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

The host humoral immune response to M. pneumoniae infection was further characterized. Examination of isogenic revertants of spontaneous nonhemadsorbing mutants led to analysis of the biochemical/immunological relatedness of selected virulence-associated M. pneumoniae proteins. Monospecific antiserum against protein P1 was examined for effects on mycoplasma viability, metabolism, and cytadsorption, and was employed for immunoferritin electron microscopy studies. Monoclonal antibodies against M. pneumoniae were produced and initial characterizations were begun. This report includes a final summary for the entire contract period.

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MOLECULAR PATHOGENESIS OF ACUTE RESPIRATORY DISEASE

ANNUAL PROGRESS REPORT AND FINAL SUMMARY

July, 1983

(For the Period 1 January 1982 to 31 December 1982)
Annual
Final March 1981 — December 1982

Joel B. Baseman, Ph.D.
Debra K. Leith, Ph.D.
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Supported by

U.S. ARMY MEDICAL RESEARCH AND DEVELOPMENT COMMAND,
Fort Detrick, Frederick, Maryland 21701

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Summary

1.	Preparing Institution	University of Texas Health Science Center, San Antonio
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4.	Number of Pages Illustrations	40 pages 21 illustrations
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6.	Supported by	U.S. Army Medical Research and Development Command, Department of the Army, Fort Detrick, Frederick, MD 21701

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

The serological response of hamsters and humans to M. pneumoniae infection was further characterized by radioimmunoprecipitation techniques. Proteins P1 and P2 were found to be antibody-accessible predominant immunogens. Spontaneous hemadsorbing revertants reacquired M. pneumoniae proteins absent in isogenic nonhemadsorbing mutants. An examination of the relatedness of HMW1-3 by peptide mapping and immunological analysis suggested that these proteins are indeed distinct. Monospecific anti-P1 antiserum inhibited M. pneumoniae cytadsorption, but had no effect on mycoplasma viability or metabolism. Immunoferritin electron microscopy performed with monospecific anti-P1 antiserum demonstrated P1 clustering at the tip of virulent M. pneumoniae, which was not observed at the terminus of avirulent organisms. Monoclonal antibodies which recognize a variety of mycoplasma proteins were generated and initially characterized. An ELISA was developed for detection of anti-M. pneumoniae antibodies. The data described will be applicable to diagnostic testing and vaccinogen development.

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, National Research Council (DHEW Publication No. (NIH) 78-23, Revised 1978).

INTRODUCTION

During this report period, work under this contract has been performed by J.B. Baseman, D.K. Leith and D.C. Krause. Results are summarized under the following headings:

Molecular Pathogenesis of Acute Respiratory Disease

A. Analysis of M. pneumoniae Immunogens by Radioimmunoprecipitation (RIP)

The previous Annual Report presented data on radioimmunoprecipitation of [^{35}S]methionine-labeled M. pneumoniae proteins by pre- and post-immune hamster sera and acute and convalescent human sera. Two predominant immunogens, now known to be proteins P1 and P2, were identified with immune hamster sera, while convalescent human sera precipitated protein P1 most readily (1). These observations led to further investigation of the host humoral immune response to M. pneumoniae infection.

1. Identification of Cell Surface, Antibody-Accessible Immunogens by Whole Cell RIP

Mycoplasma proteins which stimulate a potentially protective antibody response in vivo are most likely exposed on the cell surface, and thus would be accessible to antibodies on intact organisms. A modification of the RIP assay which uses intact M. pneumoniae, rather than detergent-solubilized mycoplasma proteins, as targets for antibody binding was employed to examine accessibility of immunogens on the cell surface of M. pneumoniae. Briefly, [^{35}S]methionine-labeled mycoplasmas were suspended in phosphate-buffered saline (PBS) and combined with test sera. After 90 min incubation at 4°C, unbound antibody was removed by washing with PBS. Intact, antibody-coated M. pneumoniae were then detergent-solubilized and immune complexes precipitated with formalinized protein A-bearing Staphylococcus aureus (Staph A). The results of a representative whole cell RIP assay are shown in Fig. 1. Both postinfection hamster sera and convalescent human sera strongly precipitate proteins P1 (165,000 MW) and P2 (110,000 MW), indicating that these two proteins are accessible to host antibody on intact M. pneumoniae.

In theory, a whole cell RIP done with [^{35}S]methionine-labeled mycoplasmas should identify the same predominant immunogens as a soluble-antigen RIP performed with radioiodinated M. pneumoniae. Figure 2 demonstrates that both techniques precipitated the same two predominant immunogens (P1 and P2). These data confirm the selective precipitation of surface immunogens by the whole cell RIP assay. In addition, the results indicate that detergent-solubilization of either predominant immunogen had no deleterious effect on antibody recognition of antigenic determinants. Trypsin treatment of M. pneumoniae prior to whole cell RIP abrogated precipitation of proteins P1 and P2 (Fig. 3), confirming the identity of these trypsin-sensitive proteins (2).

2. Time Course Examination of Military Recruit Sera

The availability of sequential serum samples obtained from military recruits infected with M. pneumoniae (provided by Drs. J. Tully and L. Senterfit) allowed us to evaluate the host humoral immune response. The sera were tested by soluble-antigen RIP (Fig. 4) and the antibody

response to protein P1 was observed to increase and then decrease with time. Two of the representative patients (A and C) exhibited a selective response to protein P2 and a 70,000 MW protein that followed the same time course as the anti-P1 response. These data suggest that during convalescence anti-P1 antibody is made in response to infection, but extended convalescence results in a drop in anti-P1 titers. The actual time periods required for maximal antibody response are hard to define because of difficulties in determining time of infection. Also, variations among individual antibody responses to M. pneumoniae would be expected.

3. Time Course Evaluation of Experimentally Infected Hamster Sera

To further characterize the host serological response to mycoplasma pneumonia, sequential serum samples were obtained from hamsters intranasally infected with M. pneumoniae M129-B15. The sera were analyzed by soluble-antigen RIP. Infected hamsters produced antibody to both proteins P1 and P2 which could be detected at weeks 4 through 8 postinfection (Fig. 5). In addition, anti-P2 appeared to peak later and remained elevated through week 12 postinfection. The significance of this extended host response to P2 is unknown.

As an extension of these studies, and in conjunction with previous characterization of mutant and revertant strains of M. pneumoniae (see previous Annual Report), groups of hamsters were tagged, bled, and intranasally infected with 1-3 x 10^7 colony-forming units (CFU) of wild-type (B25C), mutant [HA1 (3); Class I, II, III, IV (4)], or revertant [HA1-R(5)] strains of M. pneumoniae. Control hamsters received an intranasal dose of Hayflick medium only. At weeks 1, 2, 4, 8, and 12 postinfection blood was removed by cardiac puncture. At week 13 postinfection all remaining animals were intranasally challenged with 5 x 10^6 CFU of wild-type M. pneumoniae. None of the hamsters infected with mutant or revertant strains of M. pneumoniae demonstrated a specific antibody response to infection. Wild-type-infected hamsters consistently revealed an antibody response to P1 and P2, as described above. However, reinfection of these hamsters with wild-type M. pneumoniae at week 13 after initial infection produced a very prominent response to P2 and a less notable serological response to protein P1 (data not shown). This may be due to the fact that anti-P2 antibodies were still detectable at the time of challenge, which may in turn have produced a stronger memory response to P2 when the organism was presented again.

B. Characterization of Revertant Strains of Spontaneous Hemadsorption-Negative Mutants

Examination of spontaneously-arising hemadsorption-negative (HA^-) mutants of M. pneumoniae permitted identification of several proteins which appear to play a role in cytadsorption and virulence (4). Subsequently, hemadsorption-positive (HA^+) revertants were isolated from each of 4 classes of spontaneous HA^- mutants (see previous Annual Report: 6). One dimensional sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) showed that revertants of class I mutants have reacquired proteins HMW 1-4.

1. Two-dimensional PAGE

The total protein profiles of wild-type strain B25C and four revertant strains of M. pneumoniae (derived from representatives of 4 different classes of HA^- mutants) were analyzed by two-dimensional PAGE (Table 1). Note that revertants of class III and class IV mutants have regained proteins A, B, and C. Since class II HA^- mutants had no detectable protein deficiencies, both class II mutant and revertant strains had two-dimensional PAGE profiles identical to that of wild-type strain B25C. The other three revertant strains also exhibited two-dimensional PAGE patterns identical to that of B25C, indicating that all revertants had reacquired specific M. pneumoniae proteins which were absent or reduced in the homologous HA^- mutants.

2. In vitro Tracheal Ring Attachment

The HA^+ revertant strains were assayed for attachment to control and neuraminidase-treated tracheal rings (Table 2). All revertants demonstrated levels of adherence equivalent to that of wild-type strain B25C, which is in contrast to the reduced tracheal ring attachment observed with the homologous HA^- mutant strains. The class III HA^+ revertant concomitantly regained proteins A, B, and C and the capacity to recognize sialic acid-containing receptors on target cells. This observation lends credence to a role for A, B, and/or C in mycoplasma cytadsorption.

3. Examination of the Relatedness of HMW 1-3

The spontaneous loss and return of a group of high molecular weight proteins in class I HA^- mutants and HA^+ revertants, respectively, was particularly interesting. If these proteins were unrelated, a significant part of the small M. pneumoniae genome would be used to code for the HMW proteins. A precursor-product relationship between some or all of these proteins would require less genetic information for expression of cytadsorption-associated proteins.

Peptide mapping of HMW 1-3 was done by the method of Cleveland et al (7). Untreated controls showed only limited proteolysis. Addition of 2.5 µg chymotrypsin to each protein produced notably different peptide patterns (Fig. 6). Similar results were observed when papain was substituted for chymotrypsin. These data suggest that HMW 1-3 are indeed distinct polypeptides.

Selected HMW proteins were also examined with respect to immunological relatedness. Antibodies against HMW1 and HMW3 were raised in rabbits by injecting excised, macerated preparative SDS-PAGE bands containing these proteins. Anti-HMW2 antiserum could not be produced by this technique. Anti-HMW1 and anti-HMW3 antisera were tested in a soluble-antigen RIP assay (Fig. 7). Anti-HMW1 antiserum precipitated both HMW1 and HMW4, but not HMW2 or HMW3. Anti-HMW3 antiserum precipitated only HMW3 and not the other HMW proteins. These results indicate that HMW1 and HMW3 are not immunologically related polypeptides. The precipitation of HMW4 by anti-HMW1 antiserum may be due to contamination of the HMW1 antigen preparation (gel slice) with HMW4, or may indeed be indicative of cross-reactivity resulting from relatedness.

C. Monospecific Antibodies

The production of monospecific antiserum against M. pneumoniae protein P1 was described in the previous Annual Report. Purified protein P1 was excised from preparative SDS-PAGE gels, macerated, combined with adjuvant, and injected into rabbits. RIP analysis of anti-P1 serum showed significant reactivity with a 165,000 dalton protein only. Monospecific anti-P1 antibodies were examined for biological activity.

1. Purification of Monospecific Antibodies

Normal rabbit serum and anti-P1 antiserum were passed over a protein A-Sepharose column, and unbound material was washed away with phosphate buffer. Bound immunoglobulin (Ig) was eluted with 1 M acetic acid, and protein was monitored spectrophotometrically at 280 nm. Fractions containing Ig were pooled, dialyzed, and concentrated. Protein content of the concentrated Ig preparations was 11 mg/ml for normal serum Ig and 20 mg/ml for anti-P1 Ig.

2. Fab Fragments of Anti-P1 Ig

The Fab fraction of anti-P1 Ig was obtained by papain digestion (150 µg, 4 h at 37°C) as described (8,9). After dialysis and centrifugation, the Fab preparation was passed over a protein A-Sepharose column to remove Fc fragments The void volume (purified Fab) was concentrated and purity of the preparation determined by SDS-PAGE and immunoelectrophoresis.

3. Anti-Metabolic Activity

The ability of anti-P1 antiserum to inhibit M. pneumoniae metabolism was examined by two techniques (8).

a. Reduction in Colony-Forming Units

The effect of anti-P1 antiserum on M. pneumoniae viability was determined by incubating mycoplasmas in heat-inactivated (HI) Hayflick medium with HI normal or immune serum or Ig, or with Fab fragments of anti-P1 Ig. After incubation at 37°C for 30 min, the M. pneumoniae suspensions were diluted and plated, and after 7 d growth the CFUs were quantitated. None of the preparations tested inhibited mycoplasma growth (Table 3), in contrast to a 10-fold decrease in viability observed when HI anti-M. pneumoniae serum was added.

b. Inhibition of [^{3}H]Thymidine Incorporation

A suspension of M. pneumoniae grown in HI Hayflick medium was aliquoted into microtiter plates (200 µl/well) and incubated overnight at 37°C. The spent medium was aspirated and replaced with 100 µl fresh HI Hayflick medium plus 50 µl HI normal or HI immune serum. After incubation for 3 h at 37°C the medium was removed and 200 µl fresh HI Hayflick medium containing 50 µCi/ml [^{3}H]thymidine were added per well. The plates were incubated 18 h at 37°C, the medium aspirated, the wells

washed once with PBS, and M. pneumoniae harvested on cotton-tipped swabs. The swabs were dried, precipitated with trichloroacetic acid (TCA), and analyzed by liquid scintillation spectrometry.

Anti-M. pneumoniae serum inhibited incorporation of [^{3}H] thymidine into TCA-precipitable counts by as much as 70% (Fig. 8). No effect on [^{3}H]thymidine incorporation was observed when mycoplasmas were preincubated with normal or anti-P1 serum. Therefore, if anti-P1 serum or Ig demonstrated anti-attachment activity, the inhibition of cytadsorption could not be attributed to metabolic impairment of mycoplasmas.

4. Inhibition of Cytadsorption

The ability of monospecific antibodies to inhibit M. pneumoniae attachment was examined with two different host cell targets - hamster tracheal rings and chicken erythrocytes. In both cases, wild-type M. pneumoniae were grown in 25 ml HI Hayflick medium containing 300 µCi [^{3}H]thymidine, washed with PBS, and resuspended in 2 ml HI Hayflick medium containing 0.05 M HEPES. The suspension was passed four times through a 25g needle, made to a final volume of 5 ml with HI Hayflick medium, and centrifuged at 250 x g for 5 min. Aliquots of this mycoplasma suspension were combined with various amounts of HI normal or anti-P1 serum, normal or anti-P1 Ig, anti-P1 Fab fragments, or a PBS-1% bovine serum albumin (PBS-BSA) control for 30 min at 37°C.

a. Tracheal Ring Attachment Inhibition

Aliquots of the mycoplasma - serum/Ig preparations were placed in microtiter wells (50 µl/well), and rinsed tracheal rings were added to each well. The plates were incubated 3.25 h at 37°C, and the rings were removed, blotted, rinsed, and solubilized with 1% SDS overnight. The rings were assayed for associated radioactivity by liquid scintillation spectrometry.

When the effect of Ig on tracheal ring attachment was examined, anti-P1 Ig showed a dose-dependent inhibition of M. pneumoniae cytadsorption, while normal rabbit serum Ig exhibited no effect (Fig. 9). Anti-P1 Fab fragments also inhibited attachment in a dose-dependent fashion (data not shown). The anti-attachment activity of the anti-P1 Ig preparation could be removed through adsorption with wild-type M. pneumoniae, but was not removed by adsorption with a P1-less mutant strain (data not shown).

b. Chicken Erythrocyte Attachment Inhibition

Aliquots (100 µl) of the mycoplasma - serum/Ig preparations were added to 50 µl volumes of a 4% (v/v) suspension of washed chicken erythrocytes. The mixtures were incubated on a gyratory shaker for 30 min at 37°C, overlaid on 150 µl of 40% sucrose, and centrifuged in a Microfuge B for 20 s to pellet erythrocytes and bound mycoplasmas. The pellets were incubated with SDS and hydrogen peroxide, and erythrocyte-associated mycoplasmas were quantitated by liquid scintillation counting. Anti-P1 Ig inhibited mycoplasma hemadsorption in a dose-

dependent manner and by greater than 90% (Fig. 10). Normal rabbit serum Ig exhibited no effect on hemadsorption.

Antibodies directed against P1 block M. pneumoniae cytadsorption to two different target cells (tracheal epithelium and chicken erythrocytes). This inhibition was not due to an effect of anti-P1 Ig on mycoplasma metabolism, but rather resulted from a disruption of ligand-receptor interaction(s), probably due to steric hindrance mediated by Ig.

5. Ultrastructural Analysis

In collaboration with Dr. Roger Cole, monospecific anti-P1 antibodies and immunoferritin electron microscopy were used to examine the topography of P1 on HA^+ wild-type and HA^- mutant strains of M. pneumoniae (10). Briefly, mycoplasmas were grown in SP-4 medium (11), glutaraldehyde-fixed, and washed. The pelleted organisms were suspended in buffer and appropriate dilutions of rabbit prebleed or monospecific anti-P1 antiserum were added. The mycoplasma-antibody mixtures were incubated 2 h at room temperature, washed, and resuspended in buffer. Diluted ferritin-conjugated IgG fraction of goat anti-rabbit IgG was added, and the suspension was incubated for 2 h at room temperature. The antibody-coated mycoplasmas were washed, suspended in 1% ammonium acetate, applied to coated grids, and examined by electron microscopy. Intact mycoplasmas were also mounted and visualized by negative staining.

Differences in P1 topography were observed when wild-type and mutant strains of M. pneumoniae were compared. Virulent mycoplasmas showed P1 clustering associated with a naplike terminal structure (Fig. 11), plus other less dense P1 regions along the surface of the organisms. Avirulent strains of M. pneumoniae which possessed P1 demonstrated no P1 clustering, only diffuse P1 localization over the entire surface (Fig. 12a). The HA^- mutant strain which does not possess P1 exhibited background immunoferritin labeling (Fig. 12b). These observations suggest that the ability to concentrate P1 in the terminal organelle of M. pneumoniae may be essential for cytadsorption and virulence.

D. Monoclonal Antibodies

Monoclonal antibodies directed against M. pneumoniae proteins were desirable for several reasons. Monoclonal antibodies are directed against native antigens, in contrast to monospecific antibodies raised against reduced, detergent-denatured mycoplasma proteins. Immune spleen cells which give rise to hybridomas can be obtained from immunized or infected mice, and monoclonal antibodies derived by different routes of antigen presentation may vary in specificity or subclass. Whole organisms can be used for immunizations necessary for hybridoma production circumventing problems with antigen purity and amount. Monoclonal antibodies obtained should recognize one epitope of immunogenic proteins, thus permitting very specific analyses of structure-function relationships and antigenicity.

1. Production of Monoclonal Antibodies

Mice (BALB/c, 6-10 wk) were immunized with intact M. pneumoniae by one of four protocols: 1) intraperitoneal (i.p.) injection of 50 µg M. pneumoniae strain B16 in saline on days 1 and 15 (MP1); 2) i.p. injection of 200 µg strain B16 in saline on day 1 and for each of two booster doses (MP2); 3) i.p. injection of 100 µg strain B16 in adjuvant on days 1 and 14 and 200 µg strain B16 in saline intravenously (i.v.) on day 30 (MP3); or 4) i.p. injection of 100 µg strain B16 in adjuvant on days 1 and 14 (MP4). Three days prior to fusing an immune mouse received 400 µg strain B16 in saline i.p. (MP1 and 2) or 200 µg i.v. (MP3). For fusion MP4 the immune mouse was boosted 3 days prior to fusion with P1-less M. pneumoniae HA$^-$ mutant strain 22 (100 µg in adjuvant i.p. and 100 µg in saline i.v.).

Spleen cells from an immune mouse were fused with SP2/0 myeloma cells in the presence of 50% polyethylene glycol, according to the procedure of Oi and Herzenberg (12). Hybrid clones were selected by growth in culture medium containing 2X hypoxanthine, thymidine, and aminopterin. Hybridoma culture supernatants were screened 7-10 d after fusion for anti-M. pneumoniae reactivity by enzyme-linked immunosorbent assay (ELISA)

2. ELISA

Freshly harvested M. pneumoniae were suspended in PBS and added to microtiter wells at a concentration of 10 µg mycoplasma protein per well. The wells were dried, the protein was ethanol-fixed to the polyvinylchloride, and the microtiter strips were stored at 4°C.

A modified ELISA (13,14) was employed for detection of anti-M. pneumoniae antibodies. Prior to use mycoplasma-coated wells were incubated with PBS-BSA to block nonspecific binding sites. The wells were washed and 50 µl of test serum diluted in PBS-BSA or hybridoma culture supernatant were added. The wells were incubated 1 h at 37°C (test sera) or overnight at 4°C (supernatants), and the wells were washed with PBS. Fifty microliters of alkaline phosphatase-conjugated sheep anti-mouse Ig diluted 1:1000 in PBS-BSA were added, and the wells were incubated 2 h at 37°C. The wells were washed and 50 µl of enzyme substrate (p-nitrophenyl phosphate; 1 mg per ml in diethanolamine buffer) were added to each well. The wells were incubated 30 min at 37°C, and anti-M. pneumoniae activity was measured spectrophotometrically by absorbance at 405 nm.

ELISA-positive hybridoma supernatants were tested in a soluble-antigen RIP assay (1). To ensure detection of all immunoglobulin isotypes reactive with M. pneumoniae proteins, the IgG fraction of rabbit anti-mouse Ig was added to antigen-hybridoma supernatant suspensions and incubated 90 min at 4°C before the addition of Staph A. The proportion of ELISA-positive supernatants which were positive by RIP assay ranged from 4-23%, depending on the immunization protocol. When all immunization procedures were grouped together, 13% of ELISA$^+$ supernatants were also RIP$^+$. The ELISA$^+$/RIP$^+$ supernatants may have contained monoclonal antibodies directed against non-protein mycoplasma

antigens (13). Table 4 presents the combined data obtained from 4 different fusions (different immunization protocols). Fusions MP1, 2, and 3 yielded several monoclonal antibodies, but all precipitated a 165,000 dalton protein identified as P1. In an attempt to circumvent this apparent immunodominance of protein P1, the mouse used for fusion MP4 was boosted with a strain of M. pneumoniae which does not possess P1. As seen in Table 4, several different protein specificities were found in RIP$^+$ hybridoma supernatants resulting from fusion MP4. The mycoplasma proteins precipitated by MP4 monoclonal antibodies ranged in size from 25,000 to 200,000 daltons. Several hybridoma supernatants precipitated more than one protein, even after single cell cloning procedures.

3. Immunoglobulin Isotyping

Isotyping of monoclonal antibodies was done by ELISA. Hybridoma supernatants were added to mycoplasma-coated microtiter wells, incubated, and washed, as described in the previous subsection. Rabbit anti-mouse IgG1, IgG2a, IgG2b, IgG3, IgM, and IgA were diluted 1:8 in PBS-BSA, 50 µl were added to designated wells, and the wells were incubated 2 h at 37°C. The wells were washed and 50 µl of alkaline phosphatase-conjugated sheep anti-rabbit Ig (1:1000 in PBS-BSA) were added. Plates were then processed as previously described. As shown in Table 4, all anti-M. pneumoniae monoclonal antibodies were IgG or IgM isotypes. Anti-P1 monoclonals were predominantly of subclass IgG2a . The majority of monoclonal antibodies from fusion MP4 were IgM or IgG1.

4. Ascites Growth of Hybridomas

Pristane-primed BALB/c mice were given an i.p. injection of 5×10^6 hybridoma cells in saline. Seven to 10 days later ascites fluid was tapped from the peritoneal cavity, and hybridoma cells were removed from the Ig-containing fluid by centrifugation.

5. Purification of Monoclonal Antibody

Hybridoma tissue culture supernatants or ascites fluid of appropriate isotypes were applied to a protein A-Sepharose affinity column and processed as previously described (see Section C.1).

6. Whole Cell RIP Analysis of Selected Monoclonal Antibodies

The antibody accessibility of target epitopes on intact M. pneumoniae was examined by whole cell RIP (as described in Section A.1). In addition, mycoplasmas were treated with trypsin (10 µg, 10 min at 37°C) just prior to whole cell RIP to determine proteolytic susceptibility of target epitopes. Figures 13, 14, and 15 show the results of such experiments. Monoclonal antibody $2G_4$, which precipitates protein P1, recognized a trypsin-sensitive epitope which was accessible to antibody on intact M. pneumoniae (Fig. 13). In contrast, the epitope recognized by monoclonal antibody $1D_{11}$ was trypsin-insensitive and antibody-inaccessible on intact cells (Fig. 14). This monoclonal antibody precipitates a 200,000 dalton protein which comigrates with HMW1. Monoclonal antibody $9G_4$ precipitates 3 proteins (155,000, 42,000, and 30,000 daltons), and whole cell RIP indicated that all 3 proteins possess an antibody-accessible

epitope on intact M. pneumoniae, but only the 155,000 dalton protein had a trypsin-sensitive epitope (Fig. 15). These data suggest that RIP techniques, in conjunction with proteolytic treatment of intact mycoplasmas, will permit specificity analysis of anti-M. pneumoniae monoclonal antibodies.

7. Assay of Inhibition of M. pneumoniae Metabolism

Since selected monoclonal antibodies bind to epitopes presented on the mycoplasma cell surface, and because anti-M. pneumoniae antiserum has been shown to exert anti-metabolic activity, selected monoclonals were examined for anti-metabolism activity according to the procedure previously described (see Section C.3b). Table 5 demonstrates that none of the monoclonals or normal mouse serum inhibited M. pneumoniae incorporation of [^{3}H]thymidine when tested at two different dilutions. In contrast, mouse anti-M. pneumoniae antiserum inhibited [^{3}H]thymidine incorporation by 55% when compared to a buffer control. It is possible that one monoclonal antibody directed against one epitope is insufficient to cause metabolic alterations, but perhaps a mixture of monoclonals which would bind to many epitopes accessible at the mycoplasma surface would mimic the inhibitory effects of mouse anti-M. pneumoniae antiserum.

E. Mycoplasma Ligand-Receptor Studies

The previous Annual Report described a technique for identification of M. pneumoniae proteinaceous surface ligands important in attachment to host cell receptors. When a preparation of detergent-solubilized M. pneumoniae was incubated with glutaraldehyde-fixed hamster trachea epithelial (HTE) cells, three mycoplasma proteins were avidly bound to the eukaryotic target cells (15). The three bound proteins were identified as P1, P2, and HMW3 (molecular weights - 165,000, 110,000, and 140,000, respectively) on the basis of electrophoretic mobility, trypsin-sensitivity, and HA mutant analysis. Hoechst staining confirmed that intact, virulent M. pneumoniae did bind to glutaraldehyde-fixed HTE cells, indicating that fixation had no adverse effect on the biological activity of HTE receptor molecules.

Competitive binding assays were performed to determine the specificity of binding of mycoplasma proteins to HTE cells. A constant amount of soluble, radiolabeled M. pneumoniae protein was combined with increasing amounts of soluble, unlabeled mycoplasma protein prior to incubation with fixed HTE cells. Figure 16 shows that as the ratio of unlabeled to radiolabeled M. pneumoniae protein increased, the percent of radiolabeled protein bound decreased linearly. These data suggest that binding of mycoplasma surface proteins to HTE cells is mediated by specific ligand-receptor interactions, and the saturability of binding indicates the existence of a finite population of HTE receptors.

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Legends to Figures

Fig. 1 Whole cell RIP of cell surface, antibody-accessible [^{35}S]methionine-labeled M. pneumoniae proteins with acute and convalescent sera. From left to right, the first lane is the SDS-PAGE profile of total, ^{35}S-labeled M. pneumoniae proteins. The second lane is a control RIP performed in the absence of serum. The remaining lanes are RIP patterns obtained with paired pre-bleed and postinfection hamster sera and paired acute and convalescent human sera, as indicated. Molecular weight markers (myosin, 200,000; phosphorylase b, 92,500; bovine serum albumin, 68,000; ovalbumin, 43,000; and chymotrypsinogen, 25,700) and proteins P1 and P2 are indicated to the right.

Fig. 2 Comparison of whole cell RIP of intrinsically labeled M. pneumoniae (left) and soluble-antigen RIP of extrinsically labeled mycoplasmas (right). From left to right, the first lane in "^{35}S-Whole Cell" is the SDS-PAGE profile of TDSET-soluble, [^{35}S]methionine-labeled M. pneumoniae proteins. Lane 2 is a control RIP performed without added serum. The next four lanes are RIP patterns obtained with paired acute and convalescent human sera and paired pre-bleed and postinfection hamster sera, as indicated. The first lane in the "^{125}I-Soluble" set is the SDS-PAGE profile of total ^{125}I-labeled M. pneumoniae proteins. The sera and controls used in the remaining lanes are identical to those described in "^{35}S-Whole Cell." Molecular weight markers are indicated.

Fig. 3 Whole cell RIP of control and trypsin-treated intact M. pneumoniae. Mycoplasmas were labeled with [^{35}S]methionine and were incubated briefly with PBS or trypsin (5 µg). The enzymatic reaction was terminated by addition of trypsin inhibitor (10 µg). Control (left) and trypsinized (right) M. pneumoniae total radiolabeled SDS-PAGE profiles are presented in the first lane of each set. Whole cell RIP patterns of control and trypsin-treated mycoplasmas obtained with paired acute and convalescent human sera are shown in the accompanying lanes, as indicated. Proteins P1 and P2 and molecular weight markers are indicated to the right.

Fig. 4 RIP analysis of sequential human serum samples. Serum was collected from patients A, B, and C at the time points indicated, with day 1 representing the time of initial serum collection. The first lane represents TDSET-soluble, ^{35}S-labeled M. pneumoniae proteins. The remaining lanes are soluble-antigen RIP profiles produced by the sequential serum samples obtained from individual patients. Molecular weight markers and proteins P1 and P2 are indicated.

Fig. 5 Examination of sequential hamster serological response to M. pneumoniae infection. Hamsters were intranasally inoculated with ~5 x 10^6 CFU of virulent M. pneumoniae. The first lane is the SDS-PAGE profile of TDSET-soluble, intrinsically labeled mycoplasma protein. The accompanying lanes are the soluble-antigen RIP patterns produced by pre-bleed (week 0) and postinfection sera drawn from a representative animal at the designated time

points. Proteins P1 and P2 and molecular weight markers are indicated.

Fig. 6 Peptide mapping of M. pneumoniae proteins HMW1-3. (a) Untreated control profiles; (b to d) profiles produced after treatment with 0.5, 1.25, and 2.5 μg of chymotrypsin, respectively. Lane: A, HMW1; B, HMW2; C, HMW3. Proteins were separated on a 12% polyacrylamide gel and visualized by silver staining.

Fig. 7 Examination of the relatedness of M. pneumoniae proteins HMW1-3 by RIP analysis employing anti-HMW1 (a) and anti-HMW3 (b) antisera. In set (a): lane A contains ^{14}C-labeled molecular weight markers; lanes B and C are RIP patterns produced by pre-bleed (lane B) and anti-HMW1 (lane C) serum; lane D, total radiolabeled mycoplasma protein. In set (b), lane A contains total ^{35}S-labeled M. pneumoniae protein; lanes B and C are RIP profiles obtained with pre-bleed (lane B) and anti-HMW3 (lane C) serum; lanes D and E are RIP patterns produced by pre-bleed (lane D) and anti-M. pneumoniae (lane E) serum; lane F contains ^{14}C-labeled molecular weight markers.

Fig. 8 Effect of heat-inactivated normal, anti-P1, or anti-M. pneumoniae serum on [^3H]thymidine incorporation by M. pneumoniae. The values represent the means and standard deviations obtained from quadruplicate samples.

Fig. 9 Influence of pretreatment of M. pneumoniae with normal rabbit serum Ig (•) or rabbit anti-P1 serum Ig (Δ) on mycoplasma attachment to hamster tracheal rings. Normal and anti-P1 Ig preparations were processed and concentrated comparably from equivalent volumes of serum. Values represent the means and standard deviations from quadruplicate samples, and are expressed as percentages of PBS-BSA control values. The background level of mycoplasma attachment to tracheal rings at 4°C is indicated by the dashed line.

Fig. 10 M. pneumoniae adherence to chicken erythrocytes following pretreatment of mycoplasmas with normal rabbit serum Ig (•) or rabbit anti-P1 serum Ig (Δ). Values represent means and standard deviations obtained from triplicate samples and are expressed as percentages of PBS-BSA control values. The dashed line represents the background level of mycoplasma attachment to erythrocytes at 4°C.

Fig. 11 (a) Negatively stained, intact virulent M. pneumoniae with characteristic nap (solid arrow) and truncated terminus (open arrow). (b) Immunoferritin labeled virulent M. pneumoniae demonstrating highly dense clustering of P1 at the terminus, with less dense P1 sites along the remaining membrane surface. Magnification: (a) ×125,000; (b) ×76,000.

Fig. 12 Immunoferritin labeling of avirulent HA^- mutants of M. pneumoniae. (a) M. pneumoniae class I mutant which possesses P1. P1 sites

can be observed along the membrane surface, but no clustering of P1 can be seen at the termini. (b) M. pneumoniae class IV mutant which lacks P1 and demonstrates little binding of immunoferritin label. Magnification: (a) x63,000; (b) x55,000.

Fig. 13 RIP analysis of monoclonal antibody $2G_4$. Intact [^{35}S]methionine-labeled M. pneumoniae were incubated with PBS (-ENZ) or trypsin (+ENZ) prior to soluble-antigen or whole cell RIP. Lane 1, total radiolabeled M. pneumoniae proteins; lanes 2 and 3, whole cell RIP of untreated (lane 2) and trypsin-treated (lane 3) mycoplasmas; lanes 4 and 5, soluble-antigen RIP of untreated (lane 4) and trypsin-treated (lane 5) mycoplasmas; lane 6, total protein profile of trypsinized, ^{35}S-labeled M. pneumoniae.

Fig. 14 RIP analysis of monoclonal antibody $1D_{11}$. The lanes are as those described in Fig. 13.

Fig. 15 RIP analysis of monoclonal antibody $9G_4$. The lanes are as those described in Fig. 13.

Fig. 16 Competitive binding analysis of HTE cell-binding M. pneumoniae proteins. Percent binding was quantitated by densitometer scanning of autoradiograms and calculation of the area under each peak, with maximum binding (100%) determined in the absence of unlabeled material. Ratios of labeled to unlabeled material were determined with a modified Lowry protein assay (16).

Figure 1

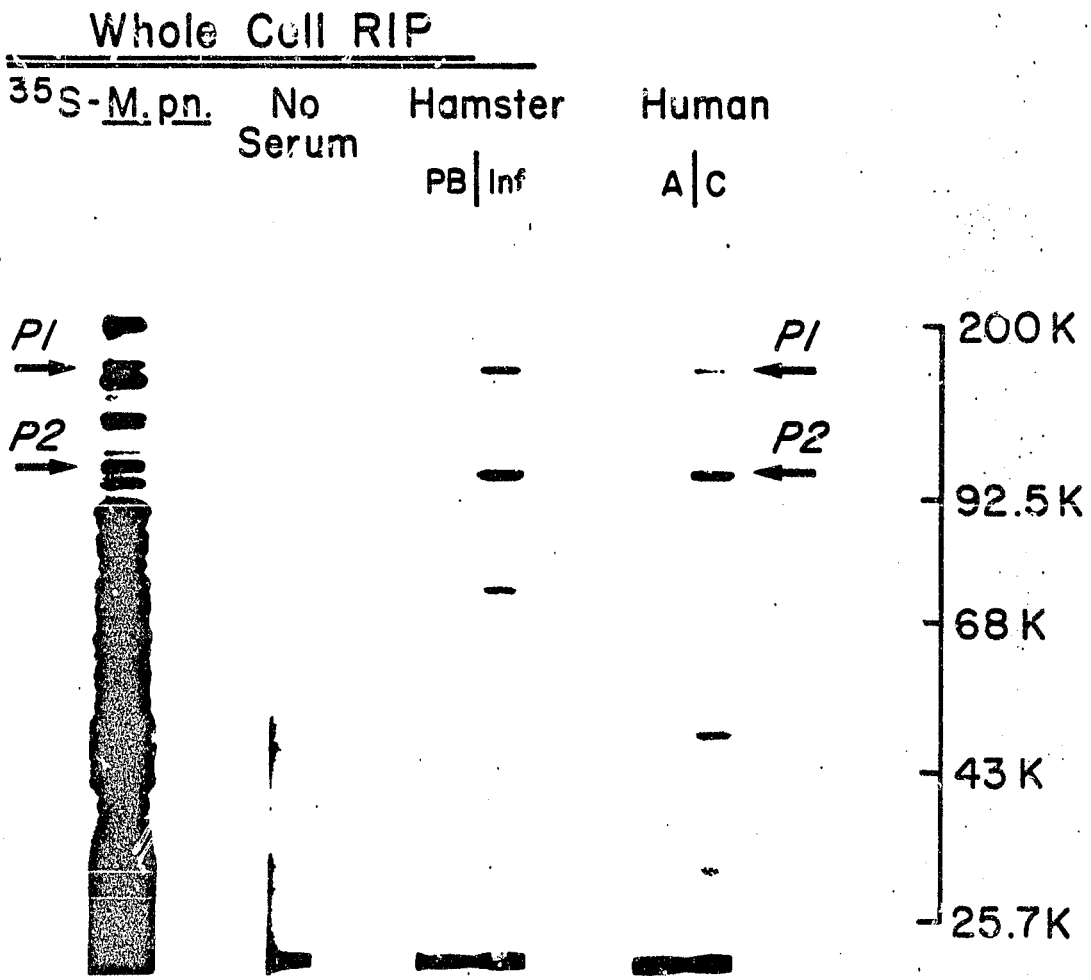

Whole Cell RIP
35S-M.pn.
No Serum
Hamster
PB | Inf
Human
A | C
P1
P2
P1
P2
200K
92.5K
68K
43K
25.7K

Figure 2

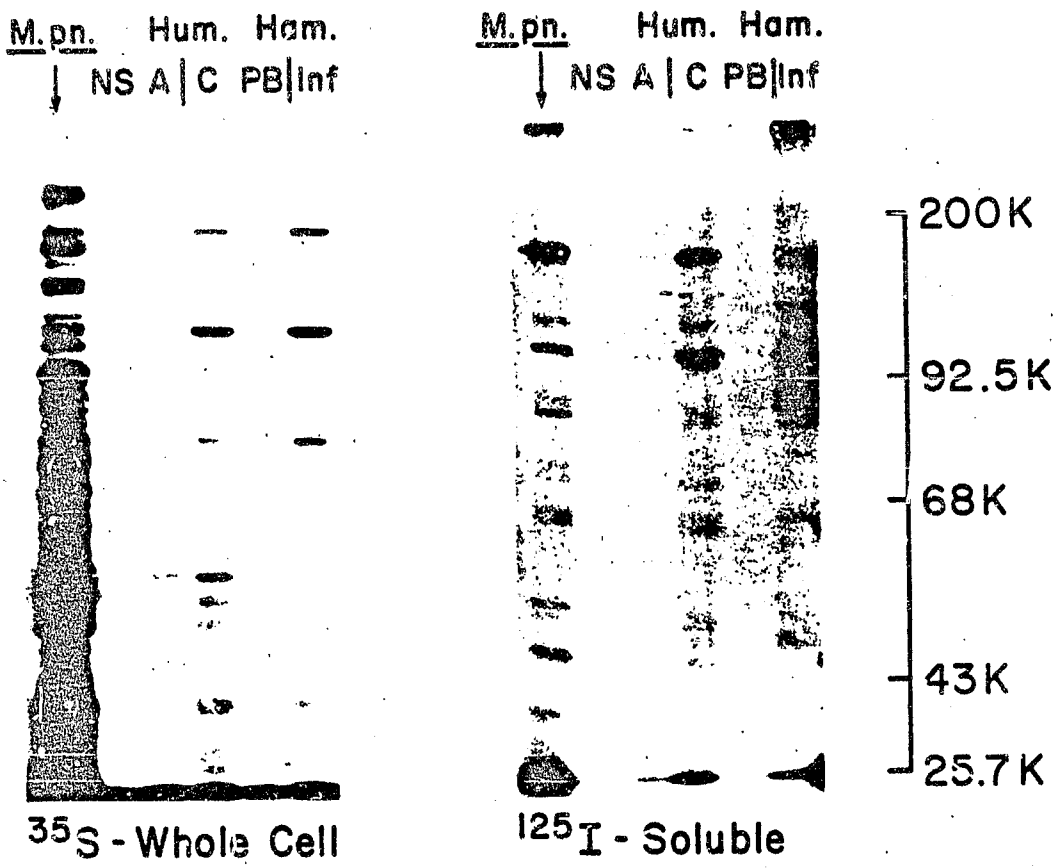

M.pn.
Hum.
Ham.
NS
A
C
PB
Inf
200K
92.5K
68K
43K
25.7K
35S - Whole Cell
125I - Soluble

Figure 3

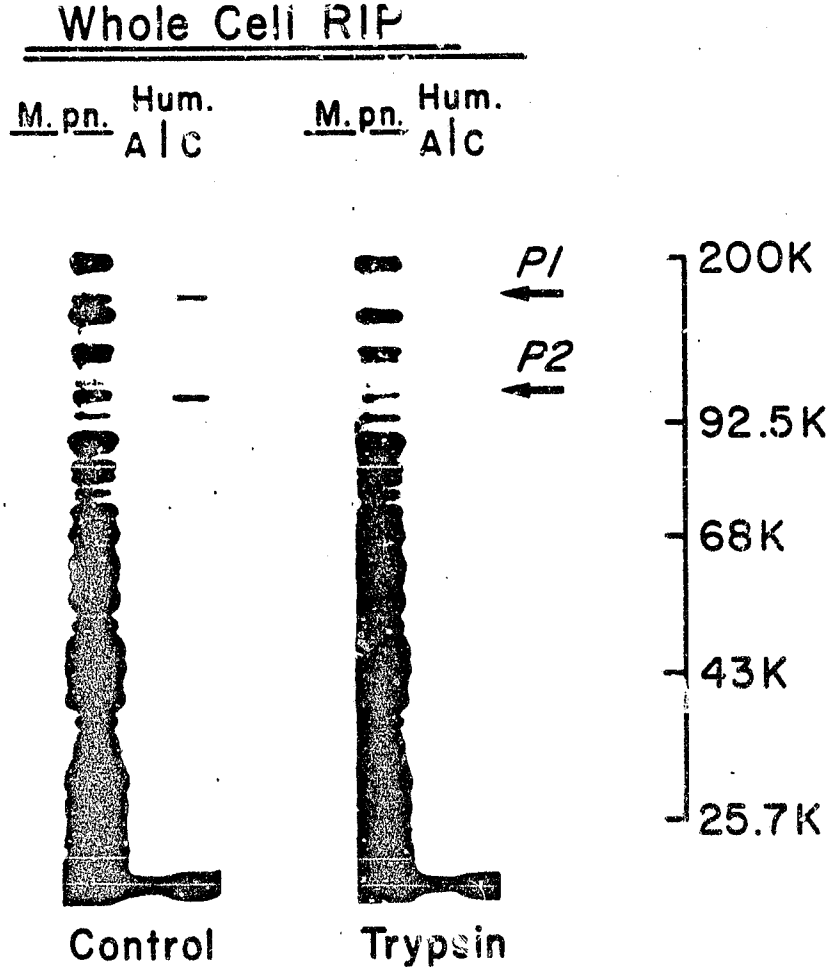
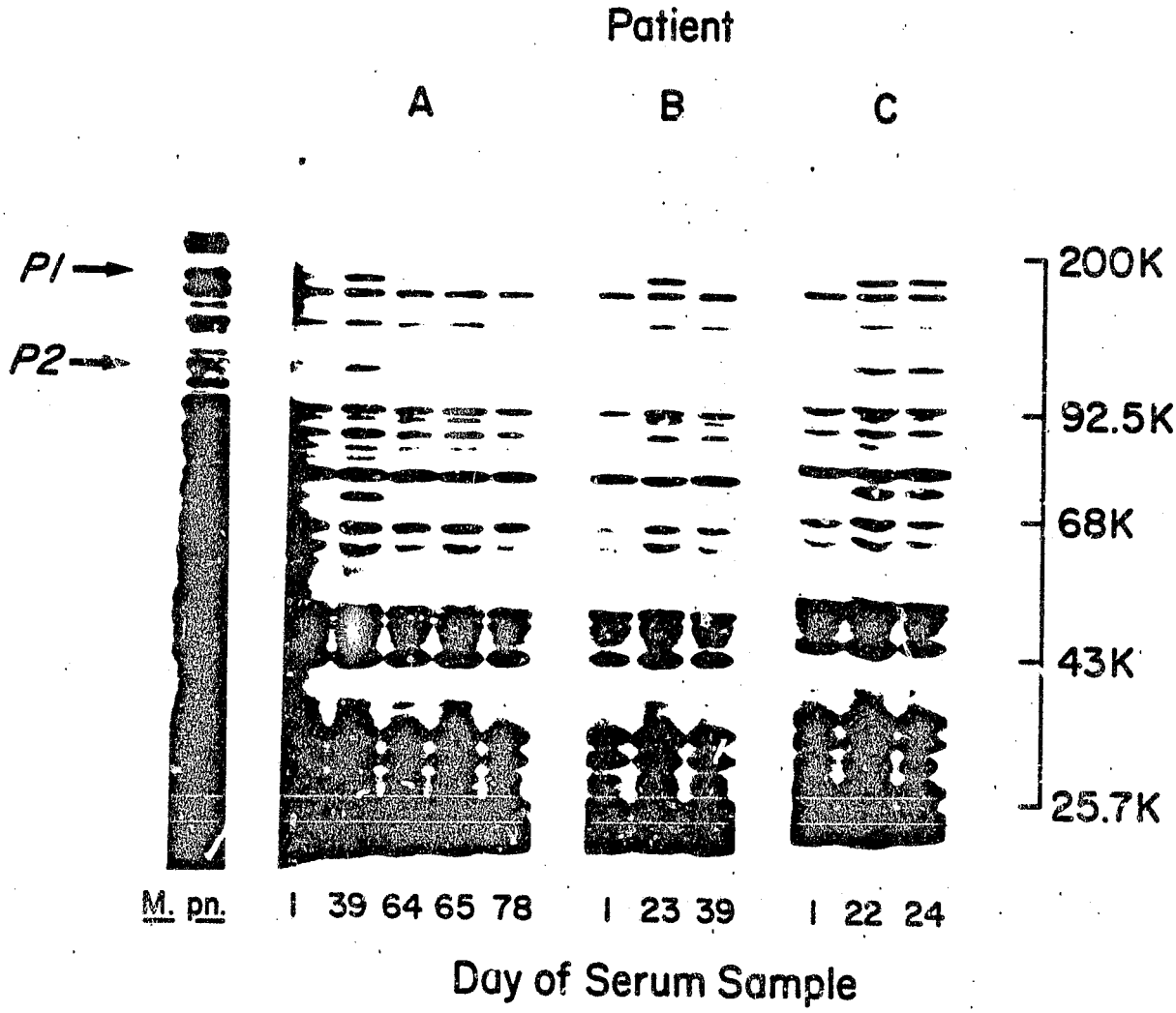

Whole Cell RIP
M.pn.
Hum. A|C
M.pn.
Hum. A|C
P1
P2
200K
92.5K
68K
43K
25.7K
Control
Trypsin

Figure 4


Patient
A
B
C
P1
P2
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92.5K
68K
43K
25.7K
M. pn.
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1 23 39
1 22 24
Day of Serum Sample

Figure 5

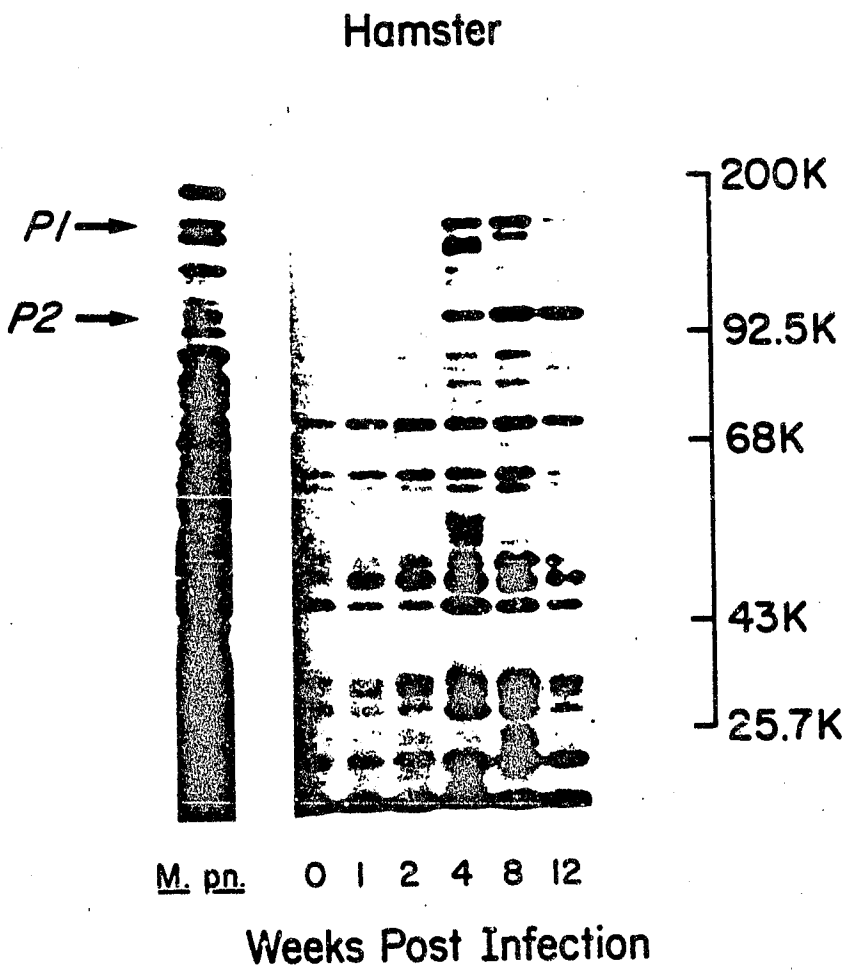

Hamster
P1
P2
200K
92.5K
68K
43K
25.7K
M. pn.
0 1 2 4 8 12
Weeks Post Infection

Figure 6

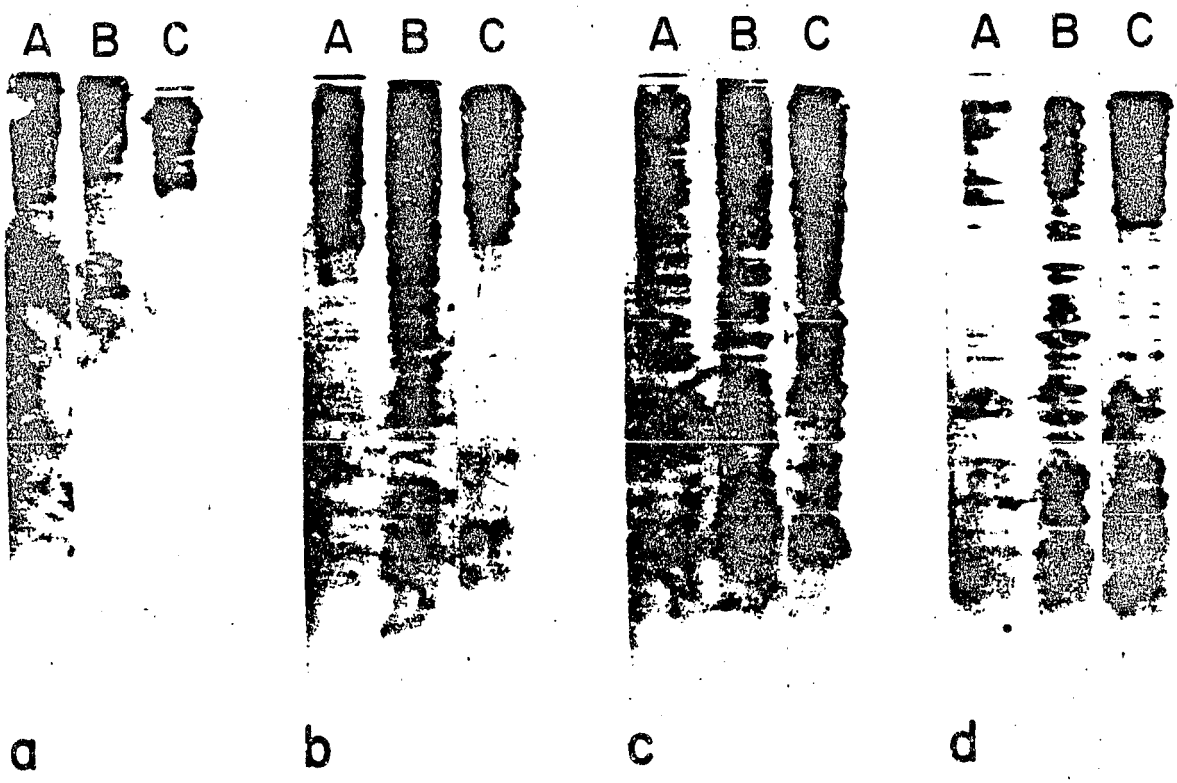
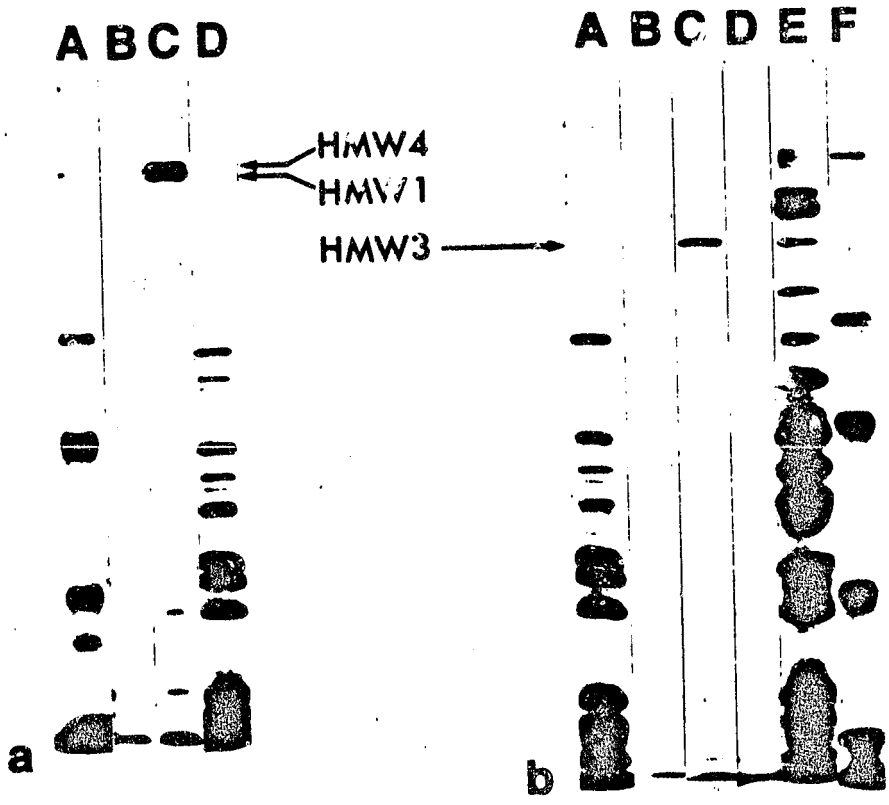


Figure 7



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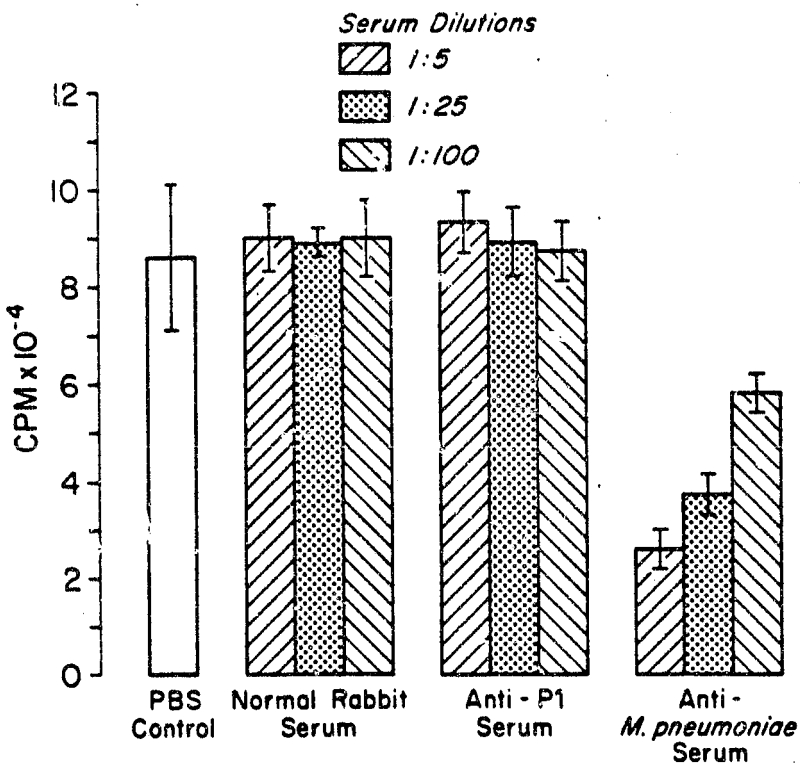
Serum Dilutions
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1:25
1:100
CPM x 10⁻⁴
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2
4
6
8
10
12
PBS Control
Normal Rabbit Serum
Anti - P1 Serum
Anti - M. pneumoniae Serum

Figure 9

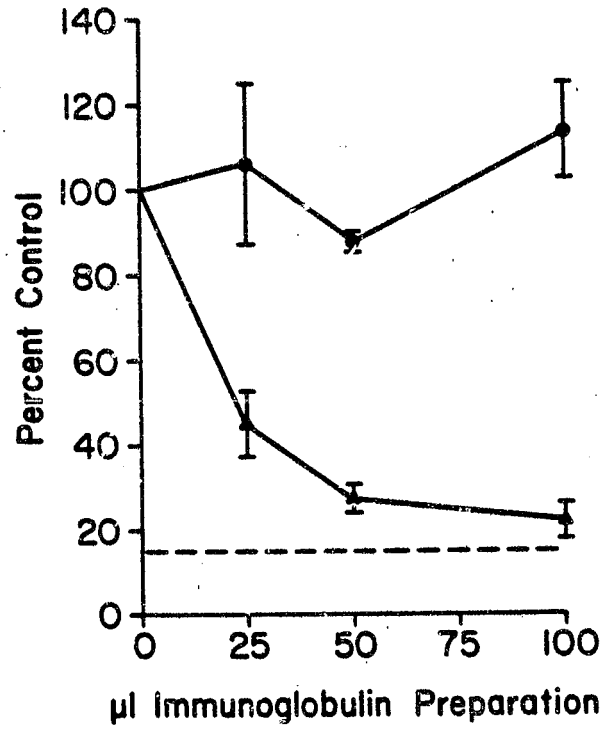
140
120
100
80
60
40
20
0
Percent Control
0
25
50
75
100
µl Immunoglobulin Preparation

Figure 10

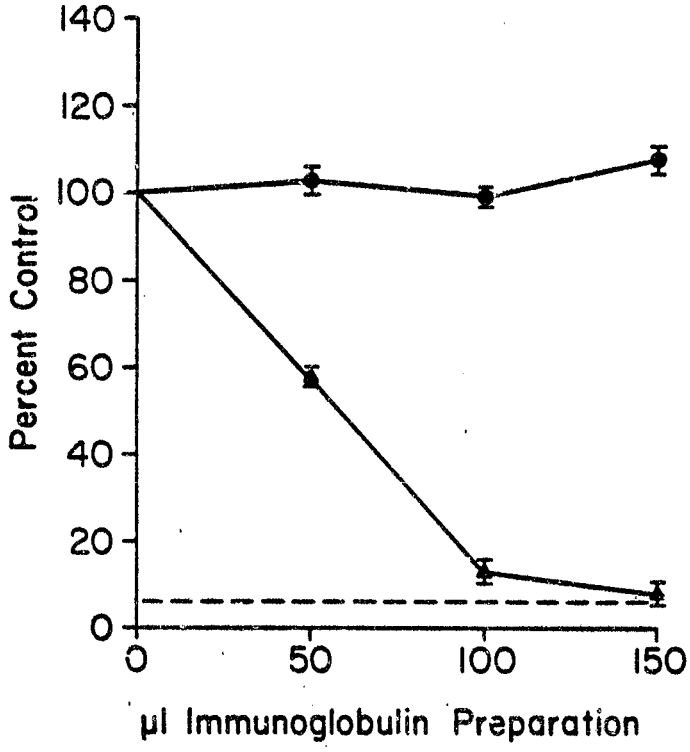
140
120
100
80
60
40
20
0
Percent Control
0
50
100
150
µl Immunoglobulin Preparation

Figure 11

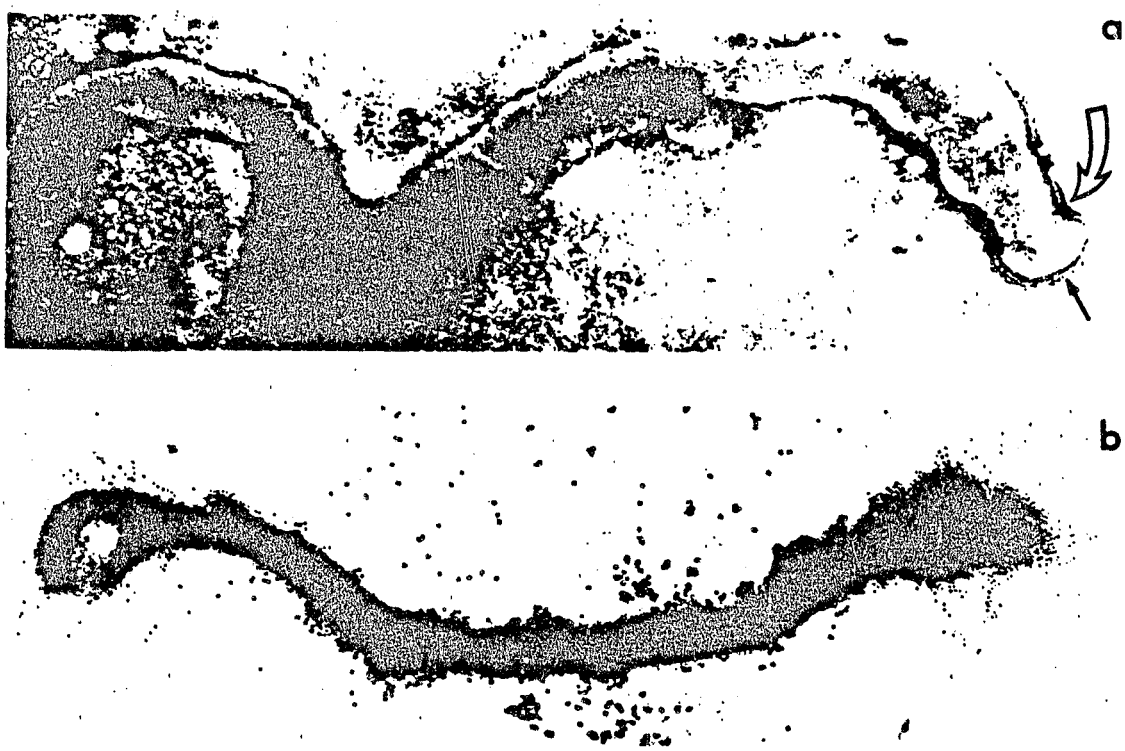

Figure 12

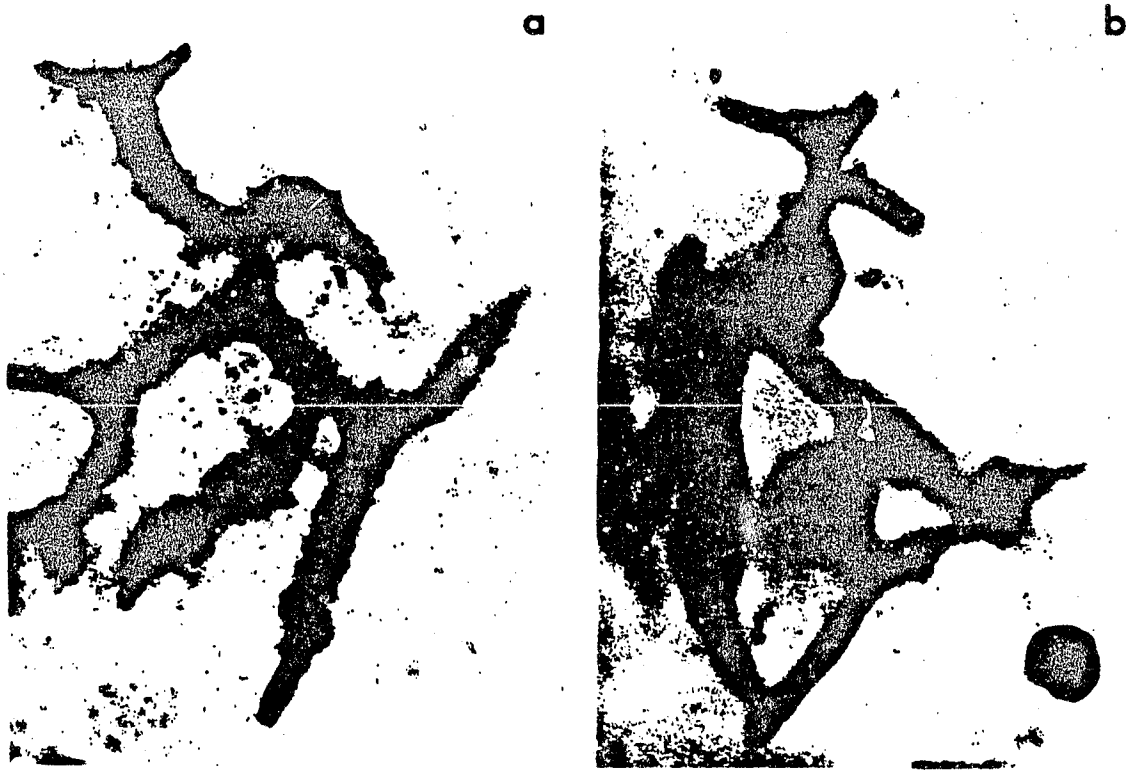

Figure 13

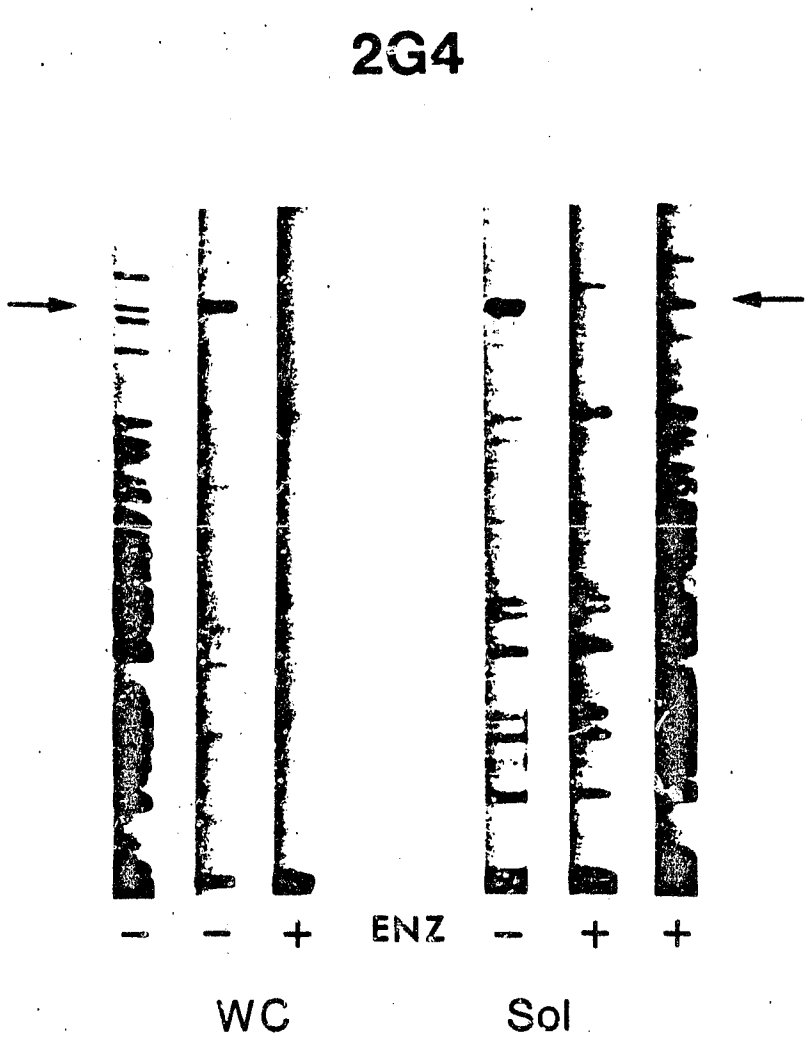

Figure 14

Figure 15

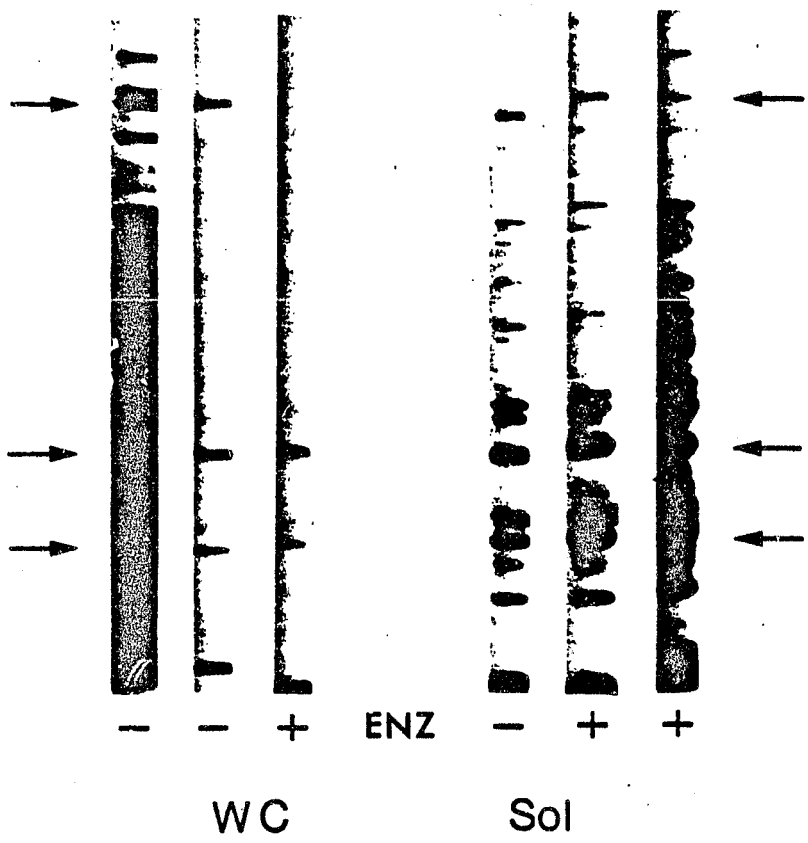

Figure 16

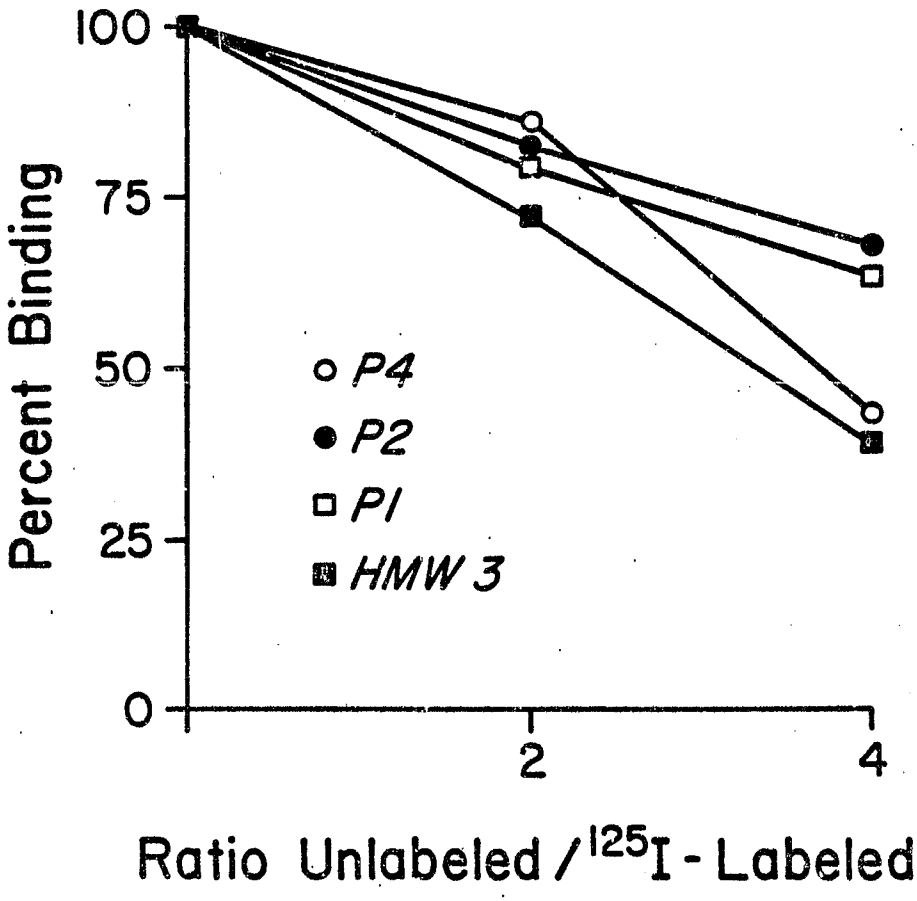
100
75
50
25
0
Percent Binding
P4
P2
P1
HMW 3
2
4
Ratio Unlabeled / 125I - Labeled

Table 1. Summary of the protein profiles of the spontaneous HA^- mutant classes and the homologous class-specific HA^+ revertants

Strain	Phenotype	Protein profile[a]							
		HMW1	HMW2	HMW3	HMW4	A	B	C	P1
M129-B25C	Wild-type	+	+	+	+	+	+	+	+
Class I	HA^-_+ Mutant	±	-	-	-	+	+	+	+
	HA^+ Revertant	+	+	+	+	+	+	+	+
Class I'[b]	HA^- Mutant	±	-	-	-	+	+	+	+
Class II	HA^-_+ Mutant	+	+	+	+	+	+	+	+
	HA^+ Revertant	+	+	+	+	+	+	+	+
Class III	HA^-_+ Mutant	+	+	+	+	-	-	-	+
	HA^+ Revertant	+	+	+	+	+	+	+	+
Class IV	HA^-_+ Mutant	+	+	+	+	-	-	-	-
	HA^+ Revertant	+	+	+	+	+	+	+	+

[a]Protein present; ±, protein markedly deficient or a minor comigrating polypeptide present; —, protein absent.

[b]No HA^+ revertant isolated.

Table 2. Adherence of wild-type and HA$^+$ revertant M. pneumoniae to control and neuraminidase-treated hamster tracheal rings in vitro[a]

Strain	Adherence	
	% of Control[b]	% Inhibition by Neuraminidase[c]
M129-B25C[d]/37°C	100	64
4°C[e]	22	-
Class I-R[f]	102	55
Class II-R	116	66
Class III-R	94	62
Class IV-R	114	56

[a] Values represent the means from two separate experiments. All assays were performed in quadruplicate. The data were evaluated using the nonparametric Kruskall-Wallis analysis of variance. Differences observed among wild-type and revertant strains (at 37°C) were statistically insignificant ($p>0.05$).

[b] % of control = [(revertant counts per minute bound/revertant counts per minute added)/(wild type counts per minute bound/wild type counts per minute added)] x 100%.

[c] % Inhibition = {1 — [(counts per minute bound to neuraminidase-treated rings/counts per minute added)/(counts per minute bound to control rings/counts per minute added)]} x 100%.

[d] Wild-type strain.

[e] Tracheal rings infected with B25C at 4°C served as a negative control.

[f] Designation for HA$^+$ revertants from each class of HA$^-$ mutants.

Table 3. Viability of M. pneumoniae following exposure to anti-P1 or normal rabbit serum immunoglobulin

Treatment	Amt (µl)	Log_{10}CFU/ml
PBS + 1% bovine serum albumin		6.75
Normal rabbit serum immunoglobulin	25 µl	6.68
	100 µl	6.64
Anti-P1 immunoglobulin	25 µl	6.64
	100 µl	6.79
Anti-P1 Fab fragments	25 µl	6.71
	100 µl	6.64

Table 4. Monoclonal antibodies to M. pneumoniae proteins

Molecular Weight of Protein Antigen	Number of Clones	Representative Clone	Representative Isotype
32K	6	$H13.4F_4$ $H13.14D_4$	IgG_1 IgM
127K	2	$H13.7H_{11}$	IgM
165K	8	$H5.3F_5$ $H5.2G_4$ $H12.24G_8$	IgG_1 IgG_{2a} IgG_{2b}
200K	3	$H13.1B_2$ $H13.1D_{11}$ $H13.18H_7$	IgG_1 IgG_3 IgM
28,25K	1	$H13.16F_{10}$	IgG_{2b}
72,42K	1	$H13.14E_7$	IgM
127,42K	1	$H13.22C_9$	IgG_{2b}
155,42K	2	$H13.18E_7$	IgM
120,55K	1	$H13.4G_4$	IgM
155,42,30K	7	$H13.9G_4$ $H13.7G_1$	IgG_1 IgM

Table 5. Effect of monoclonal antibodies on M. pneumoniae metabolism

Antibody preparation	% Control
5 µg SP 2/0	106
24 µg $2G_4$	102
5 µg $2G_4$	105
20 µg $1D_{11}$	109
4 µg $1D_{11}$	104
21 µg $9G_4$	109
4 µg $9G_4$	110
20 µg $4F_4$	104
4 µg $4F_4$	118
1:5 dilution normal mouse serum	112
1:5 dilution immune mouse serum	55

Final Summary Report

Our attempts to understand the virulence of Mycoplasma pneumoniae and to develop ways of preventing and interrupting the progression of disease have required immunologic, chemical and genetic strategies during this final contract period. We defined the key mycoplasma membrane protein components that mediated cytadherence to respiratory cells and reinforced the role of specific mycoplasma proteins by isolating spontaneous mutants and revertants of M. pneumoniae and characterizing them by in vitro and in vivo criteria for loss or reacquisition of virulence. The important interaction of multiple proteins in the activation and lateral mobility and clustering of the major mycoplasma ligand, protein P1, was demonstrated.

Identification of critical mycoplasma immunogens was accomplished by comparing prebleed, acute and convalescent sera from hamsters and humans and visualizing "immunologic conversion" of the host to specific M. pneumoniae proteins. These data are detailed in the last 2 annual progress reports and have permitted current developments regarding rapid diagnosis of primary atypical pneumonia and rationalization of vaccine candidates.

Generation of a spectrum of immunological probes including polyclonal and monoclonal antibodies has assisted in structure-function analysis of virulent and avirulent mycoplasmas, purification of membrane proteins by antibody affinity chromatography with possible application to the diagnosis of disease. In addition, the library of monoclonal antibodies has been used to assess anti-attachment, anti-metabolic and anti-growth properties of these immunoglobulins.

Our current studies on human pathogenic mycoplasmas are a direct extention of the previously supported research by the U.S. Army Medical Research and Development Command and we are indebted for research support during the past years.

Publications

1. Hu, P.C., A.M. Collier, and J.B. Baseman. 1975. Alterations in the metabolism of hamster tracheas in organ culture after infection by virulent Mycoplasma pneumoniae. Infect. Immun. 11:704-710.

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Mycoplasma pneumoniae; respiratory disease; radioimmunoprecipitations; mycoplasma immunogens; spontaneous hemadsorbing revertants; monospecific antibodies; ultrastructural analysis; monoclonal antibodies; ELISA; ligand-receptor interactions.

20. ABSTRACT (Continue on reverse side if necessary and identify by block number) The host humoral immune response to M. pneumoniae infection was further characterized. Examination of isogenic revertants of spontaneous nonhemadsorbing mutants led to analysis of the biochemical/immunological relatedness of selected virulence-associated M. pneumoniae proteins. Monospecific antiserum against protein P1 was examined for effects on mycoplasma viability, metabolism, and cytadsorption, and was employed for immunoferritin electron microscopy studies. Monoclonal antibodies against M. pneumoniae were produced and initial characterizations were begun. This report includes a final summary for the entire contract period.

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