

HOST DISCRIMINATION OF *MYCOPLASMA PNEUMONIAE* PROTEINACEOUS IMMUNOGENS*

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Mycoplasma pneumoniae is a non-invasive pathogen which colonizes the mucosal surface of the respiratory tract (1-3). The resultant primary atypical pneumonia occurs most frequently in children and young adults (4-6), but the incidence of disease is probably underestimated because the symptoms are relatively mild and diagnostic methods are suboptimal (6, 7). Infection with *M. pneumoniae* is often accompanied by (a) the appearance of cold agglutinins in the patient's serum (8, 9); (b) a rise in complement fixation (CF)¹ titer (8, 10); and (c) the production of antibody that inhibits metabolic processes of the pathogen (8, 11, 12). Still, the role of the immune response during *M. pneumoniae* infection is not understood. Studies performed with intranasally infected hamsters demonstrated immunoglobulin-producing cells in association with pulmonary infiltrates (13). Similar experiments cannot be done with human tissue because of the infrequency of mortality caused by mycoplasma pneumonia. However, some pertinent observations have been made with human patients and volunteers and with the experimental hamster model. Prior infection with virulent *M. pneumoniae* provides some protection against subsequent disease in both hamsters and humans (14-18). It is not known whether this protective effect is due to cellular or humoral immunity or both. Other reports suggest that humoral immunity may affect disease expression. For example, immunodeficient patients with B cell dysfunction are more severely ill with *M. pneumoniae* infection than normal patients, but show no evidence of radiographic pneumonia (19).

The most commonly used serological test for *M. pneumoniae* is a CF assay that uses a lipid hapten extracted from the organism as the target antigen (9, 20). However, lipids are rarely good immunogens unless complexed to protein (21). To evaluate whether specific *M. pneumoniae* proteins are immunogenic during natural infection, radioimmunoprecipitation (RIP) was used to examine infected human and hamster sera. This paper reports the identification of two predominant proteinaceous immu-

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¹ Abbreviations used in this paper: CF, complement fixation; CFU, colony-forming units; PAGE, polyacrylamide gel electrophoresis; PBI, phosphate-buffered iodide; PBS, phosphate-buffered saline; RIP, radioimmunoprecipitation; SDS, sodium dodecyl sulfate; SP buffer, 0.1 M Tris-HCl (pH 6.8), 2% SDS, 20% glycerol, 2% β -mercaptoethanol, 0.02% bromophenol blue; Staph A, formalin-treated protein A-bearing *Staphylococcus aureus*; TDSET, 10 mM Tris-HCl (pH 7.8), 0.2% sodium deoxycholate, 0.1% SDS, 10 mM EDTA, 1% Triton X-100.

nogens of *M. pneumoniae* which are precipitated by convalescent, but not by acute, sera.

Materials and Methods

Organisms and Culture Conditions. Virulent, wild type *M. pneumoniae* strains M129-B15 (22, 23) and M129-B25C (24) were used in this study. Monolayer cultures were grown in 32-oz (~960 ml) prescription bottles in Hayflick medium (25) at 37°C for 48–72 h, until the phenol red pH indicator in the growth medium became orange. The monolayers were washed four times with phosphate-buffered saline (PBS) (pH 7.2) before collection by centrifugation at 9,500 g for 15 min. For intrinsic labeling of *M. pneumoniae* with [³⁵S]methionine, log-phase cultures were washed once with PBS and pulsed for 6 h at 37°C with 250 µCi of L-[³⁵S]methionine (Amersham Corp., Arlington Heights, IL) (1,050–1,400 Ci/mmol sp act) in Hanks' balanced salts solution containing 10% dialyzed horse serum. The monolayers were washed once with PBS containing 1 mM cold methionine, chased for 1.5 h at 37°C in Hayflick medium, and then washed four times with PBS containing 1 mM cold methionine before collection as above. Mycoplasmas were extrinsically labeled in suspension by a lactoperoxidase-catalyzed radiiodination procedure (3, 26). Washed *M. pneumoniae* monolayers or cultures of *Mycoplasma orale* and *Mycoplasma salivarium* (provided by Dr. J. Tully) were suspended in 1 ml PBS, and 0.5 mCi carrier-free [¹²⁵I]NaI (Amersham Corp.) and 50 µg lactoperoxidase (E.C. 1.11.1.7; 100 IU/mg; Calbiochem-Behring Corp., San Diego, CA) were added. The radiiodination reaction was initiated by the addition of 25 µl of 0.001% hydrogen peroxide, and 10 min later another 25-µl aliquot of hydrogen peroxide was added. After another 10-min interval, the reaction was terminated by the addition of 5 ml phosphate-buffered iodide (PBI; similar to PBS, but with sodium iodide substituted for sodium chloride). The iodinated mycoplasmas were pelleted by centrifugation, and washed twice with PBI and once with PBS before use.

Antisera. Paired acute and convalescent human sera obtained from patients with *M. pneumoniae* were provided by Dr. C. Helms, University of Iowa, Iowa City, IA. These pairs of human sera were selected because they demonstrated a detectable rise in *M. pneumoniae* CF titer. Sequential serum samples obtained from military recruits participating in an *M. pneumoniae* vaccine field trial were provided by Dr. J. Tully and Dr. L. Senterfit. Only those recruits who later contracted mycoplasma pneumonia were selected for this evaluation of sequential serum response to *M. pneumoniae*. Control human serum samples collected from patients who did not have mycoplasma pneumonia were provided by the clinical pathology department of Medical Center Hospital, San Antonio, TX. Preinfection hamster sera were obtained by cardiac puncture of uninfected Syrian golden hamsters. Some hamsters were then intranasally inoculated (23, 27) with 10⁸ colony-forming units (CFU) of *M. pneumoniae* strain B15 and bled on day 26 postinfection to obtain high-dose-infected anti-*M. pneumoniae* sera. For the time course evaluation of hamster humoral immune response to mycoplasma infection, hamsters were intranasally inoculated with ~5 × 10⁶ CFU of wild type, virulent *M. pneumoniae* strain B25C. Hamsters were bled at 1, 2, 4, 8, and 12 wk postinfection. Rabbit anti-*M. pneumoniae* antiserum was obtained by subcutaneous and intramuscular injection of 10¹⁰ CFU of wild type *M. pneumoniae* in Freund's complete adjuvant, followed three weeks later by a booster of 10¹⁰ mycoplasmas in Freund's incomplete adjuvant. Blood was drawn 5–14 d after the booster injection.

Soluble-antigen RIP Assay. The RIP assay used for the identification of soluble *M. pneumoniae* immunogens was a modification (28) of the method of Baseman and Hayes (29). Fresh or frozen radiolabeled mycoplasma pellets were resuspended in 250 µl of 10 mM Tris-HCl (pH 7.8), 0.2% (wt/vol) sodium deoxycholate, 0.1% (wt/vol) sodium dodecyl sulfate (SDS), 10 mM EDTA, and 1% (vol/vol) Triton X-100 (TDSET) (30) that contained 100 mM phenylmethylsulfonyl fluoride. The suspension was incubated at room temperature for 5 min, 20 µl of ovalbumin (50 mg/ml) was added, and the suspension was incubated at 37°C for 15 min. This solution was then centrifuged over a cushion of 5% sucrose in TDSET at 100,000 g for 45 min to remove insoluble material. The supernatant was carefully removed and then diluted with TDSET to the desired volume. This soluble antigen preparation was divided into 100-µl aliquots, and 20 µl test antiserum was added. The antigen-antiserum mixtures were incubated at 37°C for 15 min before incubation at 4°C overnight. 50 µl of a washed 10% suspension of formalin-treated

protein A-bearing *Staphylococcus aureus* (Staph A) was added to each aliquot, and the suspensions were placed on a rocker platform for 90 min at 4°C. The Staph A, with adsorbed immune complexes, was washed four times with TDSET. The labeled, precipitated antigens were eluted by resuspending the Staph A pellet in 30 μ l of 0.1 M Tris-HCl (pH 6.8), 2% (wt/vol) SDS, 20% (vol/vol) glycerol, 2% (wt/vol) β -mercaptoethanol, and 0.02% (wt/vol) bromophenol blue (SP buffer) (31) and boiling the suspension for 3 min. The Staph A was pelleted and the supernatant was subjected to SDS-polyacrylamide gel electrophoresis (PAGE).

Whole-Cell RIP Assay. Hansen et al. (32) have recently described an RIP procedure which permits identification of antibody-accessible proteins on the cell surface of pathogens. This procedure, which will be referred to as a whole-cell RIP assay, was used to identify surface immunogens of *M. pneumoniae* that are accessible to antibody in their native conformation. Briefly, [³⁵S]methionine-labeled *M. pneumoniae* were suspended in cold PBS, and 125- μ l aliquots of this suspension were mixed with 40 μ l test antisera. The suspensions were placed on a rocker platform for 90 min at 4°C to allow antibody binding to accessible mycoplasma surface proteins. The mycoplasmas were pelleted in a Microfuge B (Beckman Instruments, Inc., Palo Alto, CA) and washed once with PBS to remove unadsorbed antibody. The organisms were resuspended in 1.0 ml TDSET, vortexed, and incubated at 37°C for 1 h (with periodic vortexing to ensure efficient solubilization). Insoluble material was removed by centrifugation at 45,000 g for 1 h. The uppermost 0.9 ml of supernatant was carefully transferred to another tube, and 250 μ l of washed Staph A was added to each supernatant. The suspensions were placed on a rocker platform and incubated for 90 min at 4°C. The Staph A was washed four times with TDSET, and adsorbed *M. pneumoniae* surface immunogens were eluted in 35 μ l SP buffer, as described above, before SDS-PAGE.

Trypsin Treatment of Intact *M. pneumoniae*. Intrinsically labeled ([³⁵S]methionine) *M. pneumoniae* was resuspended in 100 μ l PBS, and 5.0 μ g trypsin (type XI; Sigma Chemical Co., St. Louis, MO) was added. The suspensions were incubated at 37°C for 10 min, and the enzymatic reaction was stopped by the addition of 10 μ g trypsin inhibitor (type II-0; Sigma Chemical Co.). After incubation for 10 min at 0°C, the trypsin-treated mycoplasmas were used in a whole-cell RIP assay.

SDS-PAGE and Fluorography. One-dimensional SDS-PAGE was performed according to Laemmli (33), using a 1.5-mm slab consisting of a 3% stacking and 7.5% separating gel. ¹⁴C-labeled molecular weight standards (Bethesda Research Laboratories, Rockville, MD) were myosin (200,000), phosphorylase b (92,500), bovine serum albumin (68,000), ovalbumin (43,000), and chymotrypsinogen (25,700). Upon completion of electrophoresis, the slab was placed in fixative (methanol/acetic acid/H₂O, 45:10:45) overnight. The gel was then processed for fluorography according to the method of Bonner and Laskey (34).

Results

Soluble-antigen RIP of Hamster and Human Sera. *Mycoplasma pneumoniae* proteins that are immunogenic in intranasally inoculated hamsters and infected human patients were identified by RIP (Fig. 1). The lane labeled “³⁵S-M. pn.” is the one-dimensional PAGE profile of the TDSET-soluble fraction of total [³⁵S]methionine-labeled *M. pneumoniae* proteins. The binding of this detergent-soluble fraction to Staph A in the absence of antiserum is negligible, as demonstrated in the lane designated “No Serum.” However, two proteins with apparent molecular weights of 74,000 and 30,000 are weakly, but consistently, adsorbed to Staph A in the absence of antiserum. The lanes labeled “Hamster PB/Inf” are representative RIP patterns produced by prebleed and postinfection (day 26, high-dose inoculation) hamster sera. Preinfection hamster serum precipitates minimal amounts of radiolabeled *M. pneumoniae* proteins. Precipitation of the 74,000 and 30,000 proteins, which bind weakly to Staph A in the absence of serum, is enhanced in the presence of serum. Postinfection hamster serum demonstrates a response to numerous mycoplasma proteins not recognized by preinfection serum. Among the predominant immunogens that were precipitated postin-

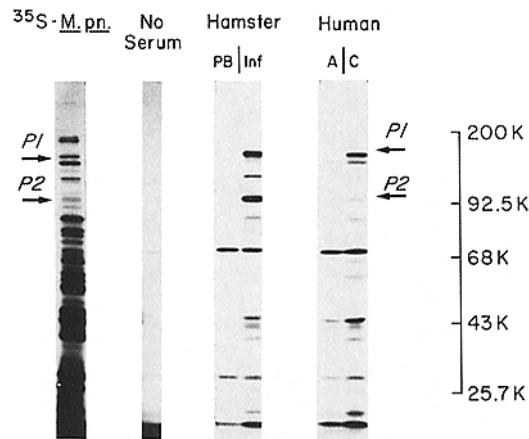


FIG. 1. Soluble-antigen RIP of intrinsically labeled *M. pneumoniae* with acute and convalescent sera. Mycoplasmas were radiolabeled with [^{35}S]methionine and RIP analysis was performed as described in the text. From left to right, the first lane displays the one-dimensional SDS-PAGE profile of the TDSET-soluble fraction of ^{35}S -labeled *M. pneumoniae* proteins. The second lane shows the minimal binding of TDSET-soluble proteins to Staph A in the absence of acute or convalescent sera (designated "No Serum"). The next two lanes are representative RIP profiles produced by paired pre-bleed and high-dose infection hamster sera, and the last two lanes are representative RIP patterns produced by paired acute and convalescent human sera. Molecular weight markers (K = 10^3) are indicated to the right, and proteins P1 and P2 are indicated on both the right and left.

fection are proteins with apparent molecular weights of 165,000 and 110,000. When human acute and convalescent sera are assayed by RIP ("Human A/C"), the resultant precipitation patterns are remarkably similar to those produced by pre- and postinfection hamster sera. Examination of paired antisera by RIP revealed that the majority of infected hamster and human sera precipitated the 165,000 protein during convalescence only. However, the 110,000 protein, which was also precipitated by a majority of postinfection hamster sera, was precipitated poorly, if at all, by convalescent human sera. When 15 serum samples obtained from patients who did not have *M. pneumoniae* were tested in the RIP assay, none precipitated the 165,000 or 110,000 protein (data not shown). The 165,000 protein co-migrates on SDS-PAGE with protein P1, and the 110,000 protein co-migrates with protein P2, both of which have been previously described by us (3). Proteins P1 and P2 have been localized on the surface of *M. pneumoniae* by ^{125}I -labeling and are sensitive to brief trypsin treatment of intact mycoplasmas (3, 35). In addition, P1 and P2 bind in vitro to respiratory epithelial cells (35).

Whole-Cell RIP. Immunogens that elicit host protective antibody in vivo are commonly exposed at the surface of the pathogen and are accessible to antibody. The whole-cell RIP assay permitted identification of antibody-accessible immunogens on intact *M. pneumoniae* (Fig. 2). The first lane is a total [^{35}S]methionine-labeled *M. pneumoniae* protein profile, and the second lane shows the binding of labeled antigens to Staph A in the absence of antiserum. The next pair of lanes is representative of whole-cell RIP patterns produced by pre-bleed and postinfection (day 26, high-dose inoculation) hamster sera, whereas the last two lanes are representative of whole-cell precipitation patterns obtained with human acute and convalescent sera. The paired sera from hamsters and humans yield almost identical whole-cell RIP profiles. Note

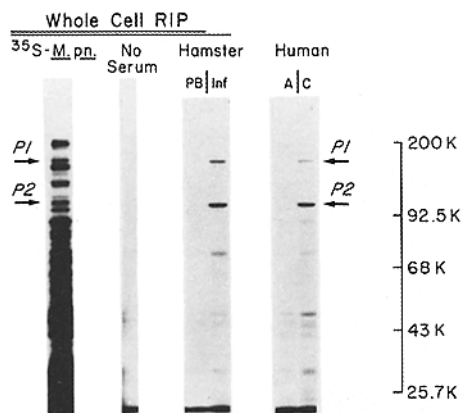


FIG. 2. RIP of cell-surface, antibody-accessible ^{35}S -labeled *M. pneumoniae* proteins with acute and convalescent sera. The whole-cell RIP assay was performed as described in the text. From left to right, the first lane is the SDS-PAGE profile of total ^{35}S -methionine-labeled *M. pneumoniae* proteins. The second lane is the whole-cell RIP assay with no serum control. The remaining lanes are representative whole-cell RIP profiles of paired pre-bleed and high-dose infection hamster sera and paired acute and convalescent human sera, as indicated. Molecular weight markers ($\text{K} = 10^3$) and proteins P1 and P2 are indicated to the right.

the decreased precipitation of labeled mycoplasma proteins by hamster preinfection and human acute sera when the whole-cell (rather than the soluble-antigen) RIP assay is used. Also, hamster and human convalescent sera strongly precipitate both proteins P1 and P2 in the whole-cell RIP assay, in contrast to the human convalescent precipitation pattern detected by the soluble-antigen RIP (Fig. 1).

Intrinsically Labeled Whole-Cell RIP vs. Extrinsically Labeled Soluble-Antigen RIP. In theory, a whole-cell RIP performed with ^{35}S -methionine intrinsically labeled *M. pneumoniae* should produce a precipitation pattern very similar to that derived from a soluble-antigen RIP employing lactoperoxidase-catalyzed, radioiodinated intact *M. pneumoniae*. Fig. 3 presents such a comparison. Although the intrinsically and extrinsically labeled *M. pneumoniae* total protein profiles are significantly different, the precipitation of predominant surface immunogens with either method (^{35}S -methionine whole-cell or ^{125}I -soluble-antigen RIP) is nearly identical. These results confirm the selective precipitation of surface proteins by the whole-cell RIP assay and indicate that detergent solubilization does not adversely affect antibody recognition of *M. pneumoniae* antigens.

*Whole-Cell RIP of Trypsinized *M. pneumoniae*.* Brief trypsin treatment of intact mycoplasmas will remove proteins P1 and P2 from the surface of the mycoplasmas (3) and thus should abolish precipitation of these proteins by convalescent sera in a whole-cell RIP assay. Comparison of control and trypsin-treated ^{35}S -methionine-labeled *M. pneumoniae* total protein profiles revealed that the band that co-migrates with protein P1 is removed upon trypsinization, whereas the band that co-migrates with protein P2 is reduced in intensity (Fig. 4). Convalescent human sera no longer precipitate either protein P1 or P2 (or other minor immunogens; see Figs. 2 and 3) when intact mycoplasmas are trypsin treated before use in a whole-cell RIP assay. The complete removal of P2 precipitation by antibody suggests that the residual protein at 110,000 remaining after trypsin treatment is probably another protein of the same or similar molecular weight.

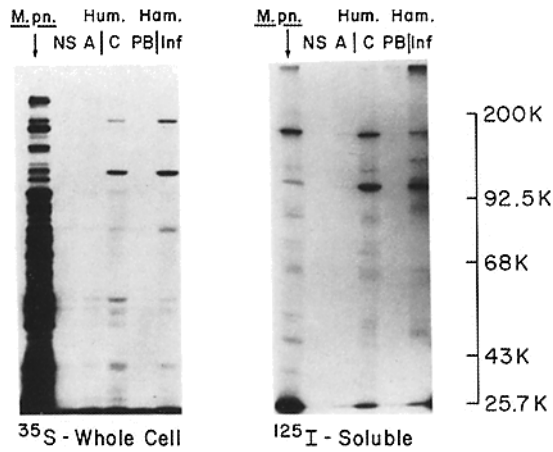


FIG. 3. Comparison of precipitation patterns produced by whole-cell RIP of intrinsically labeled *M. pneumoniae* and soluble-antigen RIP of extrinsically labeled *M. pneumoniae*. From left to right in the "³⁵S-Whole Cell" PAGE profiles, lane 1 is the SDS-PAGE profile of TDSET-soluble, [³⁵S]methionine-labeled mycoplasma proteins. Lane 2 is the whole-cell RIP assay with no serum (NS) control. The next four lanes are representative whole-cell precipitation patterns produced by paired acute and convalescent human sera and pre-bleed and high-dose infection hamster sera, as indicated. From left to right in the "¹²⁵I-Soluble" PAGE profiles, lane 1 is the SDS-PAGE profile of total ¹²⁵I-labeled *M. pneumoniae* proteins. Radioiodination of mycoplasmas was performed as described in the text. The second lane shows the ¹²⁵I-labeled, soluble-antigen RIP assay with no serum control. The remaining lanes are representative whole-cell precipitation patterns produced by acute and convalescent human sera and pre-bleed and high-dose infection hamster sera, as indicated. Molecular weight markers (K = 10³) are indicated to the right.

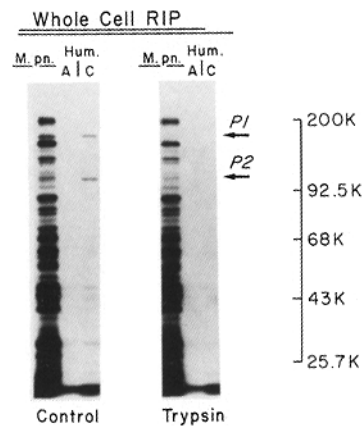


FIG. 4. Comparison of whole-cell RIP of control and trypsin-treated intact *M. pneumoniae*. Mycoplasmas were intrinsically labeled with [³⁵S]methionine and were incubated briefly with PBS or trypsin, as described in the text, before use in a whole-cell RIP assay. The control (left) and trypsin-treated (right) *M. pneumoniae* total radiolabeled protein profiles are presented in the first lane of each set. Whole-cell RIP of control and trypsin-treated mycoplasmas with a representative pair of acute and convalescent human sera produced the precipitation patterns shown in the accompanying lanes, as indicated. Molecular weight markers (K = 10³) and proteins P1 and P2 are indicated to the right.

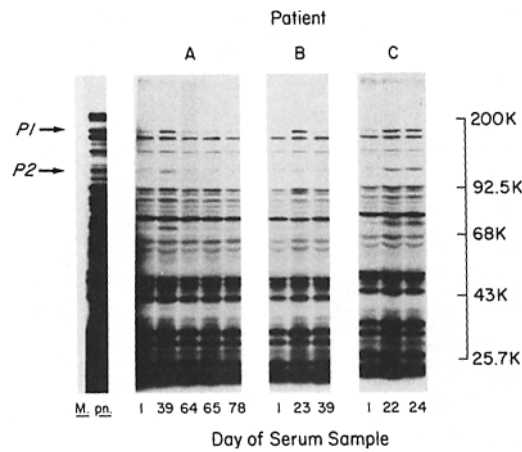


FIG. 5. Examination of sequential human serological response to *M. pneumoniae* infection. Serum was collected from patients A, B, and C at the time points indicated, with day 1 representing the time of the initial serum sample. The first lane represents the SDS-PAGE profile of TDSET-soluble, [³⁵S]methionine-labeled *M. pneumoniae* proteins. The remaining lanes show the precipitation patterns produced when the sequential serum samples obtained from patients A, B, and C are subjected to soluble-antigen RIP analysis. Proteins P1 and P2 are indicated to the left, and molecular weight markers ($K = 10^3$) are indicated to the right.

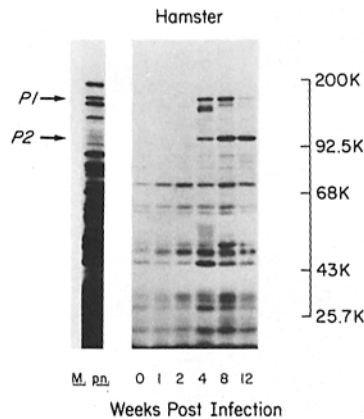


FIG. 6. Time course evaluation of the hamster serological response to *M. pneumoniae* infection. Hamsters were inoculated intranasally with virulent *M. pneumoniae* as described in the text. The first lane represents the SDS-PAGE profile of TDSET-soluble, [³⁵S]methionine-labeled *M. pneumoniae* proteins. The remaining lanes depict a soluble-antigen RIP performed with pre-bleed (week 0) and postinfection hamster sera drawn from an individual animal at the designated time points. Proteins P1 and P2 and molecular weight markers ($K = 10^3$) are indicated.

Time Course Evaluation of Human and Hamster Serological Response to Infection. To further examine the host humoral response to *M. pneumoniae*, sequential serum samples obtained from patients or infected hamsters were examined by a soluble-antigen RIP (Figs. 5 and 6). In human patients, antibody to P1 increased and then decreased with time (Fig. 5). Note that patients A and C also demonstrated a selective antibody response to protein P2 and a protein with a molecular weight of ~70,000 that followed the same kinetics as the anti-P1 response. Hamsters infected with wild type *M. pneumoniae* strain B25C similarly showed an increase in anti-P1 antibody as the

infection progressed, followed by a decrease with time (Fig. 6). The anti-P2 response maximized slightly later than the anti-P1 response, but remained high through 12 wk postinfection.

RIP Using other Mycoplasma Species. The oropharynx of most humans is colonized with at least one mycoplasma species (36). It is possible that this normal flora stimulates a host immune response that is cross-reactive with *M. pneumoniae* antigens. To examine this possibility, *M. salivarium* and *M. orale* were radioiodinated and tested in the soluble-antigen RIP assay with rabbit anti-*M. pneumoniae* antiserum. The total protein and RIP patterns were compared with those of *M. pneumoniae* strain B15 (Fig. 7). The iodinated protein profiles of the three mycoplasma species were very dissimilar. Neither oral mycoplasma possessed proteins that co-migrate with protein P1 or P2. Anti-*M. pneumoniae* serum precipitated all iodinated strain B15 proteins but recognized no *M. orale* or *M. salivarium* proteins. These data demonstrate that there is little cross-reactivity between oral mycoplasmas and *M. pneumoniae* and further suggest that RIP analysis of convalescent sera detects a specific immune response to *M. pneumoniae*.

Discussion

Several *M. pneumoniae* proteins have been associated with cytoadsorption and virulence (3, 23, 24, 28, 37), which implies that these macromolecules play an essential role in successful parasitism. The host immunologic response to mycoplasma pneumonia may be directed against such attachment factors or virulence-specific proteins. Although the literature suggests that *M. pneumoniae* stimulates cellular, humoral, and secretory immune responses (13, 18, 38-41), we chose to examine the host humoral response to specific mycoplasma proteins. The RIP technique, previously shown to be a powerful tool for analyzing the immune response to proteinaceous immunogens of pathogens (29, 30, 32, 42-44), was used to examine acute and convalescent human and hamster sera.

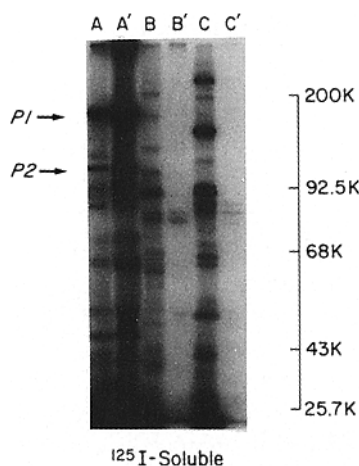


FIG. 7. Analysis of the specificity of the humoral immune response to *M. pneumoniae* immunogens. Lane A is the total, radioiodinated protein profile obtained from virulent *M. pneumoniae*. Lane A' is the soluble-antigen RIP of radioiodinated *M. pneumoniae* proteins by rabbit anti-*M. pneumoniae* antiserum. Lanes B, B' and C, C' are the corresponding profiles produced when radioiodinated *M. orale* (B, B') and radioiodinated *M. salivarium* (C, C') are substituted for ^{125}I -labeled *M. pneumoniae* (A, A'). Proteins P1 and P2 and molecular weight markers (K = 10^3) are indicated.

Preinfection hamster and acute human sera usually precipitate a small number of mycoplasma proteins in the soluble-antigen RIP assay. This precipitation may be due to cross-reactive antibodies or may result from a nonspecific binding of labeled mycoplasma proteins to Staph A that is mediated by nonimmunoglobulin serum components. For example, the 74,000 protein, which is weakly bound to Staph A in the absence of antiserum, shows enhanced precipitation in the presence of many serum samples tested. When the whole-cell RIP is used, precipitation of the 74,000 protein (and others) by serum is negligible, which indicates that this protein may be cytoplasmic. Whatever the reason for background precipitation of *M. pneumoniae* proteins by preinfection and acute sera, this did not interfere with identification of immunogens based on increased precipitation of specific mycoplasma proteins by convalescent sera as compared with matched acute sera.

Proteins P1 and P2 elicit a strong antibody response during convalescence of both hamsters and humans infected with *M. pneumoniae*. The observed anti-P1 response is compatible with the immunogenicity of this protein (45). Excellent correlation is shown between intrinsically labeled whole-cell RIP and extrinsically labeled soluble-antigen RIP, which confirms the surface location of P1 and P2 and the selectivity of the whole-cell RIP assay (Fig. 3). The epitopes of proteins P1 and P2 are apparently preserved in the detergent system used for solubilization of the mycoplasmas, and antigen-antibody complexes are readily formed and maintained in the presence of this detergent mixture. However, trypsin treatment, which removes P1 and P2 and destroys the attachment capability of *M. pneumoniae*, does abolish precipitation of these predominant immunogens by convalescent sera in both the soluble-antigen and whole-cell assays (Fig. 4 and data not shown). This suggests that most, if not all, immunogenic determinants of P1 and P2 are available at the surface of the organism.

One potential shortcoming of the RIP technique is that an immunogen that is insoluble in the detergent system used would not be detected. Although this possibility cannot be ruled out, the similarity of the TDSET-soluble [³⁵S]methionine-labeled *M. pneumoniae* profile (Fig. 1) to the total intrinsically labeled protein profile (Fig. 2) indicates that excellent solubilization of the mycoplasmas has occurred.

The RIP data obtained from sequential serum samples drawn from military recruits with mycoplasma pneumonia (Fig. 5) and experimentally infected hamsters (Fig. 6) indicate that anti-P1 antibody is formed in response to *M. pneumoniae* infection, but the level of anti-P1 in the serum decreases with continuing convalescence. The hamster anti-P2 response, in contrast, remains strong throughout 12 wk postinfection. Also, the hamster anti-P2 precipitate seems to maximize later than the hamster anti-P1 precipitate (Fig. 6). In some recruit sera, another protein with a molecular weight of ~70,000 is recognized concomitantly with P1 and P2. The observed precipitation of additional proteins by certain human sera appears to be the result of a quantitative, rather than a qualitative, phenomenon, since in a whole-cell RIP using recruit sera, the precipitation of P2 and the 70,000 protein is greatly enhanced (data not shown).

The RIP technique has demonstrated a specific host humoral immune response to certain *M. pneumoniae* proteins. Two predominant immunogens, proteins P1 and P2, stimulate an antibody response only during convalescence of humans and hamsters. These proteins do not have correlates in two common oral mycoplasmas, and anti-*M. pneumoniae* antiserum does not precipitate proteins derived from such oral mycoplasmas, which further suggests that anti-P1 and anti-P2 are specifically produced in

response to *M. pneumoniae* infection. These data, plus the absence of any false positive anti-P1 or anti-P2 responses in a normal population or in patients hospitalized for other illnesses (data not shown), suggest that proteins P1 and P2 may be useful in serodiagnosis of mycoplasma pneumonia.

Clinical diagnosis of mycoplasma pneumonia is usually based on a fourfold rise in the CF titer over a 3 wk period (9). The CF test detects antibody directed against lipid determinants extracted from *M. pneumoniae* (9, 10). However, this lipid antigen cross-reacts with many plant and animal glycolipids (9, 46). In addition, patients with pancreatitis demonstrate high titer antibody against this *M. pneumoniae* lipid antigen (47). These known potential sources of false positive CF reactions have only emphasized the need for a serodiagnostic test that measures antibody to a specific component of *M. pneumoniae*. The data reported herein suggest that, for instance, an enzyme-linked immunosorbent assay using proteins P1 and P2 as target antigens may provide an improved method for serodiagnosis of mycoplasma pneumonia.

Proteins P1 and P2 would appear to be logical components of an *M. pneumoniae* vaccine. However, controversy over the role of serum antibodies in natural disease remains unsettled (14, 16, 18, 40, 48). Experiments currently underway in this laboratory will evaluate whether anti-P1 and anti-P2 antisera have any biological effect (bactericidal, opsonic, anti-attachment, etc.) on virulent *M. pneumoniae*. Such data should indicate the efficacy of P1 and P2 as vaccine candidates. It is noteworthy that human and hamster sera examined by RIP consistently demonstrate a response to proteins P1 and P2, even though these subjects were probably infected with different strains of *M. pneumoniae*. This observation further suggests the feasibility of using P1 and P2 as tools in serodiagnosis and vaccine development.

Summary

The immune response of experimentally infected hamsters and human patients to *Mycoplasma pneumoniae* was examined by radioimmunoprecipitation in conjunction with gel electrophoresis and fluorography. Both intrinsically and extrinsically labeled mycoplasma proteins were coincubated with acute and convalescent sera in a radioimmunoprecipitation assay. Two *M. pneumoniae* proteins were selectively precipitated by convalescent sera. These predominant immunogens were trypsin-sensitive, antibody-accessible surface proteins that co-migrate on polyacrylamide gels with proteins P1 and P2, which were previously implicated by us as mediators of cytoadsorption. Anti-*M. pneumoniae* antiserum did not precipitate radiolabeled antigens derived from *Mycoplasma orale* or *Mycoplasma salivarium*. These data indicate that *M. pneumoniae* infection stimulates a specific and highly targeted host antibody response to key proteinaceous immunogens.

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