Mapping of a Surface-Exposed, Conformational Epitope of the P6 Protein of *Haemophilus influenzae*

JOHN A. BOGDAN, JR.,¹ AND MICHAEL A. APICELLA^{2*}

Departments of Medicine and Microbiology, School of Medicine and Biomedical Sciences, State University of New York at Buffalo, Buffalo, New York 14215,¹ and Department of Microbiology, University of Iowa College of Medicine, Iowa City, Iowa 52242²

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P6 is an outer membrane protein of *Haemophilus influenzae* that is antigenically conserved and considered a candidate component of future *H. influenzae* vaccines. P6 contains a surface-exposed epitope recognized by monoclonal antibody (MAb) 3B9. This epitope has been shown to be distinct from that recognized by the P6-specific MAbs 7F3 and 4G4 in a competitive inhibition enzyme-linked immunosorbent assay (ELISA). MAb 3B9 did not bind to synthetic P6-specific sequential and overlapping hexameric peptides. Five peptides made to correspond to P6 sequences with high probabilities of surface exposure did not inhibit binding of MAb 3B9 to P6. An antiserum to one of the peptides, designated SP66, inhibited binding of MAb 3B9 to P6. A rabbit antiserum to P6 bound to sequential hexameric peptides, Gly-87AsnThrAspGluArgGlyThr-94, which were in the SP66 region of P6. This antiserum inhibited the binding of P6 to MAb 3B9 in a competitive inhibition ELISA. P6 mutations with His and Ala substitutions at residues Thr-88 and Asn-89 still bound MAb 3B9. MAb 3B9 reacted with *Escherichia coli* OmpA and *Salmonella typhimurium* OmpA. Sequence comparisons of P6 with these proteins indicated that the residue in the SP66 region responsible for binding is either Gly-87, Asp-90, or Gly-93. Mercaptoethanol reduction abolished MAb 3B9 binding to *E. coli* OmpA and *S. typhimurium* OmpA. In these proteins, immediately downstream of the second cysteine, there is an ArgArg dipeptide which is identical to and aligns with Arg-147Arg-148 in P6. This dipeptide has a high probability of surface exposure in P6. Mutagenesis of the Arg-147Arg-148 to an AlaAla dipeptide in P6 abolished binding of MAb 3B9, demonstrating that it was either a portion of the epitope or important in the protein folding necessary for expression of this epitope. This study demonstrates that MAb 3B9 recognizes a conserved conformational determinant on the surface of *H. influenzae* that is composed of two discontinuous regions of P6.

Haemophilus influenzae is a common cause of childhood infections. *H. influenzae* type b is a cause of childhood meningitis, while nontypeable *H. influenzae* (NTHi) is the etiologic agent in 20 to 40% of middle ear infections (11, 12, 33). In less-well-developed countries, *H. influenzae* can be a significant cause of lower respiratory tract infection (31). The ability to generate protective antibodies in infants by use of protein conjugates of the type b polyribosyl-ribitol capsular polysaccharides has substantially reduced the incidence of *H. influenzae* type b meningitis in infants. This vaccine does not prevent disease secondary to NTHi infection, and the somatic antigens in the cell wall have been studied as possible vaccine candidates (4, 18). The P6 protein of *H. influenzae* is a candidate component for such a vaccine (43). It is a highly conserved 16.6-kDa outer membrane protein that has been found in all *H. influenzae* type b and NTHi strains tested (8, 34, 37, 39). Antibodies directed against P6 are important in the immune response to these organisms (16, 17, 32).

The P6 protein contains regions which have sequence homology to the peptidoglycan-associated lipoprotein (Pal) of *Escherichia coli* (7). *E. coli* Pal is important in maintaining the structural integrity of the outer membrane. P6 may perform a similar function in *H. influenzae* (8). P6 also has homology with a region in the carboxyl termini of the OmpA proteins of a number of gram-negative bacteria. In addition, amino acids 68 to 88 of P6 contain a helix-turn-helix sequence that is in good

agreement with the consensus sequence shared by the family of helix-turn-helix DNA-binding proteins. Recent studies have shown that P6 binds to its own gene and may regulate its own expression (44).

Portions of P6 are exposed on the bacterial cell surface (37, 38). Monoclonal antibody (MAb) 3B9 recognizes such an epitope. Studies of acute- and convalescent-phase sera from children with meningitis and middle ear infections caused by *H. influenzae* indicate that an antibody to the MAb 3B9 epitope evolves during infection. Understanding the nature of this epitope may be important in understanding the nature of the P6 protective response in human infection. In this study, we have shown that MAb 3B9 binds to a discontinuous epitope consisting of either Gly-87, Asp-90, or Gly-93 and the Arg-147 and Arg-148 residues of P6.

MATERIALS AND METHODS

Bacterial strains and recombinant constructions. NTHi strains 3198, 5657, 3524, and 7502 have been previously described (34). *Neisseria gonorrhoeae* 1291 was obtained from our own collection. A strain of *Bordetella avium* was obtained from Claudia Gentry-Weeks, Food and Drug Administration, Bethesda, Md., and a strain of *Pseudomonas aeruginosa* was obtained from Eileen Rawling, University of British Columbia, Vancouver, British Columbia, Canada. A *Salmonella typhimurium* LT2 strain was obtained from Kenneth E. Sanderson, University of Calgary, Calgary, Alberta, Canada. Plasmids expressing *E. coli* OmpA deletion mutations in an *ompA* mutant (UH203) of *E. coli* (21, 23, 29) were obtained from Ulf Henning, Max-Planck Institute for Biology, Tübingen, Germany. Strain UH203 was made competent by the method of Hanahan (described in reference 30) and transformed with *ompA*-containing plasmids (6, 13). A plasmid containing a 550-bp insert encoding the P6 gene, designated pUB100, was provided by Stanley Spinola, University of Indiana College of Medicine, and was constructed by placing the *Bam*HI fragment of pBUD5 (39) into the *Bam*HI site of pGem 3Zf+ (Promega, Madison, Wis.) and transformed into *E. coli* DH5αF'.

* Corresponding author. Mailing address: Department of Microbiology, University of Iowa College of Medicine, Iowa City, IA 52242. Phone: (319) 335-7807. Fax: (319) 335-9006. Electronic mail address: Michael.Apicella@uiowa.edu.

Synthesis of P6 peptides and development of polyclonal antisera. P6 peptides were synthesized at the Center for Applied Molecular Biology and Immunology, State University of New York at Buffalo, and by Bio-Synthesis Inc., Lewisville, Tex. (see Fig. 3A). These peptides were designated SP65 (F-36GGTSVAD LOQYNTVYFGF-55), SP66 (K-82VLVEGNTDERGTPEYNIAL-101), SP66a (K-82VLVEGNTDERGTPEYNIALGQRRADAVKGYL-113), SP67 (G-115K GVDAGKGLTGSYG-129), and SP68 (S-144KNRRVAVLAY-153). A P6-specific antiserum was developed by injecting rabbits with 50 µg of purified P6 emulsified in an equal volume of incomplete Freund's adjuvant in phosphate-buffered saline (PBS) (38). Rabbit anti-*E. coli* OmpA serum was provided by Milan Blake, The Rockefeller University. Rabbit antipeptide sera were developed by immunizing rabbits with 50 µg of SP65, SP66, or SP67 peptide by using the Ribi adjuvant system (Ribi Immunochem Research, Inc., Hamilton, Mont.) according to the manufacturer's instructions.

Synthesis and analysis of P6 overlapping peptides. Sequential and overlapping hexameric peptides of P6 were synthesized on plastic pins (Commonwealth Serum Laboratories, Victoria, Australia) and analyzed according to the manufacturer's instructions. In enzyme-linked immunosorbent assays (ELISAs), these P6 peptides were incubated with either rabbit anti-P6 serum (1:1,000), preimmune rabbit serum (1:1,000), or MAb 3B9 ascites (1:100). A_{405} s were determined on a Microtek ELISA reader (Bio-Tek Instruments Inc., Winooski, Vt.).

Immunoelectron microscopy. NTHi strains were suspended in distilled H₂O, applied to Formvar-coated nickel grids (Ladd Research Industries, Burlington, Vt.), and allowed to air dry. Undiluted MAb tissue culture supernatant was applied to the grids and allowed to incubate for 30 min at 37°C. The grids were then washed with PBS (pH 8.0) and incubated with goat anti-mouse immunoglobulin conjugated to 10-nm-diameter colloidal gold beads (Janssen Biotechnology, Piscataway, N.J.) according to the manufacturer's instructions. The grids were washed with PBS, and specimens were visualized under a Siemens Elmiskop 1A transmission electron microscope at an accelerating voltage of 80 kV (3).

MAbs. Murine MAb 3B9, made to NTHi strain 3198, and MAbs 7F3 and 4G4, made to NTHi strain 3524, were developed by a modification (2) of the method of Kennett (22). Hybridomas reactive with NTHi strains 3198 and 3524 were screened by using a whole-organism immunodot assay (2). P6-specific MAbs were detected by Western blotting (immunoblotting) of whole-organism preparations (2). MAbs from murine ascitic fluid or tissue culture supernatant were affinity purified with a cyanogen bromide-staphylococcal protein A-Sepharose 4B column (Pharmacia, Piscataway, N.J.) containing protein A purified from Cowan 1 *Staphylococcus aureus* (Sigma, St. Louis, Mo.).

CIELISAs. Purified P6 was isolated from NTHi strain 3524 by the method of Murphy et al. (35, 38) or Zlotnick et al. (46). All protein concentrations were determined by the method of Lowry et al. (27). P6 was biotinylated with *N*-hydroxysuccinimidobiotin (Sigma). A competitive inhibition ELISA (CIELISA) was designed to test the ability of antibodies to compete for binding of soluble biotinylated P6 (BioP6) to a plate-bound MAb. Antigen-containing plates were prepared by coating microtiter wells with 1 µg of each MAb diluted in sodium carbonate buffer (pH 9.0). The plates were placed at 4°C overnight and washed three times with 0.01 M PBS, pH 7.0. The plates were blocked with 3% gelatin in PBS for 1 h at 37°C. A standard curve of binding of BioP6 was established for each of the MAbs by addition of increasing amounts of BioP6 diluted in PBS and 0.5% gelatin to microtiter wells coated with 1 µg of MAb. The concentration of BioP6 that was fourfold lower than the saturation point was chosen for use in all inhibition assays. CIELISA was performed to determine whether MAbs 3B9 and 7F3 bound the same epitope on P6. To determine if immune rabbit sera contained antibodies that inhibited binding of BioP6 to MAbs 3B9 and 7F3, BioP6 was preincubated with twofold dilutions of these antisera, starting at a dilution of 1:100, for 4 h at room temperature and then added to the plate-bound MAb. The plates were washed three times with PBS containing 0.5% Tween 20. Bound BioP6 was detected with avidin-peroxidase (Organon Teknica Corp., West Chester, Pa.) diluted 1:6,000 in PBS plus 0.5% gelatin, incubated in the wells for 1 h at room temperature, and developed with *o*-phenyldiamine dihydrochloride (Sigma) in 0.1 M citric acid and 0.2 M sodium phosphate dibasic. The reaction was catalyzed by the addition of 0.003% hydrogen peroxide. The reaction was stopped after 30 min by the addition of sulfuric acid to a final concentration of 1 N (vol/vol). The optical densities were measured in triplicate at 490 nm on a Microtek ELISA reader (Bio-Tek Instruments Inc.) and converted to percent inhibitions.

Statistical analysis of the CIELISA. To standardize the CIELISA system, statistical analysis was performed on results of repetitive assays for binding of BioP6 to plate-bound MAbs. Ninety-five percent confidence limits for the control and experimental values were calculated by using a Macintosh Classic computer and a Statview 512+ program (Brainpower, Inc., Ventura, Calif.).

Whole-cell ELISA. Whole-cell ELISA was performed according to the method of Abdillahi and Poolman (1).

SDS-PAGE and Western blot. Whole-organism preparations for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) were made in the absence or presence of 2-mercaptoethanol (2-ME) as previously described (39). In Western blots, the nitrocellulose was blocked with 5% bovine serum albumin (BSA) or 2% nonfat dry milk in buffer A (39) and then probed either with undiluted MAb 3B9 in tissue culture fluid or with diluted mouse murine ascites fluid or rabbit polyclonal sera diluted in 5% BSA or 2% nonfat dry milk in buffer A. Bound antibody was detected with either protein A-peroxidase (Kirkegaard &

FIG. 1. CIELISA results which demonstrate that MAb 3B9 (●) has a binding site on P6 different from those of anti-P6 MAbs 4G4 (□) and 7F3 (■). Ascites derived from SP2 cells (○) was used as a negative control. In this assay, MAb 3B9 is bound to the microtiter plate, and inhibition of binding of BioP6 by the three MAbs is measured. The binding of BioP6 was completely inhibited by incubation with 0.05 µg of purified MAb 3B9, while purified MAbs 4G4 and 7F3 failed to inhibit at amounts as high as 1 µg and were not different from the SP2 control. Each datum point is the mean of triplicate measurements. The bars represent the 95% confidence limits of these measurements.

Perry Laboratories, Inc., Gaithersburg, Md.), sheep anti-mouse immunoglobulin G-peroxidase (Amersham Life Sciences, Arlington Heights, Ill.), or goat anti-rabbit immunoglobulin G-peroxidase (Gibco-BRL, Gaithersburg, Md.). Western blots were developed with horseradish peroxidase conjugate (Bio-Rad Laboratories, Hercules, Calif.) or Renaissance chemiluminescence reagent (DuPont NEN, Boston, Mass.) according to the manufacturers' instructions.

P6 sequence comparisons. The amino acid sequences of *H. influenzae* P6 (Heaompp6; accession number M19391), *E. coli* OmpA (Ecompa; accession number V00307), *S. typhimurium* OmpA (Stompa; accession number X02006), *N. gonorrhoeae* Rmp (NgompIII; accession number X05105), and *B. avium* OmpA (Bpeompa; accession number M96550) were compared by using Pileup and displayed with the Pretty program (9, 10, 26, 28).

Site-directed mutagenesis. Site-directed mutagenesis of the P6 gene in pBUD5 was performed by using the PCR site-directed mutagenesis system from Life Technologies (Gaithersburg, Md.) according to the manufacturer's instructions. P6 amino acids Asn-88 and Thr-89 were changed to His-88 and Ala-89, respectively, by PCR with the oligonucleotides 5' UAG ACG UAC UGG AAG ATG ATT ATG AAA TCG 3' and 5' CCA GUA CGU CUA CTT GCA GCA CCA TGT 3'. P6 amino acids Arg-147 and Arg-148 were changed to Ala-147 and Ala-148 by PCR with the oligonucleotides 5' AUG ACG UCG ACG UAA AAA TCT TAT ACG TCG 3' and 5' ACG UCG ACG UCA UAA TCG CAT GAT TAA GAA C 3'. P6 mutants were cloned into pUC18 and confirmed by double-stranded sequencing. The resulting mutants were expressed in *E. coli* DH5αF'. P6 mutants were solubilized and analyzed by Western blots with probing with MAb 3B9 and rabbit anti-P6 serum.

RESULTS

Characterization of the 3B9 epitope of *H. influenzae*. The conservation of P6 among strains of the *Haemophilus* species as determined by using MAb 7F3 and MAb 4G4 has been previously described (34, 36). We have demonstrated that MAb 3B9 binds to *H. influenzae* P6 on Western blots (data not shown) and that strains from all members of the genus *Haemophilus* reacted with MAb 3B9 in immunodot assays (except *H. parainfluenzae*, which does not contain this protein) (data not shown). In CIELISA, MAb 3B9 inhibited binding of BioP6 to plate-bound MAb 3B9, while MAb 4G4 and MAb 7F3 did not (Fig. 1). When MAb 7F3 and MAb 4G4 were bound to plates, each completely inhibited binding of BioP6 to the other.

FIG. 2. Immunoelectron microscopic analysis of NTHi strain 3524 probed with MAb 3B9. Goat anti-murine immunoglobulin G conjugated to 10-nm-diameter gold beads was used to identify the sites of MAb 3B9 binding. Bar, approximately 1 μ m.

MAb 3B9 failed to inhibit the binding of BioP6 to either MAb 7F3 or 4G4 (data not shown). This suggested that MABs 7F3 and 4G4 were binding to the same or a closely related P6 epitope, while MAB 3B9 bound to a distinct P6 epitope. A P6-specific rabbit antiserum inhibited binding of MAB 3B9 in CIELISA, indicating that this serum contained antibodies with specificities similar to that of MAB 3B9 (data not shown).

Immunofluorescence analysis of NTHi strains 3524, 3198, 2019, 7502, and 5657 with MAB 3B9 indicated that the 3B9 epitope was surface exposed. Immunoelectron microscopic studies confirmed the surface accessibility of the MAB 3B9 epitope of P6 in NTHi strain 3524 (Fig. 2). We have also performed whole-cell ELISA studies with NTHi strains 2019 and 3524 and have demonstrated the surface exposure of the MAB 3B9 epitope (data not shown).

In order to further define the specificity of MAB 3B9, we developed five P6-specific peptides (SP65, SP66, SP66a, SP67, and SP68) which had high probabilities of surface exposure on the basis of Kyte-Doolittle hydropathy (25) and surface probability plots of P6 (Fig. 3). SP66a was synthesized to include Gly-87ArgThrAsp-90 on the same peptide with the Arg-104Arg-105 region, which was immediately downstream. Both of these regions have high probabilities of surface exposure. We had considered the possibility that Gly-87ArgThrAsp-90 and Arg-104Arg105 might be the conformational components of the MAB 3B9 epitope. However, this peptide failed to inhibit MAB 3B9. MAB 3B9 did not bind to the five peptides in either CIELISA or direct ELISA (data not shown). Rabbit anti-peptide sera to three of the five surface-exposed P6-specific peptides (SP65, SP66 and SP67) were developed and used to inhibit binding of BioP6 to MAB 3B9 in CIELISAs. The rabbit anti-SP66 peptide serum inhibited binding of P6 to MAB 3B9 in CIELISA, while the preimmune sera did not (Fig. 4). Pre-immune and postimmune SP65 and SP67 peptide antisera did not inhibit binding of P6 to MAB 3B9.

FIG. 3. (A) The bars designate the domains of the five peptides which were generated to correspond to P6 regions with high probabilities of surface exposure. (B) Kyte-Doolittle hydropathy plot of P6 from residue 31 to 153. (C) Surface exposure probability analysis of P6 from residue 31 to 153.

FIG. 4. CIELISA results which demonstrate that rabbit antiserum to the SP66 peptide (■) inhibits the binding of BioP6 to MAb 3B9-coated microtiter wells. Preimmune serum (□) failed to inhibit this binding. Each datum point is the mean of triplicate measurements. The bars represent the 95% confidence limits of these measurements.

We tested sequential overlapping P6 hexameric peptides in a solid-phase epitope-mapping system for binding of MAb 3B9 and rabbit anti-P6 sera. MAb 3B9 did not react to any of the hexameric peptides. However, P6-specific rabbit antisera did react with selected peptides. The strongest reactivity was observed with two peptides in the SP66 peptide region with the overlapping sequences Gly-87AsnThrAspGluArgGlyThr-94 (data not shown). Five hexamer peptides near the carboxyl terminus also reacted, but much less strongly, with the rabbit anti-P6 antisera.

On the basis of these studies, we made the following assumptions about the 3B9 epitope. MAb 3B9 did not react either with peptides demonstrating a high probability of surface exposure or with sequential overlapping peptides spanning the entire protein. This was consistent with our opinion that the MAb 3B9 epitope was conformational. The SP66 region was considered to contain a portion of the MAb 3B9 epitope because rabbit anti-P6 sera bound to a portion of this peptide in the

display library and inhibited BioP6 binding to MAb 3B9, and finally, antisera to the SP66 peptide inhibited binding of MAb 3B9 to P6 in CIELISA.

An analysis of OmpA-like proteins, which have sequence homology with P6 and react with MAb 3B9, also suggests that the SP66 region contains a portion of the MAb 3B9 epitope. There is a high degree of homology between the carboxy termini of P6, *E. coli* OmpA, and *S. typhimurium* OmpA (Fig. 5). Four of the eight amino acids in the region of P6 from residue 87 to 94 align with and are identical to regions in the carboxy termini of *E. coli* OmpA and *S. typhimurium* OmpA (Fig. 5). MAb 3B9 does not bind the *E. coli* Pal protein. This protein has the sequence HisAla in the homologous Asn-88Thr-89 region. From this, we felt that replacement of Asn-88 with His and of Thr-89 with Ala would modify binding of MAb 3B9. This did not occur when this modification was made (Fig. 6, lanes 2). Three other amino acids in this region are identical in these three proteins: Gly-87, Asp-90, and Gly-93. Our studies suggest that one or more of these amino acids is part of the MAb 3B9 epitope.

E. coli OmpA has a disulfide bridge extending from Cys-311 to Cys-323. MAb 3B9 reacted with *E. coli* OmpA on a Western blot when the sample was prepared without 2-ME (Fig. 7). Reduction of *E. coli* OmpA with 2-ME abolished this binding. MAb 3B9 reacted with the heat-modifiable form of *E. coli* OmpA solubilized at 100°C (Fig. 7). MAb 3B9 also reacted with *S. typhimurium* OmpA in a Western blot under the same conditions (data not shown). These data suggest that the conformation necessary for MAb 3B9 reactivity is dependent upon disulfide bond formation, which may bring discontinuous portions of *E. coli* OmpA and *S. typhimurium* OmpA into position for binding. Deletion mutations of the carboxy terminus of *E. coli* OmpA, in the region of the disulfide bridge, abolished binding of MAb 3B9 (data not shown). P6 does not contain a disulfide bridge; thus, MAb 3B9 binding may be dependent upon folding of the P6 protein. On the basis of the 2-ME experiments and the deletion mutant studies, we assumed that the second portion of the MAb 3B9 epitope in *E. coli* OmpA was on the carboxy-terminal side of the disulfide bridge.

An area in this region of *E. coli* OmpA and *S. typhimurium* OmpA with a high probability of surface exposure and which was identical to and aligned to a region in P6 was the Arg-147 and Arg-148 residues immediately upstream of the second cysteine of the disulfide bridge (Fig. 5). To confirm the role of

Heaompp6	ATPAA--KVL	VEGNTDERGT	PEYNIALGQR	RADAVKGYLA	GKGVDAGKLG	124
Ecoompa	NLDPKDG-V-	VLGYTDRIGS	DAYNQGLSER	RAQSVVDYLI	SKGIPADKIS	296
stompa	NLDPKDG-V-	VLGSTDRIGA	DAYNQGLSEK	RAQSVVDYLI	SKGIPSDKIS	279
NgompIII	RTNVQ--SV-	VEGHTDFMGS	KYYNAYLSER	RAYVVANNLV	SNGVPASRIS	170
Heaompp6	TVSYGEEKPA	V-----	-----LG	HDEA--AY-S	KNRRAYLAY-	153
Ecoompa	ARGMGESNPV	TGNTCD----	-----NVK	QRAALIDCLA	PDRRVEIEVK	335
stompa	ARGMGESNPV	TGNTCD----	-----NVK	PRAALIDCLA	PDRRVEIEVK	317
NgompIII	AVGLGESQAQ	MTQVCQAEVA	KLGAKASKAK	KREALIACIE	PDRRVVDKIR	220

FIG. 5. Amino acid sequence alignments of P6 and other bacterial OmpA-like proteins. The shaded areas are the regions of the MAb 3B9 binding sites. The bar above the alignments spans the SP66 region. This alignment was generated by using Pileup and displayed with the Pretty program (9).

FIG. 6. Western blot demonstrating that the P6 His-88Ala-89 and Ala-147Ala-148 mutations are expressed in *E. coli* DH5 α (A) and that P6 containing the His-88Ala-89 mutation is recognized by MAb 3B9 while P6 with the Ala-147Ala-148 mutation is not (B). (A) Western blot probed with rabbit anti-P6 sera; (B) blot probed with MAb 3B9. Because of the reduced expression of the P6 Ala-147Ala-148 mutation, all samples were overloaded to confirm that P6 with the Ala-147Ala-148 mutation is produced in DH5 α . Lane 1, whole-cell lysate of *E. coli* DH5 α expressing wild-type P6 in a pUC plasmid; 2, lysate of *E. coli* DH5 α expressing the P6 His-88Ala-89 mutation in pUC18; 3, lysate of *E. coli* DH5 α expressing the P6 Ala-147Ala-148 mutation in pUC18; 4, lysate of *E. coli* DH5 α containing the pUC18 plasmid alone. The arrow on the right designates the position of P6. Positions of low-molecular-mass standards are shown in kilodaltons at the left.

the Arg-147Arg-148 residues, we replaced these basic amino acid residues in P6 with the uncharged amino acid Ala. P6 containing the Ala-147Ala-148 mutation showed reduced binding to rabbit anti-P6 serum (Fig. 6A, lane 3, lower band) and did not bind MAb 3B9 (Fig. 6B, lane 3), suggesting that Arg-147Arg-148 is part of the 3B9 epitope.

There is sequence homology between the *B. avium* OmpA-like protein, *N. gonorrhoeae* Rmp (15), and the carboxy termini of P6, *E. coli* OmpA, and *S. typhimurium* OmpA. MAb 3B9 does not react with either *B. avium* OmpA or *N. gonorrhoeae* Rmp (data not shown). *B. avium* OmpA lacks the disulfide bridge present in *E. coli* OmpA and *S. typhimurium* OmpA. This may prevent approximation of the MAb 3B9 epitope. *N.*

FIG. 7. Western blot of *E. coli* DH5 α F' solubilized without 2-ME (lanes 1 and 3) and with 2-ME (lanes 2 and 4) at 37°C (lanes 1 and 2) and 100°C (lanes 3 and 4) and probed with MAb 3B9. Positions of low-molecular-mass standards are shown in kilodaltons at the left.

gonorrhoeae Rmp contains a disulfide bridge similar to those of *E. coli* OmpA and *S. typhimurium* OmpA. It has 12 additional amino acids between the cysteines, which may prevent formation of the MAb 3B9 epitope.

DISCUSSION

P6 is a lipoprotein that makes up about 1 to 5% of the outer membrane proteins in *H. influenzae* (32, 34). Our inability to construct an *H. influenzae* P6 mutant suggests that P6 may be important for survival of the organism (40, 41, 44). The function of antibodies to P6 in protective immunity has recently been explored by several investigators. Harabuchi and coworkers have shown that the frequencies of nasopharyngeal carriage of NTHi (19) and of otitis media due to NTHi are inversely related to the level of anti-P6 secretory antibody (19, 20). Prevention of colonization was most evident during breast-feeding (19, 20). These studies suggest that P6 may be useful as a vaccine component for the prevention of otitis media due to NTHi. Mapping of antigenic and immunogenic sites on P6 has been performed by using a rabbit anti-P6 serum with icosapeptides and dodecapeptides conjugated to *N*-palmitoyl-S-[2,3(bis-palmitoyloxy)-(2*RS*)-propyl]-(*R*)-cysteinyl-(*S*)-serine. These studies indicated that regions in the carboxy terminus of the protein were immunogenic. Antisera made to correspond to P6 peptide Q-47ILDAHAA-54 were able to neutralize the intact bacterium (5). The significance of the MAb 3B9 epitope in the protective efficacy elicited by anti-P6 antisera is difficult to determine at this time. We have performed bactericidal studies using MAb 3B9 with both mouse and human complement sources and have been unable to demonstrate a bactericidal effect (data not shown). We have studied MAb 3B9 for the potential to block the bactericidal effect of antibodies known to participate in complement-mediated lysis of NTHi and have been unable to show that the MAb 3B9 epitope generates a blocking antibody (data not shown) (42). The studies of Kyd and coworkers indicated that antibodies to SDS-PAGE-purified P6 enhanced pulmonary clearance of NTHi in a rat infection model (24). The surface-exposed nature of the MAb 3B9 epitope may make it a potential candidate for binding of the antibodies involved in pulmonary clearance. However, additional studies are necessary to determine the role of this P6 epitope in pulmonary clearance.

CIELISA studies of acute- and convalescent-phase sera from children recovering from otitis media due to NTHi and from Hib meningitis indicated that over 70% of the children developed a significant serum antibody response to the MAb 3B9 epitope (data not shown). This observation and the fact that the MAb 3B9 epitope was exposed on the surface of NTHi (Fig. 2) has prompted us to further define this epitope.

This epitope is conserved on analogous proteins throughout *Haemophilus* species as well as in the family *Pasteurellaceae*, as MAb 3B9 reacts with an 18-kDa protein of *Haemophilus ducreyi*, whereas the anti-P6 MAb 7F3 and 4G4 do not (45). The sequence of this protein has recently been completed. The protein is very similar in size and has a high degree of amino acid homology to P6. It contains amino acids which align to positions 87, 90, 93, 147, and 148 that are identical to those in P6 (44a). MAb 3B9 binds to P6 in all NTHi biotype 4 strains tested, unlike MAb 7F3 (43a). The amino acid sequence of P6 in NTHi biotype 4 strains has five differences, at Ala-33, Ala-42, Asp-59, Thr-61, and Ala-152 (36). These results suggest that these amino acids do not participate in binding of MAb 3B9. CIELISAs confirmed that MAb 3B9 binds to an epitope that is distinct from MAb 4G4 and MAb 7F3 (Fig. 2).

MAb 3B9 did not react to a series of overlapping P6

hexapeptides or to a series of five peptides synthesized to correspond to P6 regions which showed a high probability of surface exposure. However, an antiserum to one of the putative surface-exposed peptides, the SP66 peptide, inhibited the binding of MAb 3B9 to P6 in CIELISA. A polyclonal anti-P6 antiserum reacted most strongly with two overlapping hexamers in the SP66 region, and this antiserum inhibited P6 binding in CIELISA. These studies suggested that the MAb 3B9 epitope is conformational and that the amino acids in the Gly-87AsnThrAspGluArgGlyThr-94 portion of P6 contained a component of this epitope. Sequence analysis of OmpA proteins which had homology to P6 and reacted with MAb 3B9 suggested that either Gly-87, Asp-90, or Gly-93 in this region is a part of the MAb 3B9 epitope. *E. coli* Pal contains the sequence GlyHisAlaAsp in the region homologous to SP66. The rationale for the choice of the HisAla substitution for Asn-88Thr-89 was based on the fact that we had found that MAb 3B9 did not bind the Pal protein of *E. coli*. This led us to believe that substituting HisAla residues for the Asn-88Thr-89 residues in the SP66 region would disrupt binding of MAb 3B9. This did not occur. We subsequently learned (44a) that the 18-kDa protein of *H. ducreyi* to which MAb 3B9 binds also contains GlyHisAlaAsp in the SP66 region. This confirms our observation that AsnThr is not involved in MAb 3B9 binding.

Western blot analysis of *E. coli* ompA deletion mutants indicated that MAb 3B9 reacted to a similar epitope on the carboxyl terminus of *E. coli* OmpA. The MAb 3B9 epitope was present only in the nonreduced forms of *E. coli* OmpA and *S. typhimurium* OmpA, indicating that it was dependent on formation of a disulfide bridge. The only disulfide bridge in *E. coli* OmpA is found in the carboxyl terminus between Cys-311 and Cys-323. Immediately downstream of the disulfide bridge are ArgArg residues which align with similar residues in P6. We have shown that after replacement of the Arg-147Arg-148 residues with Ala-147Ala-148 in P6, MAb 3B9 binding to the modified protein is abolished. Computer analysis (9) of the SP66 peptide and Arg-147Arg-148 dipeptide regions of P6 by using Chou-Fasman secondary structure, Emini surface probability, and Jameson-Wolf antigenicity predictions confirms that these two regions have the potential to form a discontinuous epitope on the surface of *H. influenzae*. Near or within these regions are alpha-helices and a propensity for turns. The SP66 peptide region is flanked by two potential membrane-spanning domains. Between these domains is a region of high probability of surface exposure, antigenicity, and a strong propensity for turns, suggesting that the SP66 peptide region may loop out of the membrane and then turn back into the membrane. Similarly, the Arg-147Arg-148 dipeptide region may also loop out and back into the membrane. We speculate that proper folding of P6 allows Arg-147Arg-148 to exist in close association with the SP66 peptide region, thus forming the MAb 3B9 epitope.

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