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Fine Mapping of Outer Membrane Protein P2 Antigenic Sites Which Vary during Persistent Infection by *Haemophilus influenzae*

BIRGITTA DUIM,[1†] LIESBETH VOGEL,[1] WOUTER PUIJK,[2] HENK M. JANSEN,[3] ROB H. MELOEN,[2] JACOB DANKERT,[1] AND LOEK VAN ALPHEN[1*]

Department of Medical Microbiology[1] and Department of Pulmonology,[3] University of Amsterdam, 1105 AZ Amsterdam, and Institute for Animal Science and Health, 8219 PH Lelystad,[2] The Netherlands

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Antigenic drift of the major outer membrane protein (MOMP) P2 of nonencapsulated *Haemophilus influenzae* as observed during persistent infections in patients with chronic bronchitis was mimicked in a rabbit model in which *H. influenzae* persisted in subcutaneous cages. The antigenic drift resulted from amino acid substitutions in potentially surface-exposed loops of MOMP P2. Since in a rabbit model the appearance of antigenic variants was associated with the presence of strain-specific bactericidal antibodies (L. Vogel, B. Duim, F. Geluk, P. Eijk, H. Jansen, J. Dankert, and L. van Alphen, Infect. Immun. 64:980–986, 1996), we determined the epitope specificities of these bactericidal antibodies. The eight loops of MOMP P2 of *H. influenzae* d1 were separately expressed as fusion proteins with glutathione *S*-transferase. Sera of rabbits persistently infected with *H. influenzae* reacted with the loop 5 and loop 6 fusion proteins in immunoblotting and enzyme-linked immunosorbent assay. For fine mapping of the epitopes with pepscan analysis, overlapping synthetic peptides consisting of 12 amino acids were made. Rabbit sera contained antibodies reacting with peptides derived from loop 5 and peptides containing amino acids of the side of loop 6. In addition, MOMP P2 variant-specific reactions with the amino acids located at the tip of loop 6 were detected. The rabbit sera showed variant-specific complement-dependent bactericidal activities, which were eliminated by affinity chromatography with fusion proteins of loop 6 but not of loop 5. We conclude that, during persistence of *H. influenzae* in rabbits, variant-specific bactericidal antibodies are elicited to the variable tip of MOMP P2 loop 6.

Nonencapsulated *Haemophilus influenzae* is a common cause of infections of the respiratory tract including infections in patients with chronic bronchitis (16, 27, 29). *H. influenzae* persists in the lower respiratory tract of chronic bronchitis patients despite the presence of antibodies specific for a variety of outer membrane proteins (6) including the porin MOMP P2, the most abundantly expressed protein in the outer membrane (28). MOMP P2 is a target for bactericidal antibodies elicited during the antibody response against nonencapsulated *H. influenzae* in humans (17). Bactericidal antibodies are strain specific, both in humans and in animals, and induce in an animal model protection against experimental otitis media by *H. influenzae* (15, 21).

During persistent infections in patients with chronic bronchitis, changes in the molecular weight of MOMP P2 were observed (9). This variation resulted in antigenic drift since the variants were no longer recognized by strain-specific antibodies recognizing MOMP P2 (9, 30). Antigenic drift of MOMP P2 by nonencapsulated *H. influenzae* may therefore contribute to the persistence of this bacterium in patients with chronic bronchitis (7–9). In a model of the secondary structure of MOMP P2 eight surface-exposed loops and 16 membrane spanning regions have been proposed (24). Comparison of the sequences of the P2 genes of various nonencapsulated *H. influenzae* strains revealed highly conserved sequences coding for the membrane-spanning regions and highly variable sequences coding for surface-exposed loops (3, 22). The surface-exposed loops contain strain-specific immunodominant epitopes (2, 10, 11, 24, 34). The antigenic drift of MOMP P2 observed during persistence of *H. influenzae* in patients with chronic bronchitis was associated with the changes in immunodominant surface-exposed epitopes (7). Nucleotide substitutions in the MOMP P2 gene, generating amino acid changes in the loops exposed at the bacterial surface were identified (4). Similar changes were observed in the MOMP P2 gene of *H. influenzae* persisting in cages implanted subcutaneously in rabbits (4, 32). The nucleotide changes in the MOMP P2 gene in variants isolated from sputum samples of patients with chronic bronchitis and from subcutaneous cages in rabbits were all nonsynonymous (amino acid altering). They occurred in regions coding for surface-exposed parts of MOMP P2. Especially changes in loop 6, similar to the change observed in loop 6 in an *H. influenzae* variant exposed in vitro to a bactericidal monoclonal antibody (MAb) and complement, were observed (30). In the rabbit model the appearance of MOMP P2 variants of *H. influenzae* was shown to be correlated with the development of strain-specific bactericidal antibodies (32). These observations strongly suggest the appearance of antigenic variants of MOMP P2 by immune selection.

In this study we determined whether the bactericidal antibodies elicited during persistence of *H. influenzae* are specific for the variable surface-exposed loops of MOMP P2. Separate MOMP P2 loops were expressed as fusion proteins with glutathione *S*-transferase (GST) in *Escherichia coli* (23) to analyze the loop specificities of anti-MOMP P2 monoclonal bactericidal antibodies and serum antibodies for rabbits persistently

* Corresponding author. Mailing address: Department of Medical Microbiology, University of Amsterdam, Academic Medical Center, Room L162, Meibergdreef 15, 1105 AZ Amsterdam, The Netherlands. Phone: 31 20 5664862. Fax: 31 20 6979271. Electronic mail address: a.j.vanalphen@amc.uva.nl.

† Present address: Department of Bacteriology, Institute for Animal Science and Health, Lelystad, The Netherlands.

TABLE 1. Oligonucleotide primers used for amplification of MOMP P2 loops

Primer[a]	Positions[b]	Sequence[c]
P2 1A[c]	100–114	TG GGA TCC GGT GGT CGT TTA AGC
P2 1B	183–197	GC GAA TTC GTG GAA ACG TGA ACC
P2 2A	231–245	TG GGA TCC CTA TGC ACA AGG TTA
P2 2B	340–355	GC GAA TTC TTC ACC GAA TGC TTT
P2 3A	361–376	TG GGA TCC CTT GGT CGT GCG AAA
P2 3B	455–469	GC GAA TTC GCC AAC GGT ATT ACC
P2 4A	505–519	CG GGA TCC GTA TTA GGC GCT AAT
P2 4B	600–615	CG GAA TTC CTG AAC GCC ATT GCT
P2 5A	658–671	GT GGA TCC GCT TAT GGT CGT ACA
P2 5B	750–764	CG GAA TTC AGT TGA AAG AGC ACC
P2 6A	802–816	GT GGA TCC GAT AGT GGC TAT GCA
P2 6B	898–912	GC GAA TTC ACC TGG AGA TAC GAA
P2 7A	970–983	TG GGA TCC GTA TCT CCA GGT TTC
P2 7B	997–1011	GC GAA TTC TAC TGC GTG TTC ACG
P2 8A	1059–1074	TG GGA TCC GAA GGT GCT TAC GCG
P2 8B	1126–1140	CG GAA TTC ACC TAC ACC CAC TGA

[a] Numbers 1 to 8 indicate loop specificity.
[b] Nucleotide positions in the sequenced P2 gene of *H. influenzae* d1 (3).
[c] 5′ to 3′. Recognition sites for restriction endonucleases *Bam*HI and *Eco*RI are underlined.

infected with nonencapsulated *H. influenzae*. The exact epitopes recognized by the rabbit antibodies were identified by pepscan analysis using overlapping synthetic peptides representing MOMP P2 loops. Loop-specific antibodies were purified and analyzed for complement-dependent bactericidal activity. The significance of immune selection of MOMP P2 antigenic variants for the persistence of *H. influenzae* infections in patients with chronic bronchitis is discussed.

MATERIALS AND METHODS

Bacterial strains, plasmids, and culture conditions. Nonencapsulated *H. influenzae* d1 and its variant d3 were obtained from sputum samples of a patient with chronic bronchitis who was enrolled in a longitudinal study on the persistence of *H. influenzae* infections, as described before (7). Variant b4, derived from strain d1, was isolated during persistence of *H. influenzae* in subcutaneous tissue cages in a rabbit model (4, 32). *H. influenzae* was cultured on chocolate agar plates or in brain heart infusion broth (Difco), supplemented with hemin and NAD (10 μg/ml each) at 37°C in a humid atmosphere enriched with 5% CO_2. The plasmid pGEX-2T (Pharmacia) and derivatives were propagated in *E. coli* K-12 strain DH5α (Bethesda Research Laboratories, Gaithersburg, Md.) grown at 37°C in Luria broth or on Luria-Bertani agar containing 100 μg of ampicillin per ml.

Amplification of the P2 gene. PCR was used for amplification of the parts of the P2 gene coding for the surface-exposed loops of MOMP P2. Primers (1A-1B to 8A-8B) were based on the conserved sequences of membrane-spanning regions flanking the surface-exposed loops. They contained a *Bam*HI (N-terminal primer) or *Eco*RI (C-terminal primer) restriction site at the 5′ end to facilitate in-frame ligation of the amplified DNA with the C-terminal portion of the GST gene which was part of the expression vector pGEX-2T (23). In Table 1 the primers used for amplification of the DNA of separate loops are listed. Amplification was performed on chromosomal DNA as previously described by Duim et al. (4).

Cloning of MOMP P2 loops in *E. coli*. The PCR fragments, approximately 100 bp each, were purified from 2% agarose gels (Nusieve GTG agarose; FMC Bio Products) with the Qiaex kit (Qiagen Inc.), digested with *Bam*HI and *Eco*RI according to the instructions of the manufacturer (Boehringer Mannheim Biochemicals, Mannheim, Germany), and electrophoresed through a 2% agarose gel. After purification, the PCR fragments were cloned in *Bam*HI- and *Eco*RI-digested pGEX-2T by using T4 DNA ligase (Boehringer Mannheim Biochemicals), and the ligated plasmid was transformed into *E. coli*. Plasmid DNA was isolated from recombinant clones. Proper insertion of *H. influenzae* DNA into the vector was controlled by restriction enzyme analysis. The clones with DNA coding for loops 1 to 8 of variant d1 were designated pGSTd1.1 to pGSTd1.8, and clones with loops 5 and 6 of variant d3 were designated pGSTd3.5 and pGSTd3.6. Sequence analysis of these clones confirmed the cloning of the MOMP P2 loops.

Expression and purification of GST-MOMP P2 fusion proteins. An overnight culture of *E. coli* transformed with pGEX-2T plasmids containing DNA encoding the loops of MOMP P2 of *H. influenzae* was diluted 1:10 in fresh medium and grown at 37°C for 1 h with shaking (23). GST fusion protein expression was induced with 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) (Sigma) at 28°C for 4 h. Bacteria were collected by centrifugation and resuspended in 1/50 of the original volume in NETN buffer (20 mM Tris-HCl [pH 8], 100 mM NaCl, 1 mM EDTA, 0.5% Nonidet P-40). The bacteria were lysed by sonication, and the lysate was clarified from cellular debris by centrifugation. GST fusion proteins were purified from the soluble fraction by chromatography on immobilized glutathione. Therefore, 1-ml aliquots of the bacterial supernatant obtained after centrifugation were incubated with 100 μl of preswollen glutathione-agarose beads (Sigma) at 4°C for 1 h. The beads were washed three times with NETN buffer, and GST fusion proteins were subsequently eluted in 2 × 250 μl of freshly made elution buffer (5 mM reduced glutathione in 50 mM Tris-HCl [pH 8]) at room temperature for 1 h.

SDS-PAGE and Western blotting. Fusion proteins were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using the discontinuous buffer system of Laemmli (12). Gels (4% stacking gel, 12% separating gel) were run at 100 V for 2 h (Bio-Rad Mini-Protein Gel System) and stained with Coomassie brilliant blue according to the method described by van Alphen et al. (31). Proteins were electrophoretically transferred to nitrocellulose as described by Towbin et al. (25) and incubated with MAbs or rabbit sera and then with alkaline phosphatase-labeled goat anti-rabbit immunoglobulin G (Pharmacia). Cell envelopes of *H. influenzae* were analyzed by SDS-PAGE as described by van Alphen et al. (31).

DNA sequence analysis. Templates for sequencing were obtained by PCR. Plasmids were isolated as described by Birnboim and Doly (1). Primers were directed to pGEX-2T sequences on both sides of the insert and were extended with sequences at the 5′ end which are complementary to the -21M13 and M13RP1 universal primers. PCR fragments were purified as described above. The sequencing reaction was performed with fluorescent dye-labeled -21M13 or M13RP1 primers according to the protocol provided by Applied Biosystems Inc. (Foster City, Calif.). The products were analyzed on an Applied Biosystems 373A DNA sequencer.

ELISA. Antibody reactivity with the MOMP P2 fusion proteins was determined by enzyme-linked immunosorbent assay (ELISA) using microtiter plates (Greiner Immulon) coated (16 h, 37°C) with 100 μl of antigen (0.75 μg of protein per well for bacterial cell envelopes, 0.5 μg per well for purified protein). Plates were washed with phosphate-buffered saline containing 0.05% Tween 20. The purification of cell envelopes, ELISA procedure, and dilution of MAbs were performed as described by van Alphen et al. (30, 31).

MAbs. Strain-specific murine MAbs directed against MOMP P2 of nonencapsulated *H. influenzae* have been described previously by van Alphen et al. (30) and Troelstra et al. (26). These MAbs react with whole bacteria in ELISA and with purified MOMP P2 on immunoblot and include six MOMP P2 variant-specific MAbs, two cross-reacting MAbs, and three broadly cross-reacting MAbs (30).

Tissue cage rabbit model for persistence of *H. influenzae*. The tissue cage rabbit model has been described extensively by Vogel et al. (32). In short, *H. influenzae* d1 was inoculated into cages implanted subcutaneously in rabbits ($n = 3$; designated nonvaccinated). When persistence of *H. influenzae* was established (6 to 10 weeks), some rabbits were vaccinated with formaldehyde-killed bacteria of *H. influenzae* d1 ($n = 3$) or variant d3 ($n = 3$) and persistence was monitored for several months. Colonies cultured from the cages at regular intervals were subjected to SDS-PAGE, and variants of *H. influenzae* with changes in electrophoretic mobilities of their MOMPs P2 were isolated. For analysis of the elicited antibodies against *H. influenzae* during persistence, blood samples from nonvaccinated as well as vaccinated rabbits were taken at regular intervals. In this study, sera obtained 100 days after inoculation were used. Sera were taken arbitrarily at 100 days after establishment of persistence while these sera had bactericidal activity and before the appearance of variants (32).

Pepscan analysis. Overlapping peptides of 12 amino acids each of loop 5 and loop 6 of MOMP P2 were synthesized on polyethylene rods and tested for their reactivities with MAbs and rabbit sera in an ELISA according to established procedures (5).

Bactericidal assay. The complement-dependent bactericidal assay was performed according to the assay described by van Alphen et al. (30). Bacteria of *H. influenzae* d1 grown to late exponential phase in brain heart infusion broth were suspended in 10 mM sodium barbital buffer, pH 7.8, to a density of 4×10^5 CFU/ml. Five microliters of this suspension was added to 5 μl of sodium barbital buffer containing 15 mM $CaCl_2$, 50 mM $MgCl_2$, 3% (wt/vol) bovine serum albumin, 5 μl of serum from a patient with agammaglobulinemia as complement source, and 15 μl of adsorbed rabbit sera (56°C heat inactivated for 30 min). The final volume was adjusted to 50 μl with sodium barbital buffer. After 30 and 60 min, 10-μl samples were plated on chocolate agar plates to determine the number of surviving bacteria.

Rabbit sera were obtained as described by Vogel et al. (32) and depleted for antibodies specific for loops 5 and 6 by affinity chromatography. Purified GST-MOMP P2 fusion proteins containing loop 5 or loop 6 were coupled to 1 g of CNBr-activated Sepharose 4B (Pharmacia) according to the method described by Murphy et al. (18). Serum from one rabbit inoculated with *H. influenzae* d1 was incubated overnight with the coupled Sepharose at 4°C. The nonbound fraction of the adsorbed sera was collected and tested in ELISA with the fusion proteins as antigens to determine the degree of adsorption.

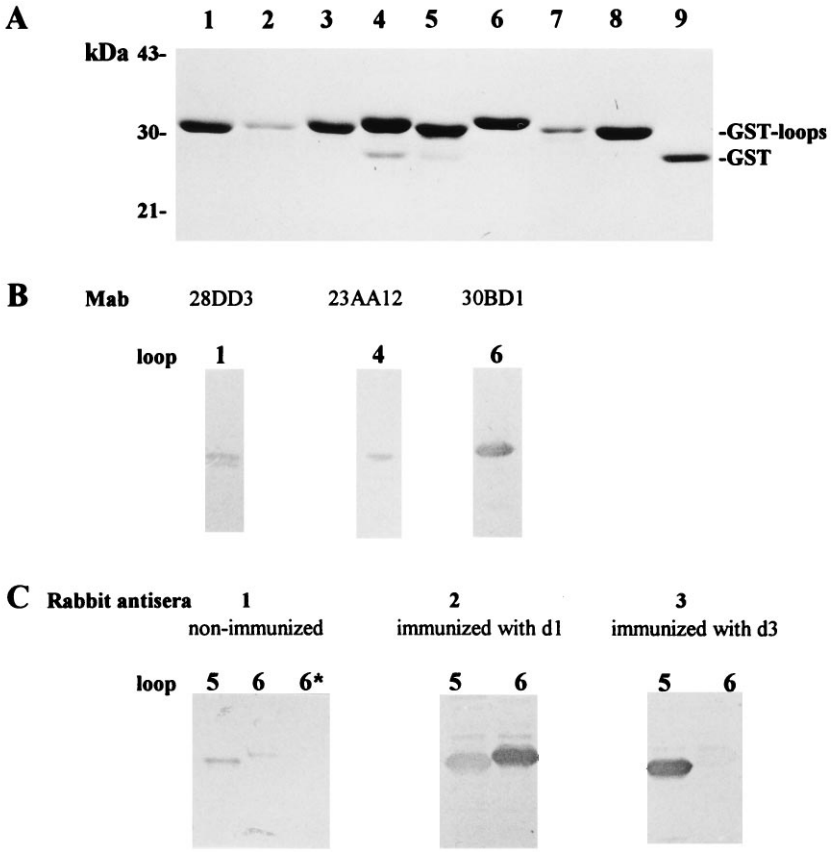

FIG. 1. (A) SDS-PAGE of purified GST-MOMP P2 fusion proteins. Separate loops of MOMP P2 were expressed as fusion proteins with GST in *E. coli* and purified by affinity chromatography. Lanes 1 to 8, GST–loop 1 to–loop 8; lane 9, GST. Molecular weight markers are indicated on the left. (B) Immunoblot analysis of purified GST-MOMP P2 loops incubated with MAbs 28DD3, 23AA12, and 30BD1 (30). Only the indicated loops reacted. (C) Immunoblot analysis of antibodies elicited against MOMP P2 during chronic infection in rabbits. Panels 1 to 3, serum sample of a rabbit inoculated with strain d1, inoculated and vaccinated with strain d1, or inoculated with strain d1 and vaccinated with variant d3, respectively. Lane 6*, GST-MOMP P2 loop 6 of rabbit variant b4, tested only with serum of the nonvaccinated rabbit from which the variant was obtained (4).

RESULTS

Cloning and expression of *H. influenzae* MOMP P2 loops in *E. coli*. For characterization of the antigenic domains on the MOMP P2 loops, fusion proteins containing GST (27 kDa) and a single loop of MOMP P2 (loops 1 to 8 of *H. influenzae* d1 and loop 6 of variant d3) were expressed in *E. coli*. After purification by glutathione-agarose affinity chromatography the relative electrophoretic mobility with SDS-PAGE was consistent with the predicted molecular mass of the fusion proteins (Fig. 1A).

Recognition of GST-MOMP P2 loop fusion proteins by MOMP P2-specific MAbs. The reactivities of MOMP P2-specific MAbs elicited against bacteria of *H. influenzae* d1 and variant d3 (26, 30) were determined by immunoblotting (Fig. 1B) and ELISA (Table 2), using purified GST-MOMP P2 loops as antigens. MAbs 27CC4 and 28DD3 reacted strongly with the fusion proteins expressing loop 1 of strain d1. The broadly cross-reactive MAb 23AA12 showed a reaction with MOMP P2 loop 4, confirming the results described by Srikumar et al. (24). This epitope was formed by the 11 conserved amino acids, encoded by PCR primer p24A, and is located within a hydrophobic beta strand which spans the membrane. MAbs 30DA11, 30DA10, 30DA5, 30BD1, and 30DD1, all specific for the MOMP P2 of *H. influenzae* variant d3, reacted with loop 6 of MOMP P2 of variant d3, the variant that was used to elicit the MAbs. A number of MAbs showed no reaction with

TABLE 2. Reactivities of MAbs with GST-MOMP P2 fusion proteins of *H. influenzae* antigenic variants in ELISA

MAb (specificity[a])	A_{492}[b]								
	d1 loops								d3 loop 6
	1	2	3	4	5	6	7	8	
30BD1[c] (d3)	−	−	−	−	−	−	−	−	++
30DA5[c] (d3)	−	−	−	−	−	−	−	−	+++
30DA10[c] (d3)	−	−	−	−	−	−	−	−	++
30DA11[c] (d3)	−	−	−	−	−	−	−	−	+++
30DD1[c] (d3)	−	−	−	−	−	−	−	−	+++
19DA11 (d1)	−	−	−	−	−	−	−	−	−
28DD3 (d1[d])	++	−	−	−	−	−	−	−	−
27CC4 (d1[d])	++	−	−	−	−	−	−	−	−
21BG2 (d1[e])	−	−	−	−	−	−	−	−	−
23AA12 (d1[e])	−	−	−	++	−	−	−	−	−
24BC9 (d1[e])	−	−	−	−	−	−	−	−	−

[a] The *H. influenzae* strain to which antibodies were elicited in mice for the production of MAbs.

[b] +++, >2.0; ++, 1.0 to 2.0; +, 0.5 to 1.0; −, <0.5.

[c] Bactericidal in the presence of complement for the homologous variant d3 (30).

[d] Cross-reactive antibodies reacting with one or a few unrelated *H. influenzae* strains.

[e] Broadly cross-reactive antibodies, reacting with 60 of 69 unrelated *H. influenzae* strains (30).

TABLE 3. Reactivities of antibodies, elicited in rabbits, with MOMP P2 loops expressed as fusion proteins with GST, as determined by ELISA

Rabbit[a]	A_{492}[b]								
	d1 loops								d3 loop 6
	1	2	3	4	5	6	7	8	
Nonvaccinated	−	−	−	−	+	+/−	−	−	+/−
Nonvaccinated	−	−	−	−	+	−	−	−	−
Nonvaccinated	−	−	−	−	++	−	−	−	−
Vaccinated with:									
d1	−	−	−	−	++	+	−	−	−
d1	−	−	−	−	++	+	−	−	−
d1	−	−	−	−	++	++	−	−	−
d3	−	−	−	−	+	+	−	−	+/−
d3	−	−	−	−	++	−	−	−	−
d3	−	−	−	−	++	−	−	−	+/−

[a] Rabbits were inoculated with *H. influenzae* d1.
[b] ++, >2.0; +, 1.0 to 2.0; +/−, 0.5 to 1.0; −, <0.5.

the fusion proteins. MAb 19DA11, specific for MOMP P2 of strain d1 (30), did not react with any of the GST-MOMP P2 fusion proteins in immunoblotting or in ELISA, despite a reaction of this MAb with *H. influenzae* d1 in a whole-cell ELISA (26, 30). Since MAb 19DA11 recognized a surface-exposed epitope as observed by immunogold electron microscopy (30), the lack of reactivity with the fusion proteins suggests that the epitope for MAb 19DA11 is incomplete or conformational. Also, the broadly cross-reactive MAbs 24BC9 and 21BG2 did not react with the fusion proteins.

The results indicate that separate loops of MOMP P2 can be used for determination of the reactivity of MOMP P2-directed antibody; at least loops 1, 4, and 6 in the fusion proteins contain linear epitopes which are recognized by antibodies generated to whole *H. influenzae* cells.

Specificities of antibodies in persistently infected rabbits. The specificities of antibodies against MOMP P2 evolved during persistence of *H. influenzae* d1 in the rabbit subcutaneous cage model were analyzed. Table 3 summarizes the ELISA results for the reactivities of GST-loop fusion proteins with antibody present in sera from nonvaccinated ($n = 3$) and vaccinated ($n = 6$) rabbits taken at 100 days after establishment of the persistent infection. All nine sera reacted with the fusion protein containing loop 5, and five of the nine sera tested reacted weakly with fusion proteins containing loop 6 of the MOMP P2 of strain d1. In one nonvaccinated rabbit a weak cross-reaction with loop 6 of variant d3 was observed. No reaction was observed with any of the other loops and GST. Vaccination of rabbits with the homologous *H. influenzae* d1 resulted in an increase of reactivity with loop 5 and a reaction with the fusion proteins containing the loop 6 peptide. An increase in reactivity with loop 5 was also observed after vaccination with the heterologous *H. influenzae* d3, but the reaction with loop 6 did not increase. In addition, a weak response to the fusion protein of loop 6 of strain d3 was elicited by the vaccination of rabbits with strain d3. Reactivities in ELISA were in all cases confirmed by reactivities of individual serum samples in immunoblots (Fig. 1C).

These results show that antibodies specific for loop 5 were readily formed in either nonvaccinated or vaccinated rabbits infected with *H. influenzae*. In contrast, antibodies specific for loop 6 were clearly detected only after vaccination and appeared strain specific.

To determine whether variant-specific antibodies were lacking before that variant appeared, we analyzed whether rabbit serum contained specific antibodies for variant b4, before this variant was detected in cultures from the rabbit tissue cages. Fusion proteins with loop 6 of the MOMP P2 variant b4 isolated from a nonvaccinated rabbit were constructed. The sequence of MOMP P2 of the rabbit variant b4 contained four amino acid substitutions in loop 6 (4). The rabbit serum reacted in immunoblotting and ELISA with the fusion protein containing loop 6 of strain d1 but not of variant b4 (Fig. 1C), indicating a variant-specific anti-loop 6 antibody response. The rabbit serum bound to the loop 5 fusion protein of strain d1 as well as the loop 5 protein of variant b4, although loop 5 of variant b4 contained one changed amino acid (not shown).

Fine mapping of epitopes on the variable MOMP P2 loops. For more precise characterization of the epitopes on MOMP P2 loop 5 and loop 6, overlapping peptides of 12 amino acids, based on the amino acid sequences of loops 5 and 6 of strain d1 and variant d3, were synthesized for pepscan analysis. Since the variant d3-specific MAbs 30DA10, 30DA11, 30DA5, 30BD1, and 30DD1 were specific for loop 6 of variant d3, the reactivities of the peptides in pepscan were assessed with these MAbs. The pepscan analysis of MAb 30DD1 is shown in Fig. 2 as a representative example. MAb 30DD1 did not react with any of the peptides of loop 5 and loop 6 of strain d1 and did react with distinct peptides of loop 6 of variant d3. Similar reactions were observed with the other MAbs specific for variant d3. In Fig. 3 the amino acids involved in binding of the variant d3-specific MAbs to the peptides are indicated. The strongest binding occurred with the peptides TDSGDGRQT ITNP and DSGDGRQITNPA, both containing amino acids located along the side and at the tip of loop 6. The tip of loop 6 contains amino acids specific for loop 6 of variant d3 (four amino acids of the tip are underlined) (4) that are present in all the peptides, except one, binding the MAbs. Since the synthesized peptides reacted with MAbs raised with whole *H. influenzae* cells (30), they probably have the native epitope conformation, thereby validating the pepscan analysis for measuring specificity of antibodies against *H. influenzae* present in rabbit sera. All sera from nonvaccinated ($n = 3$) and vaccinated ($n = 6$) rabbits reacted with all the peptides of loop 5 with an optical density of ≤ 0.5, suggesting that the reactivity of these sera with the GST–loop 5 fusion proteins is not due to binding of antibodies to distinct peptides.

The antibodies in the sera of nonvaccinated rabbits inoculated with strain d1 reacted with seven peptides in the central region of loop 6 of strain d1, while no binding occurred with

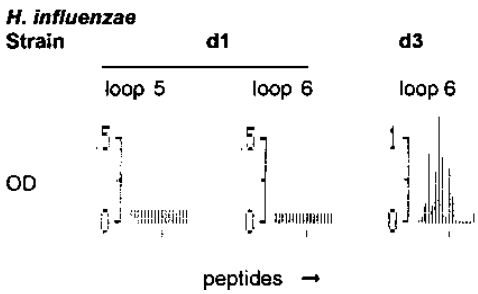

FIG. 2. Pepscan analysis with overlapping peptides consisting of 12 amino acids derived from loop 6 of MOMP P2 of *H. influenzae* d1 and d3. Individual ELISA reactivities of overlapping peptides with MAb 30BD1 are plotted. The MAb was tested at a 1:100 dilution. OD, A_{450}.

LOOP 6 variant d1	Rabbit sera 1	2	3	MAb	*LOOP 6 variant d3*	Rabbit sera 1	2	3	MAb
TTTDSSSDSQTI	-	-	++	-	TTTDSGDGRQTI	-	-	+	+
TTDSSSDSQTIT	+	+	++	-	TTDSGDGRQTIT	-	-	++	+
TDSSSDSQTITN	++	++	++	-	TDSGDGRQTITN	-	-	++	++
DSSSDSQTITNP	+	+	-	-	DSGDGRQTITNP	-	-	-	++
SSSDSQTITNPA	++	++	-	-	SGDGRQTITNPA	-	-	-	-
SSDSQTITNPAY	+	+	-	-	GDGRQTITNPAY	-	-	-	+
SDSQTITNPAYD	+	+	-	-	DGRQTITNPAYD	-	-	-	+
DSQTITNPAYDE	+	+	-	-	GRQTITNPAYDE	-	-	-	+
SQTITNPAYDEK	-	-	+	-	RQTITNPAYDEK	-	-	+	-
QTITNPAYDEKR	-	-	++	-	QTITNPAYDEKR	-	-	++	-
TITNPAYDEKRS	-	-	++	-	TITNPAYDEKRS	-	-	++	-
ITNPAYDEKRSF	-	-	+	-	ITNPAYDEKRSF	-	-	+	-

FIG. 3. Reactivities of rabbit sera with synthetic peptides of MOMP P2 loop 5 and loop 6 determined by ELISA. Rabbits were either inoculated with strain d1 (nonvaccinated; $n = 3$), vaccinated with the homologous strain d1 ($n = 3$), or vaccinated with variant d3 ($n = 3$) (columns 1 to 3, respectively). ELISA values are expressed semiquantatively: $-$, $A_{450} < 0.5$; $+$, $0.5 < A_{450} < 1.0$; $++$, $A_{450} > 1.0$. MAb 30BD1 reactivities are indicated; the same results were obtained with MAbs 30DA10, 30DA11, 30DA5, and 30DD1, specific for loop 6 of variant d3 (4, 30).

loop 6 of variant d3 (Fig. 3). Sera from rabbits vaccinated with strain d1 reacted with the peptides of loop 6 of strain d1 and not with those of variant d3. Sera from rabbits vaccinated with variant d3 cross-reacted with the peptides of loop 6 of d1 and d3, containing identical amino acids along the side of the loop. In rabbits inoculated and vaccinated with *H. influenzae* d1, strain-specific antibodies reacting with peptides containing strain d1-specific amino acids located in the tip of the loop 6 were elicited. These results indicate that after inoculation of the rabbits with strain d1 and vaccination with variant d3, antibodies were elicited against peptides containing amino acids located along the side of loop 6 which are shared by the two *H. influenzae* variants.

Bactericidal activity of the antibodies directed against MOMP P2 loop 5 and loop 6. The most important immune mechanisms of the host against bacteria are complement-dependent bactericidal activity and phagocytosis. For *H. influenzae*, both of these mechanisms are dependent on complement activation (19, 26, 30). The complement-dependent bactericidal activities of the antibodies elicited during persistence of *H. influenzae* in the rabbit model were determined. In a previous paper Vogel et al. (32) showed that serum from nonvaccinated rabbits had no bactericidal activity in the presence of complement and that, after vaccination with *H. influenzae*, strain-specific bactericidal antibodies were detected. Since the sera from these rabbits showed similar antibody reactivities (Table 3) and bactericidal activities (32), a representative serum sample from one of these rabbits was taken for this analysis. Since only antibodies specific for loop 5 and loop 6 were found, we focused on the effect of serum depletion of antibodies specific for loops 5 and 6 on the bactericidal activity. The results are summarized in Table 4. The analyzed serum strongly reacted with loop 5 and loop 6 of MOMP P2 (Fig. 1C) and killed *H. influenzae* d1 effectively in the presence of complement within 60 min. In the absence of complement no killing was observed. After this serum was run over a column with GST–loop 5, almost all anti-loop 5 antibodies remained bound to the column. Unadsorbed serum and serum adsorbed for loop 5 antibodies had similar bactericidal activities. In contrast, adsorption of antibodies specific for loop 6 resulted in a decline of bactericidal activity. The amount of residual antibodies specific for loop 6 was roughly related to the reduction in the bactericidal activity (Table 4).

Pepscan analysis of the serum adsorbed with loop 5 showed reaction with the same peptides of loop 6 as the unadsorbed serum. Similarly, the serum adsorbed with loop 6 reacted with the same peptides of loop 5 as the unadsorbed serum. All the analyzed sera were not bactericidal for the heterologous *H. influenzae* variant d3, confirming strain specificity of the loop 6-specific bactericidal antibodies. Vogel et al. (32) showed that these sera had no bactericidal activity for MOMP P2 variants appearing in the rabbit model later on.

These data show that serum depleted for antibodies to loop 5 had no effect on the complement-dependent bactericidal activity in contrast to serum depleted for antibodies to loop 6. Most of the bactericidal antibodies in unabsorbed serum containing *H. influenzae* d1 antibodies showed, therefore, strain specificity for loop 6.

DISCUSSION

MOMP P2 antigenic variants of *H. influenzae* appear during persistent infections in chronic bronchitis patients (7, 8). These variants showed antigenic drift in MOMP P2 (9). Similar antigenic drift of MOMP P2 was observed in an animal model in which *H. influenzae* persisted in subcutaneous cages in rabbits (32). Both in *H. influenzae* variants obtained from patients and in those obtained from the rabbit model the MOMP P2 variation was located in the surface-exposed loops of this MOMP, especially loop 6, which is recognized by bactericidal antibodies (4, 30, 32).

The specificities of MOMP P2 strain and variant-specific antibodies were analyzed with GST fusion proteins and synthetic peptides. The presence of MAb against the variable loops and antibodies in sera from the rabbit model showed that

TABLE 4. Complement-dependent bactericidal activities of adsorbed serum from a rabbit vaccinated with nonencapsulated *H. influenzae* d1 against the homologous *H. influenzae* strain

Adsorption[a]	Antibodies (%) to:		% Killing[b]			Killing index[c]
	Loop 5	Loop 6	With complement		No complement (30 min)	
			30 min	60 min		
None	100	100	59 ± 4	100 ± 0	0	1.0
Loop 5	11	100	56 ± 19	100 ± 0	0	0.97
Loop 6	100	75	47 ± 3	98 ± 2	0	0.62
Loop 6	100	42	7 ± 7	98 ± 2	0	0.12

[a] By GST-loop fusion protein affinity chromatography.
[b] 100 − average CFU at 30 or 60 min/average CFU at 0 min × 100. The data are means of triplicate experiments.
[c] Killing for nonadsorbed sera was assigned a value of 1.0.

the fusion proteins containing certain loops of MOMP P2 exposed at the outside of the outer membrane had a composition and conformation which still reacted with antibodies raised against bacteria (Fig. 1). The cross-reacting MAbs not binding to the loop fusion proteins may recognize conformational epitopes of MOMP P2 or bind to extended sequences not present in the MOMP P2 fusion proteins. The synthetic peptides of loop 5 and loop 6 had a conformation similar to that of the GST-MOMP P2 fusion proteins, since rabbit antibodies reacting with the fusion protein also reacted with the synthetic peptides derived from these loops. The major difference between these two methods was that the sensitivity of the pepscan analysis was higher than that of the ELISA with fusion proteins (Fig. 3; Table 3). From these data we are rather confident that our analysis detected a variety of antibodies specific for MOMP P2.

Sera from rabbits inoculated with strain d1 reacted with fusion proteins of MOMP P2 loop 5, but weakly with loop 6 of the homologous strain, and not with any of the other loops. With pepscan analysis using synthetic peptides of loop 5 of strain d1, the rabbit sera appeared to react with all the peptides of loop 5, suggesting either strong antigenicity of this loop or aspecific binding of immunoglobulins to these peptides. Aspecific binding of immunoglobulins is not very likely, since after vaccination of rabbits with the strain used to inoculate the rabbit cage model the reactivity with loop 5 increased. In addition, other groups have also demonstrated strong immune response to loop 5 (11, 34).

Pepscan analysis revealed that antibodies elicited to MOMP P2 loop 6 of *H. influenzae* during bacterial persistence in rabbits reacted with peptides containing amino acids located along the side of the loop as well as the tip of loop 6. After vaccination with strain d1, used to inoculate rabbits, the generation of strain-specific antibodies continued, since they did not react with the peptides derived from variant d3 differing in amino acids on the tip of loop 6 (Fig. 3). Vaccination with the heterologous strain of strain d1-inoculated rabbits resulted in the appearance of cross-reactive antibodies, binding to peptides covering the sides of loop 6 of strain d1 as well as d3. These peptides have identical amino acid sequences. These data indicate that triggering the immune response with a heterologous strain results in cross-reacting anti-loop 6 antibodies.

Although the pepscan data showed that the epitope of the variant-specific MAbs and the antibodies elicited in rabbits are contained within a linear epitope in loop 6, we cannot exclude the possibility that the epitope includes additional amino acids located elsewhere in MOMP P2. The amino acids essential for the binding of strain-specific antibodies may be defined by using intact MOMP P2 proteins in which amino acids are systematically deleted or replaced.

The complement-mediated bactericidal activity of rabbit serum decreased by adsorption with fusion proteins with loop 6 but not after adsorption with loop 5 fusion proteins. This implies that in this serum the loop 6-specific antibodies are the predominant antibodies in promoting antibody- and complement-mediated killing of *H. influenzae*. Antibodies specific for loop 6 require activation of complement to promote lysis of the bacteria (32). Bactericidal antibodies are elicited mainly after vaccination of the rabbits (32), indicating that bacterial persistence is poorly stimulating these loop 6-specific bactericidal antibodies. The contribution of other antibodies to the bactericidal activity of the serum cannot be excluded. However, the strain-specific anti-loop 6 antibodies raised during persistent infection in rabbits are of major importance for immune selection, since rabbit serum had no bactericidal activity against MOMP P2 variants, appearing in the rabbits, containing amino acid alterations in loop 6 (32). Also, the observations that MAbs specific for loop 6 promoted strain-specific complement-dependent killing of *H. influenzae* in the absence of other antibodies and that a variant with an amino acid alteration in loop 6 survived during selection in the presence of this MAb and complement support the immune selection by antibodies specific only for loop 6 (4, 30). During infection by *H. influenzae* strain-specific opsonizing antibodies also develop (19, 26). Opsonophagocytosis of nonencapsulated *H. influenzae* is strain specific (26) and has been described to be poor (33). In addition, in subcutaneous tissue cages phagocytosis was shown to be impaired (35). Therefore, the stimulation of immune selection of variants by opsonizing antibodies in rabbits may be of less importance than stimulation by bactericidal antibodies. However, during chronic infection in patients with *H. influenzae*, opsonizing antibodies may very well contribute to immune selection of *H. influenzae* variants.

The strong effect of the single amino acid changes in loop 6 on the reactivity with bactericidal MAb and rabbit antibodies is striking. The changes at the tip of loop 6 involve amino acids with a helix turn capacity and charge (SSDS [d1]→GDGR [d3]→SSGN [b4]). Therefore, amino acid changes in loop 6 strongly affect the conformation of the loop. In contrast, in loop 5 the turn-inducing amino acids are randomly distributed and most changes are neutral (4).

Haase et al. (11) described bactericidal MAbs specific for loop 5 and loop 8. Yi and Murphy (34) also reported bactericidal MAb specific for loop 5 and showed that a mutant with minor amino acid changes in loop 5 lost bactericidal activity. Combining our data with their results, it appears that the bactericidal antibodies were in all cases specific for the longest exposed loop in the MOMP P2 molecule; this is loop 6 of the MOMP P2 of *H. influenzae* strains described in this paper and loops 5 and 8 in the strains analyzed by Haase et al. (11) and Yi and Murphy (34). Therefore, it appears that *H. influenzae* strains vary in terms of which loop is the longest loop of MOMP P2 and that only the longest extended loop enhances proper assembly of complement into the bacterial cell envelope.

There are now a number of examples of protein epitopes in which single mutations and localization of the epitope affect the biological activity of antibodies. These include MOMP P2 of *H. influenzae*, the gp160 loop of HIV-1 (13), and the class 1 protein of *Neisseria meningitidis* (14, 20). A striking observation was that all DNA point mutations in the MOMP P2 gene resulted in amino acid substitutions (nonsynonymous mutations). Since in bacterial genes in general only 1 of 20 random mutations is nonsynonymous, this predominance of nonsynonymous substitutions in the MOMP P2 gene indicates strong selection for amino acid changes (4).

Concluding, antibodies to MOMP P2 loop 5 appeared commonly cross-reactive and not bactericidal. In contrast, antibodies to MOMP P2 loop 6 were strain specific and promoted a bactericidal effect. The specific bactericidal antibodies which develop following persistent infection are obviously not enough to facilitate eradication of nonencapsulated *H. influenzae* but may promote the selection of escape mutants with changes in the surface-exposed strain-specific epitopes during persistence in patients with chronic bronchitis.

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