA by transformation. The experiments described in this paper on the selection of bacteria with an altered OMP P2 by bactericidal MAbs and complement showed that these mutants occur spontaneously at a very low rate (10^{-9} to 10^{-11}). Whether this frequency is high enough for the selection of variants in vivo is not clear, but the numbers of bacteria in sputum of patients with COPD may exceed 10^9/ml (12). The OMP P2 of the laboratory mutant which was resistant to killing by one of the bactericidal MAbs had a different electrophoretic mobility on SDS-polyacrylamide gels and did not react with that MAb but still did with MAbs specific for other parts of the protein. We conclude that new variants in a particular epitope can emerge spontaneously. It is tempting to speculate that the variation in OMP composition of nonencapsulated *H. influenzae* is the evolutionary outcome of many of these spontaneous mutations in combination with immunologically selective pressure in patients.

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