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Lieutenant Colonel, MS
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MOLECULAR PATHOGENESIS OF ACUTE RESPIRATORY DISEASE

ANNUAL PROGRESS REPORT

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January, 1982

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20. ABSTRACT (Continue on reverse side if necessary and identify by block number)

Spontaneous nonhemadsorbing mutants of M. pneumoniae and their revertants were further characterized. Development and improvement of an in vitro binding assay to identify the molecular basis for M. pneumoniae attachment provided additional evidence regarding the functional role of key mycoplasma proteins. The process of raising monospecific antibody against these important M. pneumoniae proteins was begun. Radioimmunoprecipitation-fluorography techniques using acute and convalescent sera detected sequential differences in humoral response. Purification and analysis of M. pneumoniae released proteins were continued.

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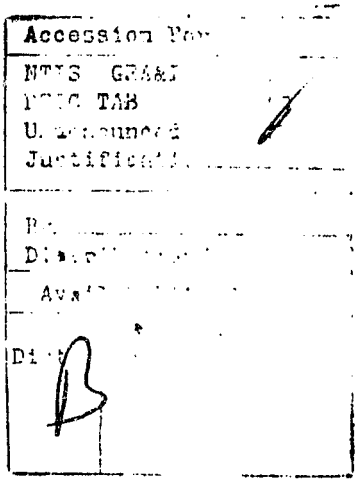
SUMMARY

Annual Report to the U.S. Army Medical
Research and Development Command

Spontaneous nonhemadsorbing mutants of M. pneumoniae and their corresponding revertants were used to further identify virulence determinants. Nonhemadsorbing mutants proved to be avirulent based upon decreased persistence in lung tissue of experimentally infected hamsters with no associated histologic pneumonia. Employing an in vitro binding assay, several mycoplasma proteins previously implicated as surface molecules were shown to have functional roles in attachment to host cells. Key mycoplasma immunogens were detected using radioimmunoprecipitation and fluorography, and a sequential humoral response in the infected host was observed. The process of raising monospecific antibody against these and other important mycoplasma immunogens was initiated. Also, an improved procedure for the isolation of extracellular proteins synthesized and released by virulent M. pneumoniae was established. These data provide the impetus for the development of appropriate diagnostic probes and vaccinogens.



FOREWORD

In conducting the research described in this report, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, Nation Research Council (DHEW Publication No. (NIH) 78-23, Revised 1978)."

TABLE OF CONTENTS

Molecular Pathogenesis of Acute Respiratory Disease

A. Characterization of Spontaneous Hemadsorption-Negative Mutants of Mycoplasma pneumoniae

In the annual report for Contract No. DADA17-73-C-3097, submitted January, 1981, we presented a preliminary characterization of 22 spontaneously arising mutants of M. pneumoniae deficient in hemadsorption (HA). We felt the potential presence of secondary mutations in nitrosoguanidine-derived mutants warranted the isolation and characterization of spontaneous HA^- mutants. Based upon consistent differences in their protein profiles in one- and two-dimensional polyacrylamide gels, the spontaneous HA^- mutants have been grouped into 4 classes (Table 1). All of the mutants exhibit similar reductions in adherence to the respiratory epithelium of hamster tracheal rings in vitro. However, significant differences were observed among the mutant classes in their ability to attach to neuraminidase-treated tracheal rings. The presence of three specific mycoplasma proteins correlated with adherence of M. pneumoniae to neuraminidase-sensitive receptors. Over the last contract period we have further characterized the spontaneous HA^- mutants.

1. Analysis of Radiolabeled M. pneumoniae Protein Profiles

A limiting factor in the detection of protein differences among mycoplasma strains is the sensitivity of Coomassie blue staining of protein bands in acrylamide gels. The incorporation of a radioactive label ([^{35}S]-methionine) into mycoplasma proteins has enabled us to increase the sensitivity of protein detection. Fluorography of [^{35}S]-labeled one-dimensional protein profiles verified the differences among mutant classes found using Coomassie blue staining (Fig. 1a and 1b). In addition, this more sensitive technique revealed a protein migrating just above HMW-1 in the wild-type strain which is absent in mutants lacking HMW 1-3 (Fig. 1b, track B). Finally, metabolic radiolabeling established that the alterations observed involve mycoplasma proteins, and do not reflect differences in binding of proteins from the growth medium.

2. Virulence of Spontaneous HA^- Mutants

The virulence of representative spontaneous HA^- mutants was examined in the hamster model. Groups of hamsters were inoculated intranasally with approximately 10^6 colony forming units (CFU) of the respective mutant and wild-type strains. Four hamsters per strain per time point (days 4, 14, and 28 post-infection) were utilized. Uninfected hamsters were included for control purposes. Virulence was defined according to two parameters: 1) persistence and/or multiplication of mycoplasmas in hamster lungs, and 2) the degree of histologic pneumonia produced. As shown in Table 2, only the virulent strain B25C persisted in the lungs at high levels over the 4 week study. The mutant categorized in class II survived at titers slightly above the limits of detection (10^3 CFU/gram lung tissue). The titers of the other mutants examined were below detectable levels.

Despite the low level survival of the class II mutant in infected hamsters, lung cytopathology scores for animals inoculated with this strain were no greater than the scores for the other mutants or for the uninfected controls (Fig. 2). This is in contrast to the extensive lung cytopathology observed by day 14 post-infection in hamsters infected with wild-type B25C. By day 28 post-infection, however, the histologic pneumonia was partially resolved.

Radioimmunoprecipitation (RIP) analysis of sera from mutant-infected hamsters is currently in progress. While the results are incomplete, preliminary data indicate little antibody response to mutant infection. This, in conjunction with borderline survival of mutant strains in hamsters, suggests that the mutants have minimal protective potential. When the RIP data are more complete, a final decision will be made concerning protection trials.

B. Isolation and Characterization of Revertants of Spontaneous HA^- Mutants

Our studies using spontaneously arising HA^- mutants have reinforced the importance of specific mycoplasma proteins to both attachment and virulence. During the current contract year we have isolated revertant strains of representative spontaneous HA^- mutants and have begun their characterization.

1. Isolation of Revertant Strains

Preliminary screening of mutant colonies for hemadsorbing revertants proved unsuccessful and indicated an enrichment procedure would be required. Revertant strains were selectively enriched using a procedure previously developed by our laboratory (1). Mutant mycoplasmas were incubated with chicken erythrocytes (CRBCs); RBC-adsorbed mycoplasmas were then separated from unattached mycoplasmas by centrifugation over a methocel/hypaque gradient. Following expansion of the "adherent" population the procedure was repeated in its entirety using fresh CRBCs. For most of the spontaneous HA^- mutant strains, this enrichment produced populations which were <10% HA^+. Individual HA^+ colonies were cloned as described previously for their HA^- "parent" strains (see previous Annual Report). Revertant strains were obtained from each class except Class I, which yielded no revertants despite an additional enrichment.

2. Sodium Dodecylsulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Equivalent whole cell preparations of the revertant strains and HA^+ B25C were analyzed by discontinuous SDS-PAGE using a 3% stacking and 5% separating gel (2). As seen in Figure 3, the Coomassie blue-stained one-dimensional protein profiles of the revertant strains are essentially identical to that of the wild-type strain. In particular, the revertant of the class I mutant has regained the high molecular weight proteins HMW 1-3 (Fig. 3B), and the revertant of the class IV mutant has regained protein P1 (Fig. 3E). Additional characterization of revertant strains will include two-dimensional PAGE and <u>in vitro</u> attachment studies.

C. Mycoplasma Ligand-Receptor Studies

The involvement of surface proteins in the adherence of M. pneumoniae to host cells has been established previously (3). Much of our research effort has been directed towards the identification of specific proteins which mediate cytadsorption. Using an approach first described for the identification of receptor binding proteins of Treponema pallidum (4), we have identified several surface proteins of M. pneumoniae which bind avidly to both respiratory epithelial cells and chicken erythrocytes.

A detergent system consisting of 16 mg/ml sodium deoxycholate (DOC), 1 mg/ml SDS, 0.25M NaCl, and 1 mM phenylmethylsulfonyl fluoride (PMSF) in 35 mM Tris, pH 8.2, was used to solubilize [^{125}I]-labeled M. pneumoniae. Following removal of insoluble material by high speed centrifugation, the detergent-soluble fraction was combined with glutaraldehyde-fixed hamster trachea epithelial (HTE) cells (5) and incubated for 50 min at 37°C. Loosely associated material was removed by a series of washes, and the avidly bound fraction was removed by boiling the cells for 4 min in 100 mM Tris, pH 6.8, containing 2% SDS, 2% β-mercaptoethanol, 10% glycerol, 2.5% sucrose, and 0.02% bromophenol blue. The cells were then pelleted by centrifugation and the resulting supernatant analyzed by SDS-PAGE and autoradiography.

Using the procedure described above we have identified two proteins, Py and P1, which bind avidly to HTE cells (Fig. 4B). When chicken RBCs were substituted for HTE cells, there was no enrichment for protein P1 and a reduced enrichment for Py (Fig. 4C). Analysis of HA$^-$ mutant attachment to tracheal rings has enabled us to make a distinction between the processes of hemadsorption and adherence to epithelial tissue. Whether the differences in binding of solubilized mycoplasma proteins to these two cell types is a reflection of the distinction between whole cell mycoplasma attachment to RBCs and epithelial cells requires further investigation.

Previous examinations of the HTE cell-bound fraction of solubilized M. pneumoniae included SDS-PAGE using a 7.5% acrylamide gel. Proteins HMW-3 and Py exhibit very similar relative mobilities, and in a 7.5% gel HMW-3 migrates just above Py. To clarify the identity of the protein in that molecular weight range which binds to HTE cells, we examined the avidly bound fraction on a 5% polyacrylamide gel, and found both proteins present (Fig. 5B). This figure also reveals the presence of a previously undetected band in the avidly bound fraction. This protein comigrates with a mycoplasma protein previously identified by our laboratory as being accessible on whole organisms to cleavage by trypsin or labeling by lactoperoxidase-catalyzed radioiodination (3). The presence of this protein, designated P2, in the avidly bound fraction varies, a phenomenon which may relate to efficiency of solubilization of different mycoplasma preparations or variability in HTE cell populations from one batch or passage level to another.

In order to confirm the identity of proteins P1 and HMW-3 in the HTE cell bound fractions, we have taken advantage of the availability of HA$^-$ mutants lacking these proteins. When we compared binding of solubilized proteins from HA$^+$ B25C (Fig. 6E) and a class IV mutant

which lacks protein P1 (Fig. 6F), we found no protein band corresponding to P1 in the avidly bound fraction from the mutant. In contrast, P1 was clearly present in the wild-type preparation. A similar experiment for the verification of HMW-3 as an HTE cell-binding protein is in progress.

Current efforts to characterize host cell binding of mycoplasma proteins center around evaluation of the specificity and significance of binding. While other detergent combinations appeared equally efficient at solubilizing M. pneumoniae, only the SDS/DOC system described resulted in enrichment of specific mycoplasma proteins. In view of the data obtained from mutant analysis, it is possible that these proteins are involved in attachment of intact organisms to host cells. Virulent M. pneumoniae readily adhere to glutaraldehyde-fixed HTE cells and hemadsorb fixed chicken erythrocytes. Thus, the fixation process has not destroyed the host cell receptors. Nevertheless, additional characterization of binding of mycoplasma proteins to HTE cells will be necessary to more firmly establish their relevancy to mycoplasma attachment. At this point the identification of proteins P1 and HMW-3 as selective ligands in the binding assay, along with their biologic role in mycoplasma attachment based upon mutant analysis and surface topography, reinforce the usefulness of this experimental approach.

D. Radioimmunoprecipitation (RIP) of Important Immunogens

Radioimmunoprecipitation, in conjunction with SDS-PAGE and fluorography, has permitted identification of immunogenic proteins of M. pneumoniae. Radiolabeled, soluble proteins are combined with test antisera (or immunoglobulin G), and antigen-antibody complexes are precipitated with protein A- Staphylococcus aureus. The RIP assay detects proteins which have stimulated a humoral immune response in the host, and such immunogens may be important in vaccine development.

A buffer containing Tris-sodium deoxycholate-SDS-EDTA-Triton (TDSET) was used to solubilize [^{35}S]-labeled M. pneumoniae. The radiolabeled supernatant is divided into 100 µl aliquots, and 20 µl of various dilutions of immune or control sera are added. After 15 min at 37°C, the sera plus radiolabeled proteins are placed at 4°C overnight. Fifty microliters of washed, formaldehyde-treated, protein A-bearing S. aureus (Cowen I)(Staph A) are then added to each mixture, and the mixtures incubated at 4°C for 1.5 hours. The Staph A-bound antigen-antibody complexes are pelleted and washed 4 times with TDSET buffer. The radiolabeled antigens are recovered by resuspending the Staph A pellet in solubilizing buffer (0.1M Tris, pH 6.8, 2% SDS, 20% glycerol, 2% β-mercaptoethanol, and 0.02% bromophenol blue), then boiling this preparation for 3 min. The Staph A are pelleted and the supernatants applied to SDS-polyacrylamide gels.

Hamsters (male Syrian golden, approx. 1 yr old) were bled to obtain pre-immune sera, then were intranasally inoculated with ~ 10^8 CFU M. pneumoniae strain M129-B15 per animal. At various times the hamsters were bled (cardiac puncture) to obtain immune sera. The pre- and post-immune sera were tested in the RIP assay. Figure 7 shows the [^{35}S]-methionine labeled M. pneumoniae proteins which were precipitated

with sera from 2 different hamsters. Pre-immune sera apparently contained antibodies that reacted with M. pneumoniae proteins, suggesting preexposure of hamsters to cross-reactive components from other microorganisms which comprise the normal flora. Alternatively, some of this reactivity might be considered "nonspecific background". Nonetheless, two predominant immunogenic proteins (165K, 100K) could be identified in sera obtained post-infection. The 165K protein comigrates with protein P1, a trypsin sensitive protein previously implicated in attachment (3). The other major immunogen (100K) has not been associated with either attachment or hemadsorption. Several other mycoplasma proteins (87K, 85K, 62K, 59K, 40K, 28K) were precipitated by post-infection sera. Two of these immunogens have calculated molecular weights similar to those of virulence-specific proteins B(85K) and C(37K) (6). It should be noted that most, but not all, of the post-infection hamster sera precipitated the majority of the immunogens described. This might be due to variations among hamsters with respect to immune responsiveness, and/or different efficiencies of inoculum delivery among animals.

Pairs of acute and convalescent sera obtained from M. pneumoniae patients were also examined by RIP (Figure 8). The predominant bands precipitated with convalescent sera are P1 (165K) and two unnamed bands (68K, 66K). Other proteins (100K, 40K, 28K) are recognized less efficiently by convalescent, but not acute, sera. The amount of P1 precipitated seems to correlate directly with serum complement fixation titers previously determined.

It is noteworthy that similarities exist between the human convalescent and hamster post-infection serum responses. Humans do not demonstrate equivalent humoral responses to M. pneumoniae infection. Likewise, hamster sera show variable antibody responses post-infection. Several immunogens, in particular protein P1, are recognized by both infected hamster and human sera during convalescence. These data indicate that intranasal infection of hamsters not only produces histologic pneumonia (7), but also stimulates a serum response in hamsters which closely resembles that seen in human M. pneumoniae patients.

Last year we reported the isolation and characterization of spontaneous mutants deficient in hemadsorption. One mutant (isolate 22) lacked protein P1 on 1-D polyacrylamide gels (as well as 3 other virulence-specific proteins on 2-D gel analysis). To confirm that the 165K immunogen is P1, and that mutant 22 is lacking this immunogenic protein, an RIP was performed with soluble, labeled proteins derived from mutant 22. Figure 9 shows that when the same serum is used to precipitate proteins from the wild-type or mutant strain, the PAGE patterns are identical except for the missing band at 165K in the mutant 22 preparation. Therefore, we conclude that P1 is a major immunogen and it is missing in mutant 22. Furthermore, the observation that antibodies do not detect additional differences between the wild-type and mutant profiles suggests that no other immunogens have been substantially altered in the mutant.

E. Released Proteins from Virulent M. pneumoniae

Virulent M. pneumoniae release proteins into the growth medium, but these proteins have been difficult to isolate from a complex serum-containing medium such as Hayflick's. Earlier studies in the laboratory utilized a serum-free medium (designated Medium 199/EYE) for growth of mycoplasmas, and released proteins were isolated through many steps involving centrifugation and filtration. This previous technique for isolation of released proteins had several disadvantages: 1) the procedure involved many manipulations; 2) a large amount of time was required to obtain small amounts of released protein; 3) the mycoplasmas were not grown under optimal conditions, which may have resulted in suboptimal release of proteins; and 4) there was the possibility of variation among preparations. Therefore, an anti-released protein (anti-RP) affinity column was constructed to provide an improved method for preparing large, consistent quantities of released protein.

1. Preparation and RIP Analysis of Anti-RP Immunoglobulin

A rabbit was injected subcutaneously and intramuscularly with released protein (prepared from Medium 199/EYE) emulsified in Freund's complete adjuvant, and was boosted 3 weeks later with released protein in Freund's incomplete adjuvant. The rabbit was bled 10 days later to obtain immune serum.

The anti-RP antiserum was applied to a protein A-Sepharose 4B column, and anti-RP immunoglobulin G (IgG) was eluted with 1M acetic acid. The fractions absorbing at 280 nm were pooled, dialyzed, and concentrated. One ml of protein A-Sepharose 4B swollen gel yielded approximately 15 mg of anti-RP IgG.

The anti-RP IgG isolated from several passes of immune serum over the protein A column was examined in an RIP assay (Figure 10). Four consecutive IgG preparations have essentially identical precipitation patterns. This demonstrates that the IgG prepared by affinity chromatography is functionally intact. When the anti-RP IgG precipitation pattern is compared to the pattern produced by convalescent human serum, the protein profiles appear quite different. Anti-RP IgG precipitates only minimal amounts of P1. However, this correlates with evidence that P1 is an integral membrane protein, and thus would not be readily released. Quantitatively, anti-RP IgG precipitates more proteins of MW <50,000 than does human convalescent serum. However, some immunogens recognized by convalescent human serum appear to comigrate with released proteins (MW 100K, 40K, 28K). This suggests that released proteins interact with the host during a natural infection, and indicates they may play a role in the disease process.

2. Anti-RP Affinity Column

The anti-RP IgG (isolated as above) was coupled to cyanogen bromide activated-Sepharose 4B (CNBr-Sepharose) according to the manufacturer's suggested procedure. Briefly, the IgG preparation was dialyzed against coupling buffer (0.1M $NaHCO_3$, pH 7.8, containing 0.5M NaCl), then reacted with CNBr-Sepharose (previously washed with 1mM

HCl and equilibrated in coupling buffer). The coupling reaction was carried out on a gyratory shaker at room temperature for 2h. (Coupling was >95% efficient.) Unreacted IgG was washed from the coupled gel, and any excess reactive groups were blocked with 1M ethanolamine. The coupled gel was then washed sequentially with coupling buffer, 0.1M acetate (pH 4) containing 0.5M NaCl, and 0.1M Tris (pH 7.5) containing 0.15M NaCl (Tris-saline).

Spent Hayflick medium was filtered to remove intact mycoplasmas and applied to the anti-RP column. The unbound protein was washed from the column with Tris-saline, and the bound protein eluted with 0.2M glycine (pH 2.8) containing 0.5M NaCl. Fractions were monitored at A_{280}, and protein-containing fractions neutralized with 1M Tris, pH 8.8. The neutralized fractions were pooled, concentrated, and dialyzed against distilled water. The anti-RP column was reequilibrated in Tris-saline buffer. A typical column yields approximately 3 mg released protein per 3 ml swollen gel (or per 50 ml spent medium).

Using this approach, enough released protein can be isolated and pooled to provide a homogeneous preparation for multiple experiments. Unlabeled released protein will be assayed for cultured hamster tracheal epithelial cell toxicity, and will be used as the antigenic stimulus for production of more anti-RP antibodies. The anti-RP serum will be examined for its effect on M. pneumoniae metabolic activity. Labeled released protein will be employed in RIP assays and in host cell binding studies.

F. Monospecific Antisera Against Key Mycoplasma Proteins

Previous studies have identified several specific mycoplasma proteins which appear to be important in pathogenesis (3,6,8,9). In the course of natural or experimental infections, humans and hamsters develop a humoral immune response against several of these proteins. In the current contract period we have initiated the production of monospecific antisera directed against certain of these proteins.

1. Production of Monospecific Antisera

Proteins were prepared for injection using a modified procedure of Tjian et al. (10). Milligram quantities of M. pneumoniae total protein were fractionated by preparative SDS-PAGE using a 3% stacking and 4% separating gel. Proteins were stained with Coomassie blue in fixative. After destaining, the bands of interest were sliced from the gels, macerated by forceful passage through a 19g needle, and lyophilized. Equivalent lyophilized bands from several gels were ground to a fine powder using a mortar and pestle and aliquoted into initial and booster doses. Each dose (containing approximately 150 µg protein) was resuspended in water to approximately 0.7 ml and emulsified 1:1 with Freund's complete adjuvant (or incomplete adjuvant for booster doses). Adult New Zealand white rabbits were injected subcutaneously in the neck and thigh regions and intramuscularly in the thigh on days 1, 15, and 29. Rabbits were bled from the ear beginning 5 days after the final booster injection.

2. Evaluation of Antisera

Specificity of pre-bleed and immune sera was established by radio-immunoprecipitation in conjunction with SDS-PAGE and fluorography (see section D). The specificity of immune serum prepared against protein P1, as compared to pre-bleed serum, is shown in Fig. 11. Currently antisera against other important mycoplasma proteins are being prepared and evaluated.

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Legends to Figures

Fig. 1 Electrophoretic analysis of total protein from wild-type and representative spontaneous HA$^-$ mutant strains of M. pneumoniae; (a) stained protein profile and (b) equivalent fluorogram. (A) HA$^+$ M129-B25C; (B) HA$^-$ mutant lacking proteins HMW 1, HMW 2 and HMW 3 (the fluorogram reveals the absence of an additional high molecular weight protein - see text); (C) HA$^-$ mutant with a one-dimensional protein profile identical to that of B25C; (D) HA$^-$ mutant lacking protein P1; (E) ^{14}C-labeled molecular weight standards:myosin (200K), phosphorylase b (92.5K), and bovine serum albumin (BSA) (68K).

Fig. 2 Lung histopathology in hamsters following intranasal inoculation with wild-type and mutant M. pneumoniae. Each bar represents the mean value of lung lesion scores from 3 or 4 hamsters. Positive standard deviations are given.

Fig. 3 SDS-PAGE analysis of total protein from the wild-type and HA$^+$ revertant strains of M. pneumoniae. (A) HA$^+$ B25C; (B-E) Revertants of HA$^-$ mutants from Clases I-IV, respectively; and (F) molecular weight standards:myosin (200K), β-galactosidase (130K), phosphorylase b (92.5K), and BSA (68K).

Fig. 4 Binding of detergent-solubilized M. pneumoniae proteins to HTE cells and chicken erythrocytes. (A) Detergent-soluble fraction of [^{125}I]-labeled M. pneumoniae; (B) avidly bound fraction using HTE cells; (C) avidly bound fraction using chicken erythrocytes. Protein bands P1 and Py are indicated.

Fig. 5 Electrophoretic analysis on (A) 7.5% and (B) 5% polyacrylamide gels of detergent-solubilized mycoplasma proteins which bind avidly to HTE cells. Protein bands P1, HMW 3, Py, and P2 are indicated.

Fig. 6 Mutant analysis of HTE cell-binding proteins of solubilized M. pneumoniae. Chloramine T/[^{125}I]-labeled protein profiles (A and B), detergent-soluble fractions (C and D), and HTE cell-bound fractions (E and F) for B25C and a class IV mutant, respectively. G) [^{14}C]-labeled molecular weight standards.

Fig. 7 Radioimmunoprecipitation (RIP) assay of intranasally infected hamster sera. (A) [^{35}S]-labeled M. pneumoniae proteins; (B,E) hamster pre-immune sera; (C,F) hamster sera drawn 25 days after infection with M. pneumoniae; (D,G) hamster sera drawn 39 days post-infection; (H) [^{14}C]-labeled molecular weight standards. Sera (B,C,D) and (E,F,G) were obtained from two different infected hamsters. Lines indicate proteins precipitated to a greater extent with post-infection sera (MW-165K, 100K, 87K, 85K, 62K, 59K, 40K, 28K). Molecular weight standards: myosin (200K), phosphorylase b (92.5K), BSA (68K), ovalbumin (43K), and chymotrypsinogen (25.7K).

Fig. 8 RIP assay of paired acute/convalescent human sera. (A) [^{35}S]-labeled M. pneumoniae proteins; (B) no serum; (C-N) acute/ convalescent paired human sera. Acute/convalescent pairs are from patient 1 (C,D), patient 2 (E,F), patient 3 (G,H), patient 4 (I,J), patient 5 (K,L), and patient 6 (M,N).

Fig. 9 RIP assay of human and hamster sera employing [^{35}S]-methionine labeled M. pneumoniae wild-type strain B15 or mutant strain 22. (A,A') [^{35}S]-labeled M. pneumoniae proteins from B15 and mutant 22, respectively; acute/convalescent sera from patient 7 vs. B15 (B,C) and vs. 22 (D,E); acute/convalescent sera from patient 3 vs. B15 (F,G) and vs. 22 (H,I) pre-bleed, day 25 post-infection, and day 39 post-infection hamster sera, respectively, vs. B15 (J,K,L) and vs. 22 (M,N,O); (F) [^{14}C]-labeled molecular weight standards. The line indicates protein P1 (165K) which is present in the wild-type strain, but absent in mutant strain 22.

Fig. 10 RIP assay of anti-released protein immunoglobulin G. (A) [^{35}S]-labeled M. pneumoniae proteins; (B-E) four consecutive preparations of rabbit anti-RP IgG eluted from a protein A-Sepharose column; (F) rabbit anti- M. pneumoniae antiserum; (G,H) two different samples of unfractionated rabbit anti-RP antiserum; (E) [^{14}C]-labeled molecular weight standards.

Fig. 11 RIP assay of monospecific anti-P1 antiserum. (A) Pre-bleed and (B) immune rabbit anti- M. pneumoniae; (C) pre-bleed and (D) monospecific anti-P1 antiserum; (E) [^{35}S]-labeled total protein profile of M. pneumoniae; (F) [^{14}C]-labeled molecular weight standards.

FIGURE 1

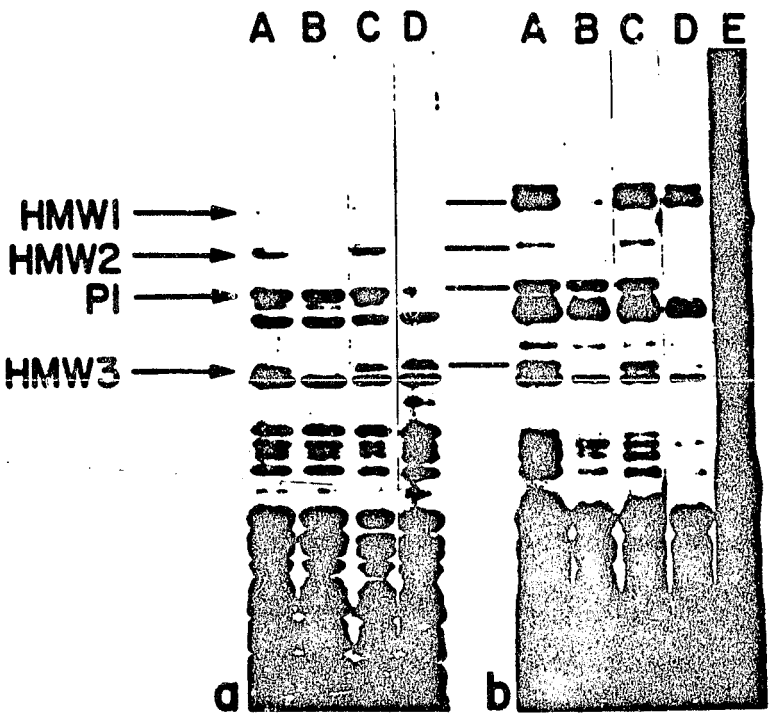


FIGURE 2

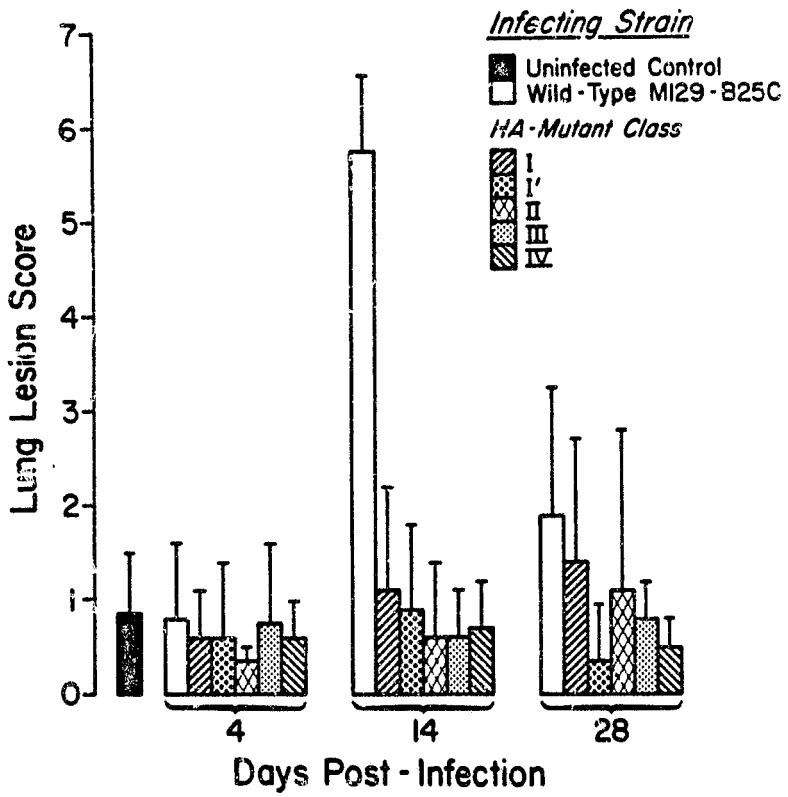

Infecting Strain
Uninfected Control
Wild-Type M129-825C
HA-Mutant Class
I
I'
II
III
IV
Lung Lesion Score
0
1
2
3
4
5
6
7
4
14
28
Days Post-Infection

FIGURE 3

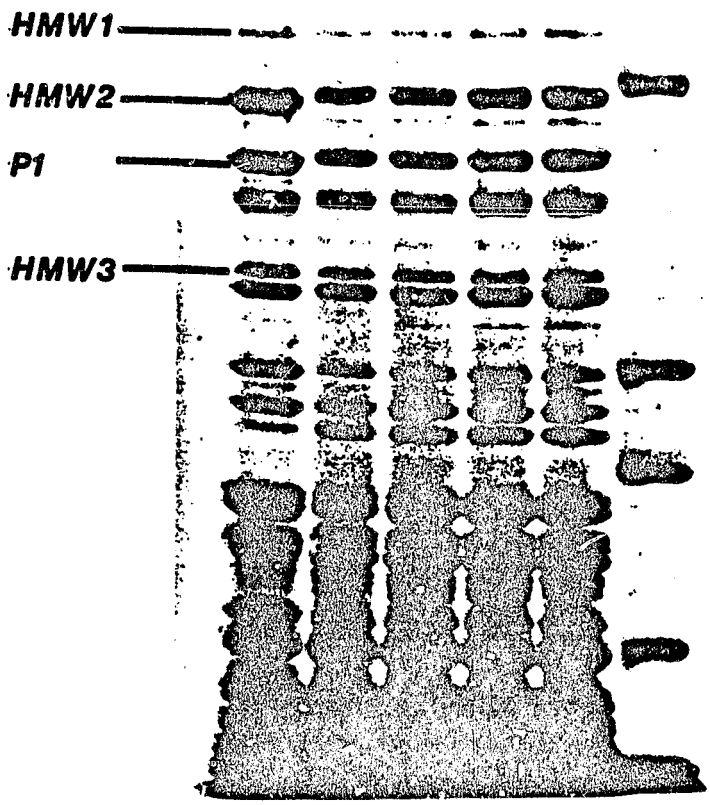
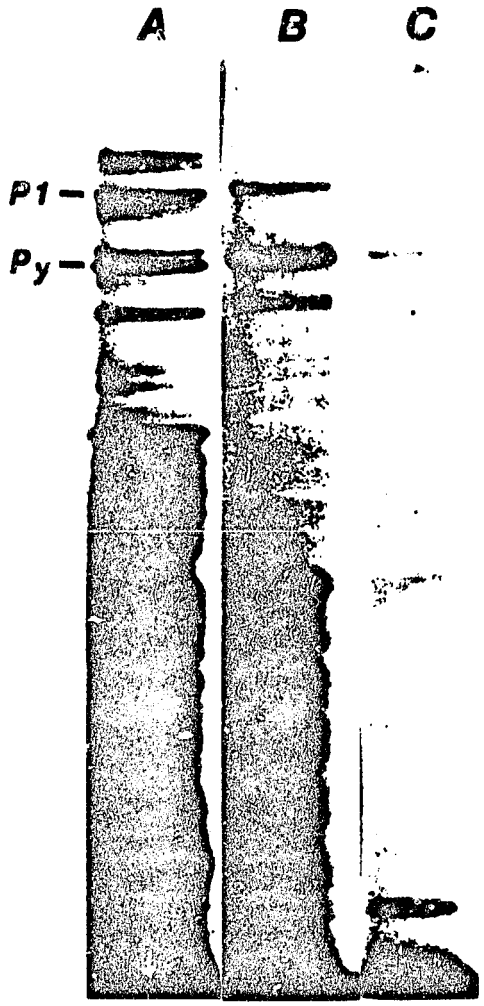


FIGURE 4



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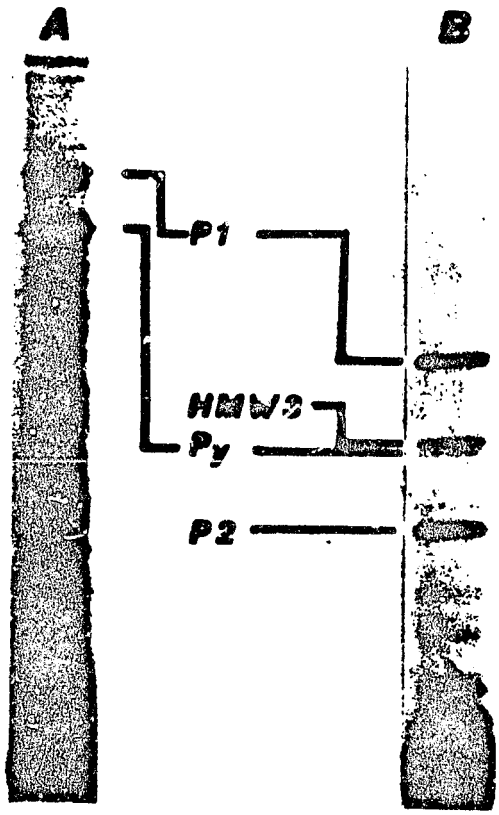

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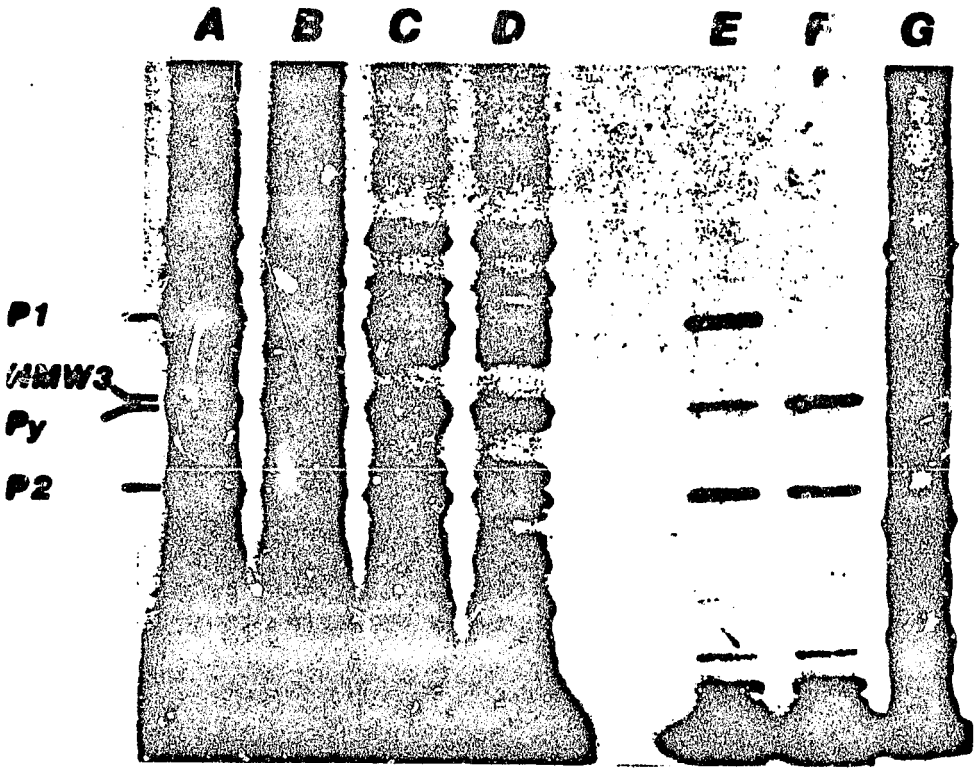

FIGURE 7

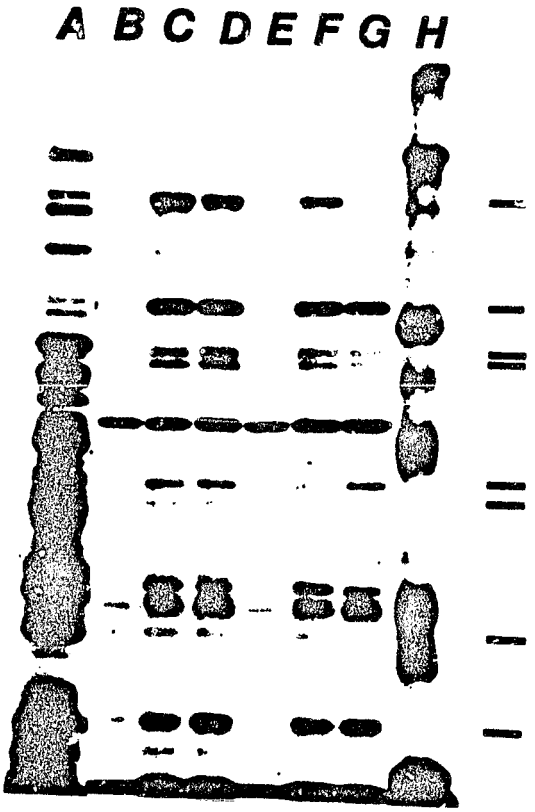

FIGURE 8

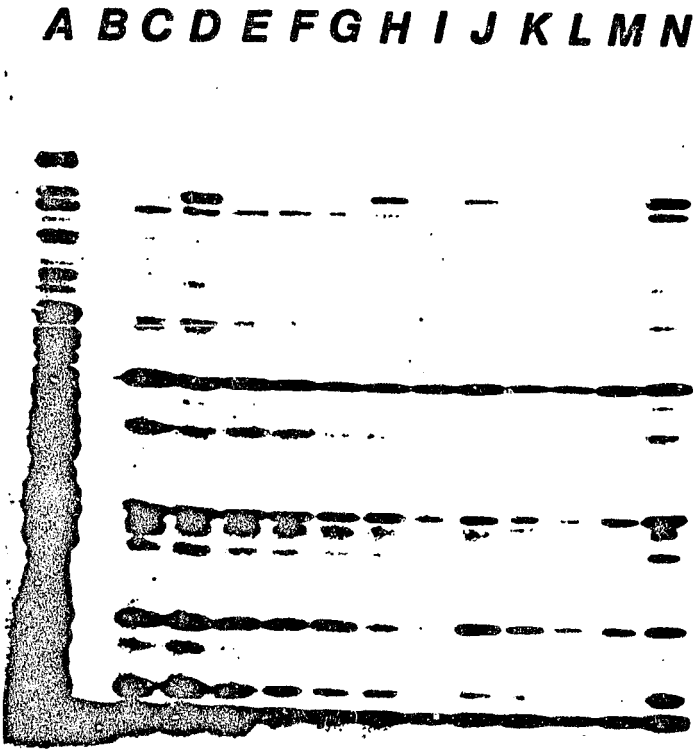

FIGURE 9

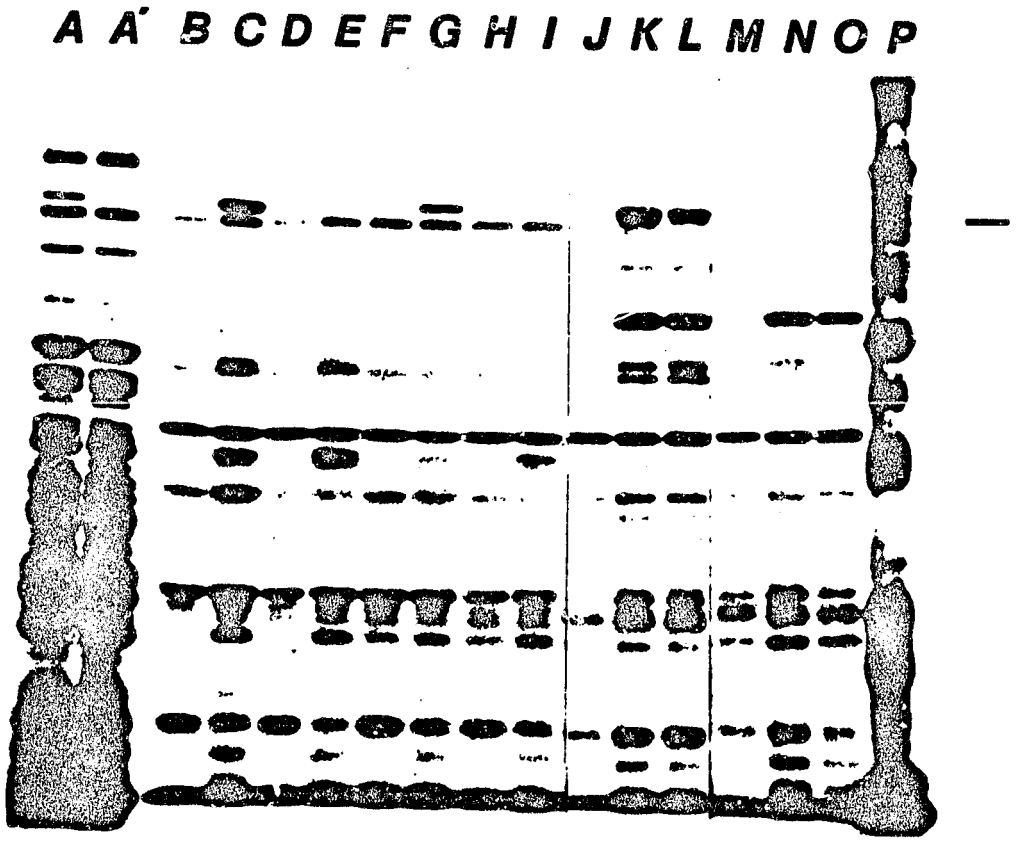

FIGURE 10

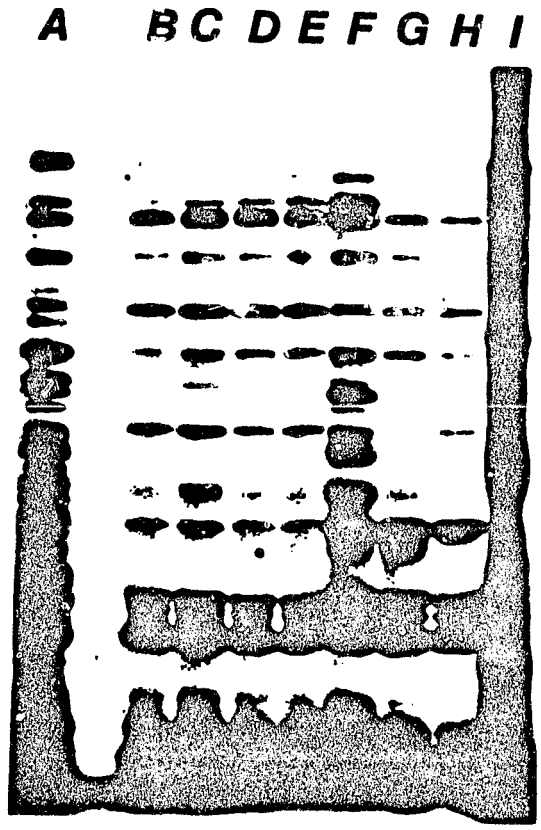

FIGURE II

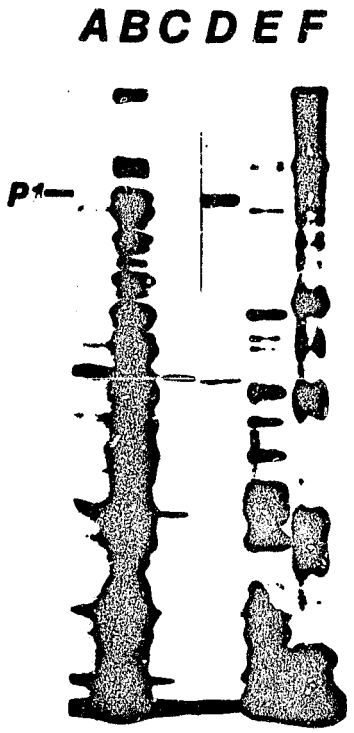

TABLE 1. Comparison of protein differences between wild-type M. pneumoniae strain B25C and the spontaneous HA^- mutants

	Mutants[a]	Proteins						
		HMW 1	HMW 2	HMW 3	A	B	C	P1
B25C	----	+[b]	+	+	+	+	+	+
Class I	2,5,6,8,9,10,11, 12,14,16,17,18,21	-[c]	-	-	+	+	+	+
Class II	1,3,7,15	+	+	+	+	+	+	+
Class III	4,13,19,20	+	+	+	-	-	-	+
Class IV	22	+	+	+	-	-	-	+

[a] Designation of individual isolates within each class.

[b] Protein present.

[c] Protein absent.

TABLE 2. Quantitation of viable mycoplasmas in hamster lungs following intranasal inoculation with wild-type or HA$^-$ mutant M. pneumoniae

Class	Inoculum[a]	Days Post-Infection		
		4	14	28
I	6.0	N.D.[b]	N.D.	N.D.
I	5.9	N.D.	N.D.	N.D.
II	6.2	3.3[c]	3.0	3.9
III	6.3	N.D.	N.D.	N.D.
IV	6.2	N.D.	N.D.	N.D.
Wild-type M129-B25C	6.0	5.3	6.3	4.8

[a] Log_{10} CFU/hamster

[b] Not detectable (<10^3 CFU/gram lung tissue).

[c] Log_{10} CFU/gram lung tissue. Each number represents the mean value obtained from 3 or 4 hamsters.

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