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Characterization of a Major Hemagglutinin Protein from *Mycoplasma gallisepticum*

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Mycoplasma gallisepticum **cell membranes were used to immunize mice to produce monoclonal antibodies to cell surface proteins. Three monoclonal antibodies were chosen for further characterization. All three reacted in immunoblots with an *M. gallisepticum* protein band of M_r approximately 67,000 (designated pMGA). By using immunoelectron microscopy, pMGA was shown to be located on the cell surface. When *M. gallisepticum* whole cells were treated with up to 250 μg of trypsin per ml for 30 min, the only major protein lost from the cell surface as judged by sodium dodecyl sulfate-polyacrylamide gel electrophoresis followed by Western immunoblot transfer was pMGA. Two of the pMGA-specific monoclonal antibodies inhibited hemagglutination of chicken erythrocytes by *M. gallisepticum* S6, suggesting a role for pMGA in the attachment of *M. gallisepticum* to chicken erythrocytes. Sequencing the amino terminus of pMGA yielded 17 amino acids with no significant homology with the *Mycoplasma pneumoniae* attachment protein P1 or any other protein in the GenBank, Swiss-Prot, and EMBL data bases.**

The avian pathogen *Mycoplasma gallisepticum* is one of a number of mycoplasmas that cause respiratory disease. The physicochemical similarities of host cell receptors in *M. pneumoniae* (a cause of atypical pneumonia in humans) and *M. gallisepticum* have been noted previously (reviewed by Razin [20]). The organisms share a morphologically distinct region, a terminal structure located at one end of the cell which is physically involved in the pathogenicity of the organism by promoting intimate contact between the mycoplasma and the host cell. In both, mycoplasma attachment is mediated predominantly through sialic acid residues residing on the host cell (reviewed by Razin [20]). Roberts et al. (21) have recently shown that *M. pneumoniae* specifically attaches to sialic acids that are α2-3 linked. The process of adhesion in *M. pneumoniae* has been shown to involve a number of proteins (8, 17) including P1, a surface polypeptide of 170 kDa that is clustered at the tip structure (6).

The designation of P1 as the primary adhesion of *M. pneumoniae* was based on two major observations. First, treatment of intact *M. pneumoniae* cells with trypsin resulted in the selective disappearance of P1, as determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), with an accompanying reduction in attachment to tracheal organ explants (12). Second, monoclonal antibodies (MAbs) and monospecific polyclonal antiserum directed to P1 were shown to block attachment of *M. pneumoniae* to tracheal organ explants (11) and chicken erythrocytes (16), respectively, suggesting that these reagents block a specific P1 site(s) involved in attachment to host cell receptors. The P1 gene has been molecularly cloned (13, 23) and shown to encode a mature polypeptide of 1,577 amino acids, which is appended by an additional 59 amino acids located amino terminal to the start of the mature sequence.

The mechanisms responsible for the adherence of mycoplasmas to host cells are fundamental in understanding the disease process. This knowledge may lead to pharmacological and immunological strategies aimed at alleviating or preventing respiratory disease. For these reasons we chose to identify the adhesin molecules of *M. gallisepticum*. The strategy used to do this was to select MAbs reactive with plasma membrane structures of the pathogenic *M. gallisepticum* S6 and, by using a hemagglutination assay, to evaluate their ability to inhibit attachment of *M. gallisepticum* cells to erythrocytes. The MAbs were used to isolate and characterize a major *M. gallisepticum* adhesin, pMGA. Seventeen amino acids at the amino terminus of pMGA were determined by protein sequencing.

MATERIALS AND METHODS

Mycoplasma strain and culture conditions. To produce antigen for the enzyme-linked immunosorbent assay (ELISA) and immunization, we grew *M. gallisepticum* S6 in modified Frey's broth medium (7) supplemented with 10% fetal calf serum (Flow) (10). For growth of *M. gallisepticum* F and R, 10% swine serum was substituted for fetal calf serum. For growth of *M. synoviae*, 0.001% (wt/vol) NAD (Sigma) was also added to the fetal calf serum-containing medium. Cultures of each organism were grown to late log phase and harvested as described previously (10). The membrane fractions of *M. gallisepticum* and *M. synoviae* were prepared by sonication and by osmotic lysis, respectively. The protein concentrations in each membrane solution were determined by the method of Hartree (9), and the final protein concentrations were adjusted to 1 mg/ml. The samples were aliquoted and stored at −70°C until needed.

Production of MAbs for species-specific epitopes of *M. gallisepticum*. Six BALB/c mice were given intraperitoneal inoculations with 1 mg of *M. gallisepticum* membrane suspension mixed 1:1 in Freund's complete adjuvant. Mice were given six more boosters (at approximately 1-week intervals) with 1 mg of *M. gallisepticum* antigen mixed 1:1 in Freund's incomplete adjuvant and finally injected intravenously with 1 mg of *M. gallisepticum* antigen without Freund's adjuvant. The mouse with the highest-titer serum as determined by ELISA was selected as the spleen donor. The donor mouse spleen cells were fused with mouse myeloma P3-NS-1/1-Ag-4-1 cells as described by Kohler and

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Milstein (15), using 50% polyethylene glycol. Supernatants from hybridoma colonies were screened by ELISA for reaction against *M. gallisepticum* and *M. synoviae*.

ELISA method used for determining species-specific MAb to *M. gallisepticum*. We used previously described ELISA procedures to monitor mouse immunoglobulin G (IgG) levels in serum and to screen hybridoma antibody production (10), with minor variations. When testing for mouse immune sera, 100-μl volumes of doubling dilutions of mouse serum were added to individual wells of an antigen-coated plate. Alternatively, 100 μl of neat hybridoma cell supernatant was added. The diluent used for mouse serum was 0.15 M sodium chloride–0.15 M sodium phosphate (pH 7.4) (NaOH) (PBS) containing 0.05% (vol/vol) Tween 20 (PBS-T). Reactivity to the ELISA antigens was detected by using rabbit anti-mouse Ig–horseradish peroxidase conjugate (Dakopatts) diluted 1:1,000 in PBS-T, and binding was detected by using chromogen 5-aminosalicylic acid.

Purification, labeling, and typing of MAbs. The isotype of each MAb was determined with a mouse MAb typing kit (Amersham) as specified by the manufacturer. Approximately 10 mg of selected MAbs was affinity purified by passing the cell-free supernatant over a protein A-conjugated Sepharose column as specified by the manufacturer (Pharmacia, Uppsala, Sweden). Approximately 5 mg of each purified MAb was conjugated to horseradish peroxidase (Sigma) by the method of Wilson and Nakane (26) and stored in 50% glycerol at −20°C.

Immunoaffinity chromatography. An affinity column was prepared by coupling 7 mg of MAb 86 to 2 g of cyanogen bromide-activated Sepharose 4B beads (Pharmacia) as specified by the manufacturer. *M. gallisepticum* cells were lysed for 30 min at 37°C in 10 mM Tris (pH 7.8) (HCl) containing 0.2% (wt/vol) sodium deoxycholate, 0.1% (wt/vol) SDS, 10 mM EDTA, 1% (vol/vol) Triton X-100 (Sigma), and 0.01 M iodoacetamide (Sigma). The lysate solution was spun at 100,000 × *g* for 1 h, and the supernatant was reacted with the MAb affinity column for at least 1 h. Unbound protein was washed from the column with 0.1 M Tris (pH 7.4) (HCl). The bound protein was eluted with 0.1 M acetic acid (pH 2.4) (NaOH). Aliquots were taken from the column and immediately neutralized to pH 7.4 with a pretitrated amount of 2 M Tris. After elution, the column was equilibrated in 0.1 M Tris buffer and stored at 4°C with the addition of 0.1% sodium azide. The eluate was dialyzed overnight at 4°C against PBS containing 0.1% sodium azide, and samples were examined by SDS-PAGE.

SDS-PAGE and immunoblotting. The system of Laemmli (18) was used for SDS-PAGE. *M. gallisepticum* and *M. synoviae* proteins were separated in a 10% (wt/vol) polyacrylamide gel for 5 h at 1.5 mA · cm^{-1}. Gels were stained with Coomassie brilliant blue (0.1% [wt/vol] in 50% methanol–10% acetic acid) and destained in 7% acetic acid. For Western immunoblot analysis, SDS-PAGE slabs, without staining or fixation, were transferred by the method of Towbin et al. (24) to an Immobilon (Millipore) membrane at 70 V overnight at 4°C with a Bio-Rad Western transfer instrument. The Immobilon membrane was then incubated with 5% (wt/vol) bovine serum albumin (BSA) overnight, cut into strips, and placed into troughs of a Perspex tray. The strips were washed three times (5 min each) in 1.5 ml of PBS-T containing 0.1% (wt/vol) BSA. Appropriate dilutions of MAbs were added, and the strips were incubated for 1 h with gentle rocking. The strips were washed as before, a dilution of 1:200 rabbit anti-mouse Ig–horseradish peroxidase in PBS-T containing 0.1% (wt/vol) BSA was added, and the mixture was incubated with rocking for 1 h. The strips were washed three times, and the conjugate binding was visualized by the addition of a substrate solution containing 0.17 mg of 3,3′-diaminobenzidine (Sigma) in 0.1 M citric acid (pH 5.0) (NaOH) containing 0.03% (vol/vol) H_2O_2. Color development was stopped by extensive washing with distilled water.

IEF. Approximately 5 μg of affinity-purified protein was focused for 4 h in an isoelectric focusing (IEF) gradient (pH 3.5 to 5.85) containing 2% Triton X-100 by using a Bio-Rad IEF minigel system. The second dimension was run in SDS-PAGE under reducing conditions and treated in the same manner for protein staining and Western transfer as described above.

Electron microscopy and immunostaining. *M. gallisepticum* was grown in Frey's broth medium and harvested when the medium pH had dropped to 6.9. The osmolarity of the growth medium (325 mosM) was measured by using an Advanced Instruments osmometer. To ensure that the organisms were maintained under similar osmolar conditions during fixation, we made a 4% (vol/vol) glutaraldehyde solution in double-glass-distilled water (DGDW). Harvested cells were fixed in 1.5 volumes of the 4% (vol/vol) glutaraldehyde solution at 4°C for 2 h. The fixed organisms were centrifuged at 9,000 × *g* for 30 min and resuspended in PBS. This washing step was repeated twice. The cells were centrifuged as above and resuspended in 50 μl each of MAb 66 and MAb 86 diluted 1:100 in PBS containing 1% (wt/vol) BSA. The suspensions were incubated overnight at 4°C and then washed as above and resuspended for 2 h at room temperature in a 1:20 dilution of biotinylated sheep-anti-mouse Ig (Amersham) dissolved in PBS. The cells were pelleted, washed as above, and resuspended in a 1:10 dilution of 5-nm-diameter colloidal gold-streptavidin (Amersham) in PBS for 2 h at room temperature. They were washed and fixed by the addition of 2% glutaraldehyde in PBS overnight. Finally, they were treated with 1% osmium tetroxide, dehydrated in acetone, and embedded in Spurr resin (Bio-Rad). Thin sections were cut and stained with uranyl acetate and Reynold's lead citrate. The sections were viewed under a Phillips 300 electron microscope.

Trypsin treatment of intact *M. gallisepticum* cells. *M. gallisepticum* cells were grown overnight as described previously and harvested at pH 6.8. The culture was centrifuged and washed three times in 50 mM Tris–0.145 M sodium chloride (TS) buffer (pH 7.4) (HCl). The cells were resuspended as six 100-μl aliquots in TS buffer, graded doses of trypsin (Sigma) were added, and the mixtures were incubated at 37°C for 30 min. Enzymatic reactions were stopped by the addition of 200 μl of 0.125% (wt/vol) trypsin inhibitor (Sigma) in TS buffer. The cell suspensions were then pelleted by centrifugation and resuspended in TS buffer. One aliquot of each cell suspension was run on SDS-PAGE and subjected to Western transfer.

Hemagglutination inhibition assay. *M. gallisepticum* cells were grown overnight as described previously and harvested at the late logarithmic phase of growth. The cells were centrifuged, washed three times in PBS, and finally resuspended in PBS to 1/50 of the original broth volume. In rows of a V-bottomed microtiter plate, duplicate serial twofold dilutions of the mycoplasma cell suspension were made in 50 μl of PBS. To each of these wells and control wells containing 50 μl of PBS was added 25 μl of a 0.5% suspension of chicken erythrocytes in PBS. The plate was incubated at 4°C overnight or until the chicken erythrocytes in the control wells had pelleted (at least 1 h). The first, second, and third

highest dilutions of mycoplasma cells that showed hemagglutination of chicken erythrocytes were assigned 1, 2, and 4 hemagglutination units (HA units), respectively. To assess MAb hemagglutination inhibition, duplicate serial twofold dilutions of each MAb (starting concentration, approximately 100 μg/ml) were made in 25 μl of PBS buffer and added to three V-bottomed microtiter plates. To these wells and to hemagglutination control wells (containing 25 μl of PBS) was added 25 μl of 1, 2, or 4 HA units of washed viable *M. gallisepticum* cells in PBS. After a 1-h incubation at room temperature, 25 μl of a 0.5% suspension of chicken erythrocytes in PBS was added to each well, including negative control wells containing 50 μl of PBS. The plate was incubated as described above. The titer of each MAb was taken as the highest dilution that showed HA inhibition.

Labeling of pMGA with ^{125}I and binding to erythrocytes. Approximately 20 μg of affinity-purified pMGA was iodinated with Na^{125}I (400 μCi; Amersham) by using Iodobeads (Pierce) as the oxidizing agent and using the method supplied by the manufacturer. Labeled protein was separated from unreacted Na^{125}I by gel filtration (PD10 column; Pharmacia). To two Eppendorf tubes, 40 μl of chicken erythrocytes in 100 μl of Elsevier's solution was added. Five 10-fold dilutions of erythrocytes (in duplicate) were made in 100 μl from the original suspension. To one set of tubes, 20,000 cpm of ^{125}I-pMGA-radiolabeled protein in 100 μl of Elsevier's solution was added. To the other duplicate set of tubes, 20,000 cpm of an unrelated ^{125}I-radiolabeled protein (of approximately the same concentration) was added as a negative control. The tubes were incubated at 4°C for 2.5 h. The erythrocytes were pelleted by centrifugation and washed gently with PBS–1% BSA. This procedure was repeated twice. The level of radioactivity in each tube was detected by using a Hewlett-Packard gamma counter.

Amino-terminal sequencing. *M. gallisepticum* S6 proteins were separated by SDS-PAGE and transferred to Immobilon membranes as described above; a sample was immunostained by using the MAb described above, and the remaining portion was stained with Coomassie blue. The band corresponding to the immunstaining of the MAb was excised from the Coomassie blue-stained membrane, and the stain was removed with a number of washes in methanol. Affinity-purified material was prepared for sequencing in a similar manner, except that immunostaining was omitted. The Immobilon membranes with attached protein and the affinity-purified protein were subjected to automated Edman degradation by using an ABI (model 471A) sequencer equipped with a Brownlee Laboratories microgradient delivery system.

RESULTS

Production of MAb for species-specific epitopes of *M. gallisepticum*. The criterion used in selecting hybridomas producing *M. gallisepticum*-specific MAbs was their ability to react with membranes from *M. gallisepticum* cells but not *M. synoviae* cells as determined by ELISA. Hybridomas selected by this method were cloned three times by limiting dilution and retested in ELISA for continued reactivity. Three different hybridoma cell lines secreting MAbs 66-29-2-4 (MAb 66; isotype IgG1), 71-9-30-1 (MAb 71; isotype IgG2a), and 86-5-9-6 (MAb 86; isotype IgG2a) were selected for further characterization.

Biochemical analysis of epitope-bearing structures in *M. gallisepticum*. Whole cells and an aliquot of MAb 86 affinity-purified protein of *M. gallisepticum* were subjected to SDS-PAGE. Staining of the gel with Coomassie brilliant blue revealed that the affinity-purified band corresponded to a strongly staining single band of M_r 67,000 in the *M. gallisepticum* protein profile (Fig. 1A). The M_r 67,000 band was designated pMGA and was the first major protein of *M. gallisepticum* described in our research. Western transfer of *M. gallisepticum* proteins onto an Immobilon membrane followed by immunostaining with all three MAbs and an irrelevant MAb revealed that MAbs 66, 71, and 86 reacted with a single band of M_r 67,000 (Fig. 1B). To further investigate the reactivity of the MAbs to other strains, we subjected *M. gallisepticum* F and R to SDS-PAGE, Western transfer, and immunostaining with the three MAbs. MAb 66 failed to react with either the F or R strain (data not shown), whereas MAbs 71 and 86 reacted, respectively, with a single band in the R and S6 strains (M_r 67,000) and a single band in the F strain (M_r 75,000) (Fig. 2A and B, respectively). The above results and, in particular, the failure of MAb 66 to react with protein structures of strains F and R could be interpreted in three ways. First, S6 may express a single pMGA protein containing epitopes for MAbs 66, 71, and 86; the MAb 66 epitope is missing from the equivalent proteins of the F and R strains. Second, MAb 66 reacts with an unrelated comigrating peptide that is not present in either F or R. Third, the S6 strain may express pMGA and proteins of similar molecular weight each containing a single MAb epitope; the F and R strains may lack protein that reacts with MAb 66 while expressing proteins containing epitopes for MAbs 71 and 86. To determine whether MAb 66 reacted to pMGA and whether pMGA of S6 consisted of one or more polypeptides, we subjected pMGA to IEF and then SDS-PAGE, followed by Western transfer with MAbs 66, 71, and 86 as detection reagents. Two major and two minor pMGA

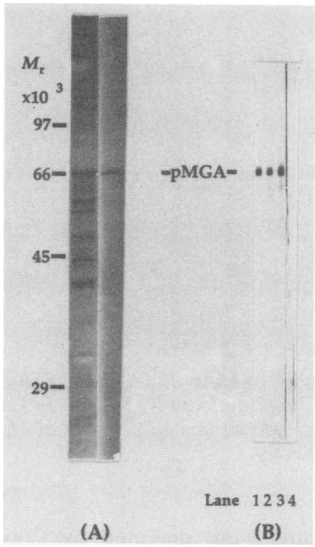

FIG. 1. Immunochemical analysis with *M. gallisepticum* reactive MAbs. (A) A sample of eluate from immunoaffinity chromatography of *M. gallisepticum* cells on a MAb 86-Sepharose matrix was run in SDS-PAGE, and the gel was stained with Coomassie brilliant blue (right channel): a sample of unfractionated *M. gallisepticum* proteins was run in the left channel for comparison. (B) Whole *M. gallisepticum* cells were run in SDS-PAGE and subjected to Western transfer. Individual channels were probed with MAb 66 (lane 1), MAb 71 (lane 2), MAb 86 (lane 3), and a MAb of irrelevant specificity (lane 4). The gel conditions were a 3% stacker–10% separater.

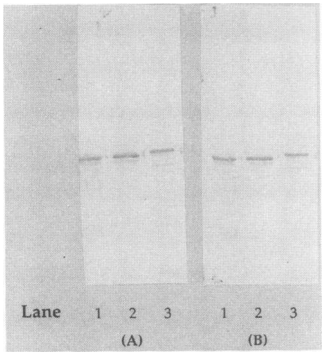

FIG. 2. Reactivity of MAb 71 (A) and MAb 86 (B) in Western immunoblot to *M. gallisepticum* R, S6, and F. Lanes 1, 2, and 3 of each panel contain unfractionated proteins of *M. gallisepticum* R, S6, and F, respectively.

isoelectric variants were detected by Coomassie brilliant blue staining (Fig. 3A). Immunostaining with all three pMGA-specific MAbs detected the same major pMGA isoelectric variants with no evidence of selective discrimination between them (Fig. 3B to D). These results are most compatible with the expression by *M. gallisepticum* S6 of a single pMGA polypeptide chain which, before or during purification, suffers minor covalent modifications that result in the limited charge heterogeneity revealed by IEF. Another possibility, that pMGA is a population of polypeptides all identical in molecular size and expressing the same three epitopes but encoded by different genes, has not been formally eliminated but seems unlikely. All existing protein sequence data on pMGA (data not shown) favor the expression in *M. gallisepticum* S6 of one pMGA polypeptide.

Surface location and attachment function of pMGA. The basic morphology of *M. gallisepticum* is shown by electron microscopy of thin sections on negatively stained cells (Fig. 4A). The cells are a rounded flask shape with a terminal structure (Fig. 4A, indicated by arrow). *M. gallisepticum* cells incubated with MAbs 66 and 86 and labeled with a colloidal gold (5 nm) conjugate (Fig. 4B) show a diffuse staining pattern on the cell membrane.

The location of pMGA on the cell surface of mycoplasmas and results of previous studies showing the sensitivity of the *M. gallisepticum* hemagglutinin moiety to trypsin led us to investigate the role, if any, played by pMGA in hemagglutination. To this end, whole *M. gallisepticum* cells were treated with increasing concentrations of trypsin or enzyme buffer alone. Duplicate sets of treated and untreated cells were concurrently subjected to SDS-PAGE. One gel was stained with Coomassie brilliant blue, while the other gel was subjected to Western transfer and immunostained with MAb 66. The band corresponding to pMGA in the Coomassie blue-stained gel was the only major band visibly reduced by increasing trypsin concentrations (Fig. 5A, lanes 2 to 6). A band which appeared at M_r 62,000 in lanes 2 to 5 was found to be a tryptic product of pMGA as demonstrated by immunostaining with MAb 86 (data not shown). This substantial loss of pMGA seen in Coomassie staining was also confirmed by a decrease in intensity of immunostaining when MAb 66 was used in the corresponding immunoblot (Fig. 5B).

To further establish the surface location of pMGA and its possible role as a hemagglutinin, we tested each of the three MAbs for their ability to inhibit hemagglutination of chicken erythrocytes. The test was conducted with 2 and 1 HA units, using strain S6 as the antigen. The pMGA MAb 86 at a concentration of up to approximately 100 μg/ml failed to inhibit hemagglutination, whereas MAb 66 and MAb 71 inhibited hemagglutination when diluted to concentrations of 1 and 2 μg/ml, respectively, and at all higher concentrations tested. When 4 HA units of S6 antigen were used, no inhibition with either MAb 66 or MAb 71 at 100 μg/ml could be demonstrated.

To assess whether the purified protein pMGA alone could attach to erythrocytes, affinity-purified pMGA labeled with

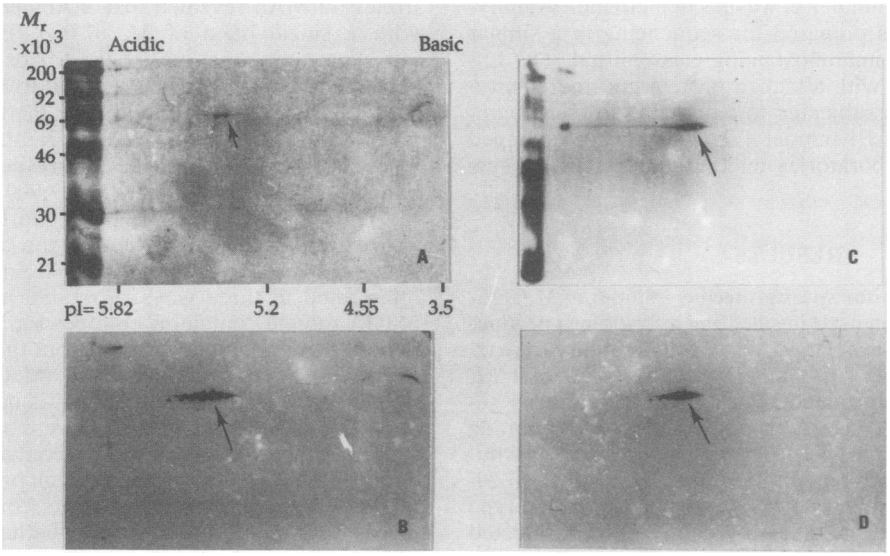

FIG. 3. Two-dimensional gel electrophoresis of pMGA. Aliquots (10 mg) of purified pMGA were subjected to IEF (horizontal dimension) and then SDS-PAGE (vertical dimension). The gels were either stained with Coomassie brilliant blue (A) or transblotted onto Immobilon membranes for immunodetection by MAb 66 (B), MAb 71 (C), or MAb 86 (D). The vertical arrows in each panel indicate the corresponding electrophoretic species with a calculated isoelectric point of pI 5.53.

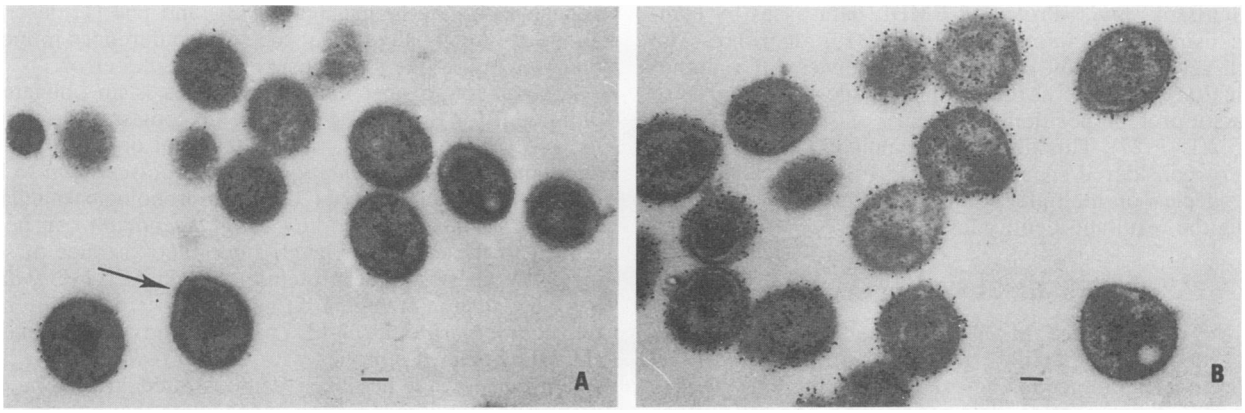

FIG. 4. Immunoelectron microscopy of thin sections of *M. gallisepticum* S6. Binding of MAbs was detected by using a 1:20 dilution of 5-nm sheep anti-mouse IgG–colloidal gold conjugate. (A) Cells incubated with conjugate only were used as negative control. Arrow, terminal structure of a cell. (B) Whole cells were incubated with 50 μg each of MAb 66 and MAb 86 and detected with conjugate as described in the text. Bars, 0.1 μm.

^{125}I was incubated with dilutions of chicken erythrocytes. At all dilutions of erythrocytes used, no significant difference could be seen in the counts per minute between control and ^{125}I-pMGA tubes. These data cannot necessarily be interpreted as negating a role for pMGA as an adhesin (see Discussion).

Immunogenicity of pMGA. A Western blot of whole *M. gallisepticum* protein was immunostained with serum collected over a number of weeks from a specific-pathogen-free hen infected with *M. gallisepticum*. The antisera reacted with a number of *M. gallisepticum* protein bands in the immunoblot (Fig. 6, lanes 2 to 4), whereas preinfection serum showed no reaction (lane 1). The relative position of pMGA was determined by immunostaining a section of the Western blot with MAb 66 (lane 5). An immune response to a protein in the same relative position as pMGA was seen by 2 weeks postinfection (lane 2) and was strongest at weeks 3 and 4 (lanes 3 and 4). Preliminary results (data not shown) with affinity-purified pMGA as an antigen in ELISA have demonstrated an immunological response to pMGA in *M. gallisepticum*-infected chickens.

Sequencing of pMGA. The amino acid sequences of the amino termini of both Western-transferred and affinity-purified pMGA were determined by using an ABI (model 471A) protein sequencer and were found to be identical. In single-letter code, the amino-terminal sequence, which begins with cysteine, is CTTPTPSPAPNPNPPSN. All amino acid assignments were unique, and there was no evidence of multiple amino acids at any position. The amino-terminal sequence was compared with all sequences contained within

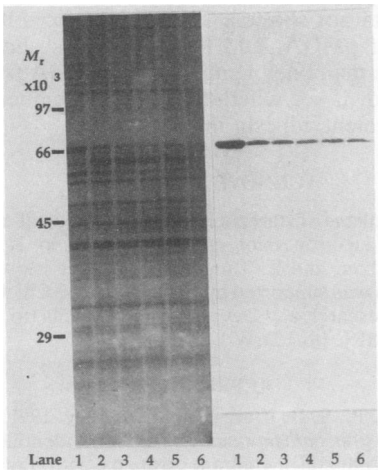

FIG. 5. Trypsin digestion of intact *M. gallisepticum*. Intact mycoplasma cells were suspended in digestion buffer (1 mg/ml) with increasing trypsin concentrations of 0 (lane 1), 15 (lane 2), 31 (lane 3), 62 (lane 4), 125 (lane 5), and 250 (lane 6) μg/ml. Digestions were terminated after 30 min and then run in SDS-PAGE (3% stacker–10% separating gel). (A) Samples stained with Coomassie brilliant blue; (B) samples subjected to Western transfer with MAb 66 as the immunodetection reagent.

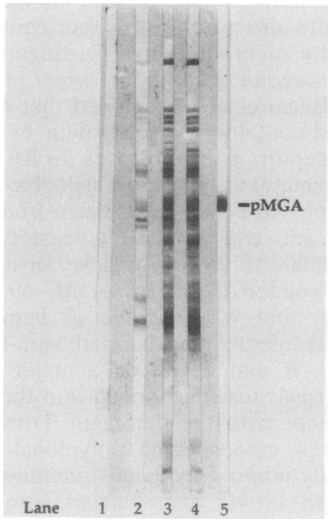

FIG. 6. Immunoreactivity of pMGA protein. Western blot of *M. gallisepticum* S6 probed with serum collected over a 5-week period from a monospecifically infected chicken and with MAb66. The chicken antisera reacted with a number of mycoplasma protein bands in the immunoblot (lanes 2 to 4), whereas preinfection serum showed no reaction (lane 1). MAb 66 (lane 5) reacted with a 67-kDa band that also reacted with the chicken antisera.

the GenBank, Swiss-Prot, and EMBL data bases by computer, using the Pearson and Lipman (19) algorithm. The search revealed a 60% identity level between the amino-terminal sequence of pMGA and the endo-1,4-β-glucanase precursor protein (positions 150 to 165) of *Cellulomonas fimi* (EC 3.2.1.4) (27). This degree of homology (although high) was not considered significant owing to the occurrence in both species of multiple proline and threonine residues, biasing the resultant identity level.

DISCUSSION

In this study three MAbs that reacted with a single homogeneous polypeptide of molecular weight 67,000 (pMGA) were produced. They recognize different epitopes of the pMGA polypeptide (data not shown). The cell surface location of pMGA was demonstrated by the use of MAbs in immunoelectron microscopy.

The hemagglutinin activity of *M. gallisepticum* has been shown in a previous study to be reduced, but not completely eliminated, by trypsin treatment (25). When whole *M. gallisepticum* cells were treated with increasing concentrations of trypsin, only pMGA was substantially removed when viewed by SDS-PAGE (Fig. 3A and B). These results, together with the ability of both MAb 66 and MAb 71 to block hemagglutination, support the proposal that pMGA is a major hemagglutinin of *M. gallisepticum*. Studies implicating P1 as an adhesin in *M. pneumoniae* were based on similar observations (12, 16). Attempts to demonstrate direct binding between pMGA and chicken erythrocytes were conducted by using immunoaffinity-purified material, labeled with ^{125}I as a ligand. No specific binding to erythrocytes could be demonstrated, although several explanations may account for this negative finding. For example, the binding affinity between isolated, single pMGA molecules and their erythrocyte receptors might be so low as to preclude a detectable level of binding. Alternatively, iodination may have impaired the attachment site on pMGA molecules. A third alternative could be that pMGA associates loosely on the *M. gallisepticum* cell surface with another protein and that the composite structure (but neither constituent alone) binds to a receptor on erythrocytes. Distinguishing between these alternatives requires additional experiments.

Research by Banai et al. (2) showed that glycophorin (a major sialic acid-containing glycoprotein of erythrocytes) was the main receptor on erythrocytes for *M. gallisepticum* attachment. Kahane et al. (14) used sialoglycopeptide affinity chromatography to isolate an adhesin from *M. gallisepticum* S6. The adhesive fraction consisted primarily of protein of M_r 70,000 to 75,000. Bradley et al. (3), using a lectin column, isolated a protein of M_r 69,000 from *M. gallisepticum* S6 that was capable of hemagglutination. Avakian et al. (1) infected specific-pathogen-free fowl with *M. gallisepticum* R and described a major immunogenic band of 64 kDa (p64) that partitioned into the hydrophobic phase on treatment with the detergent Triton X-114, and they noted that a monospecific polyclonal antiserum to electrophoretically homogeneous p64 immunostained single polypeptides from 19 *M. gallisepticum* strains tested, although differences in immunological reactivity and molecular weight between strains were noted. The p64 antiserum also immunostained a band of similar molecular weight in *M. pneumoniae*. We recently demonstrated the ability of MAb 86 to specifically bind the electrophoretically purified p64 protein of Avakian et al. (1) (kindly provided by A. P. Avakian) (results not shown). It is possible that pMGA, the adhesin (14), the hemagglutinin (3), and p64 (1) described above are identical and that the small differences in molecular weight are due to intrinsic experimental error.

Attachment to host mucosal surfaces is an obligate requirement for the pathogenesis of mycoplasma respiratory infections. The essential role of terminal organelles in the attachment of *M. pneumoniae* and *M. gallisepticum* to host cells has been emphasized (20). The morphological similarity between *M. pneumoniae* and *M. gallisepticum* can be extended to the molecular level, since the existence of serologically cross-reactive proteins to P1 in *M. gallisepticum* has been demonstrated (4, 5) whereas a serologically reactive counterpart to the 64-kDa protein band of Avakian et al. (1), which shares epitopes with the *M. gallisepticum* hemagglutinin pMGA, has been demonstrated in *M. pneumoniae*. Although it is tempting to speculate that both proteins may have a functional role in both species, there is as yet no experimental evidence which demonstrates a 64-kDa protein adhesin in *M. pneumoniae* or a P1-like adhesin in *M. gallisepticum*.

Recent molecular cloning experiments in our laboratory have revealed an unexpected complexity, which may complicate the future structural and functional analysis of pMGA. Specifically, these experiments revealed that the *M. gallisepticum* S6 genome contains a repertoire of genes, each with the potential to encode related members of a family of pMGA proteins. This finding, together with differences in molecular weight of pMGA structures seen in *M. gallisepticum* S6, F, and R, may be due to expression by different genes of antigenically related products of different sizes. Studies of surface lipoproteins in *M. hyorhinis* (22) and other mycoplasmas have revealed high-frequency variation in these structures. Three variant lipoprotein genes have been identified for *M. hyorhinis*; they are expressed independently, and each is capable of independent variation based on loss or gain of repetitive intragenic sequences (28). Although both the Vlp and pMGA systems are possibly analogous in their contribution to immune evasion, there appears to be little similarity at the molecular level. Only a single gene copy of pMGA appears to be expressed, and sequence analysis shows no similarity to the Vlp genes of *M. hyorhinis* (data not shown). These differences highlight the uniqueness of pMGA, and further studies should resolve whether more than one member of the pMGA family can be expressed and, if so, whether different members are functionally equivalent adhesin molecules.

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