

Purification of a *Mycoplasma pneumoniae* Adhesin by Monoclonal Antibody Affinity Chromatography

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A 165,000-dalton surface protein of *Mycoplasma pneumoniae*, designated protein P1, appears to be the major attachment ligand of the pathogen. We employed monoclonal antibody affinity chromatography to obtain purified protein P1.

Protein P1 is a trypsin-sensitive polypeptide which mediates attachment of *Mycoplasma pneumoniae* to target cells (8, 9). *Mycoplasma* cytoadsorption is inhibited if protein P1 is enzymatically cleaved (8) or coated with monospecific anti-P1 antibody (10). *M. pneumoniae* organisms bind to host cells through a specialized tip structure, and this terminal organelle is greatly enriched in P1 (3, 5, 7). In addition, protein P1 has been identified as a predominant immunogen during *M. pneumoniae* infection (12). These data are indicative of a major role for protein P1 in cytoadsorption and virulence of *M. pneumoniae*. More detailed structure-function analysis of protein P1 requires purification of this adhesin. Data suggest that protein P1 is a hydrophobic, integral membrane protein (2, 3), and attempts by workers at this laboratory and others to purify P1 by conventional biochemical methods have been unsuccessful. Therefore, we used antibody affinity chromatography for the purification of protein P1.

Monoclonal antibodies directed against protein P1 of *M. pneumoniae* were produced by the method of Oi and Herzenberg (15). Briefly, BALB/c mice were given multiple injections of intact, wild-type *M. pneumoniae* (100 to 200 µg of mycoplasma protein per injection, intraperitoneal and intravenous routes). The humoral immune response of the mice was monitored by radioimmunoprecipitation (RIP) as described previously (12). Immune spleen cells were fused with SP2/0 myeloma cells, and hybrid cell supernatants were analyzed for anti-*M. pneumoniae* activity by an enzyme-linked immunosorbent assay with whole *M. pneumoniae* as the antigen (14). Hybrid clones producing anti-P1 antibody were identified by RIP. Anti-P1 monoclonal antibodies which bound to protein A-bearing formalinized *Staphylococcus aureus* were expanded by ascitic growth of hybridomas in mice (15). The ascites fluids were passed over a protein A-Sepharose column to purify immunoglobulin G fractions of the anti-P1 preparations.

Previous studies demonstrated that protein P1 can be effectively solubilized in 10 mM Tris-hydrochloride (pH 7.8)-0.2% (wt/vol) sodium deoxycholate-0.1% (wt/vol) sodium dodecyl sulfate-10 mM tetrasodium EDTA-1% (vol/vol) Triton X-100 (TDSET) (12) and that anti-P1 antibodies and P1 form a stable complex in this detergent mixture. We theorized that soluble P1 would bind to immobilized antibodies in the presence of TDSET and that P1 could be eluted with a dissociating reagent that permitted solubility of a hydrophobic membrane protein.

Four anti-P1 monoclonal antibodies secreted by independently isolated hybridomas were combined in equal proportions to increase the probability of binding of multiple P1

epitopes. This pool of anti-P1 monoclonal antibodies was examined by RIP, and Fig. 1 demonstrates that the pooled antibodies recognize a single protein with an apparent molecular weight of 165,000. The low-molecular-weight band that appears in panel B migrates with the dye front and binds nonspecifically to protein A-bearing formalinized *S. aureus* in the absence of antibody. The pooled protein A-Sepharose-

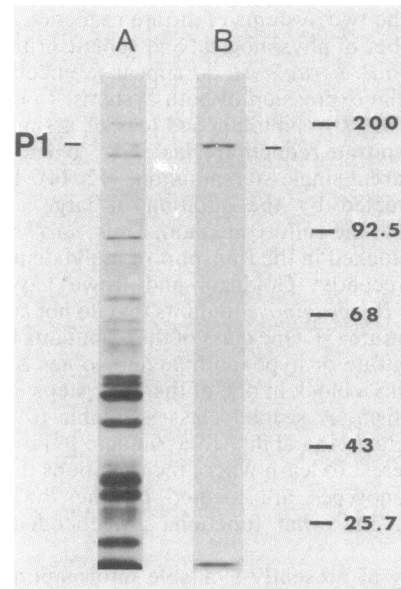


FIG. 1. RIP of *M. pneumoniae* protein P1 by a pool of four monoclonal anti-P1 antibodies. Wild-type *M. pneumoniae* M129-B16 (13) was grown in 32-oz (ca. 960-ml) prescription bottles in 70 ml of Hayflick medium (6) for 72 h at 37°C. Mycoplasmas were intrinsically labeled with [³⁵S]methionine as previously described (12). Radiolabeled *M. pneumoniae* proteins were solubilized in TDSET that contained 1 mM phenylmethylsulfonyl fluoride as described elsewhere (12). Insoluble material was removed by ultracentrifugation (100,000 × g, 45 min), and the supernatant was diluted appropriately. This soluble antigen preparation was incubated with a pool of four anti-P1 monoclonal antibodies, and the resultant complexes were precipitated with protein A-bearing formalinized *S. aureus*. The radiolabeled, precipitated antigens were eluted in sample preparation buffer (12), electrophoresed on 7.5% polyacrylamide gels (11), and processed for fluorography (4). Lane A, TDSET-soluble fraction of ³⁵S-labeled *M. pneumoniae* proteins; lane B, RIP of protein P1 by a pool of four anti-P1 monoclonal antibodies. Molecular weight markers are indicated at the right (myosin, 200,000; phosphorylase b, 92,500; bovine serum albumin, 68,000; ovalbumin, 43,000; chymotrypsinogen, 25,700).

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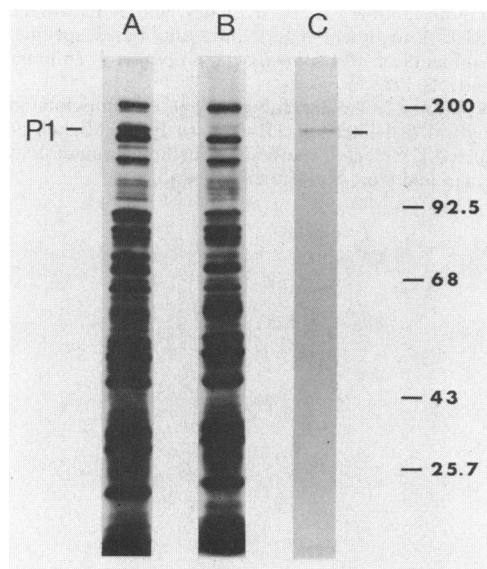


FIG. 2. Purification of *M. pneumoniae* protein P1 by antibody affinity chromatography. A preparation of unlabeled and ^{35}S -labeled TDSET-soluble *M. pneumoniae* proteins was applied to an anti-P1 affinity column. Unbound material was removed with TDSET, and the bound fraction was eluted with 50% (vol/vol) ethylene glycol (pH 11.5) (1). The described *M. pneumoniae* protein preparations were subjected to gel electrophoresis and fluorography. Lane A, TDSET-soluble preparation of *M. pneumoniae*; lane B, *M. pneumoniae* proteins that did not bind to the anti-P1 affinity column; lane C, concentrated, purified protein P1 that was bound to and eluted from the anti-P1 affinity matrix. Protein P1 and molecular weight markers are indicated.

purified anti-P1 monoclonal antibodies (34 mg) were covalently bound to 7 ml of cyanogen bromide-activated Sepharose 4B. Briefly, the anti-P1 immunoglobulin G preparation, which was eluted from protein A-Sepharose with 1 M acetic acid, was dialyzed against coupling buffer (0.1 M NaHCO_3 [pH 7.8] containing 0.5 M NaCl). Immunoglobulin aggregates were removed by centrifugation, and the anti-P1 antibodies were reacted with cyanogen bromide-activated Sepharose 4B (previously washed with 1 mM HCl and equilibrated in coupling buffer). The coupling reaction was performed for 2 h at room temperature. Unreacted immunoglobulin G was washed from the coupled gel, and excess reactive groups were blocked with 0.1 M Tris-hydrochloride (pH 7.5) containing 0.15 M NaCl (Tris-saline). The antibody affinity column was washed sequentially at room temperature with coupling buffer, 0.1 M acetate (pH 4) containing 0.5 M NaCl, Tris-saline, and TDSET.

Unlabeled *M. pneumoniae* (16 mg of protein) and [^{35}S]methionine-labeled *M. pneumoniae* (2 mg of protein) were solubilized in TDSET containing 1 mM phenylmethylsulfonyl fluoride. Insoluble material was removed by ultracentrifugation ($100,000 \times g$, 45 min), and the soluble preparation was applied to the anti-P1 affinity column. After extensive washing with TDSET, the radioactivity in the collected fractions reached a plateau level of low (essentially background) counts. The bound protein was eluted with 50% (vol/vol) ethylene glycol (pH 11.5) (1), and the elution was monitored by quantitation of radioactivity in each fraction. The peak fractions were adjusted to pH 8 to 8.5 with HCl, and sodium deoxycholate was added to a final concentration of 0.1% before pooling of the fractions. The P1 pool was

concentrated by vacuum dialysis against Tris-saline (pH 8.2) containing 0.1% sodium deoxycholate.

The results of a representative affinity purification experiment are shown in Fig. 2. Lane A contains a sample of TDSET-soluble *M. pneumoniae* proteins which were applied to the anti-P1 column. Material which did not bind to the anti-P1 affinity matrix was depleted of P1 (Fig. 2, lane B). The fraction of TDSET-soluble mycoplasma proteins which bound to the anti-P1 column and was eluted with ethylene glycol contained a single intense protein band with a molecular weight of 165,000 (Fig. 2, lane C). After dialysis and concentration, protein P1 remained soluble in Tris-saline containing 0.1% sodium deoxycholate and retained immunoreactivity with anti-P1 antibodies. Initial data indicate that ca. 1% of the soluble, intrinsically labeled *M. pneumoniae* protein preparation applied to the column was recovered in the purified P1 fraction.

A one-step procedure for enrichment of *M. pneumoniae* protein P1 has been developed. This purified ligand will be useful for further dissection of *M. pneumoniae* attachment to host cells, for identification of host cell receptor(s), and for examination of P1 as a rational vaccinogen. In addition, biochemical and immunochemical analyses of P1 are now possible.

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