

## *Pneumocystis carinii* Glycoprotein A Binds Macrophage Mannose Receptors

DEIRDRE M. O'RIORDAN,<sup>1</sup> JOSEPH E. STANDING,<sup>1</sup> AND ANDREW H. LIMPER<sup>1,2\*</sup>

*Thoracic Diseases Research Unit, Departments of Internal Medicine<sup>1</sup> and Biochemistry and Molecular Biology,<sup>2</sup> Mayo Clinic and Foundation, Rochester, Minnesota 55905*

Received 2 June 1994/Returned for modification 11 July 1994/Accepted 20 December 1994

*Pneumocystis carinii* causes life-threatening pneumonia in patients with impaired immunity. Recent studies suggest that alveolar macrophages interact with *P. carinii* through macrophage mannose receptors. However, the ligand(s) on *P. carinii* that is recognized by these receptors has not been fully defined. *P. carinii* contains a major mannose-rich surface antigen complex termed glycoprotein A (gpA). It was therefore hypothesized that gpA binds directly to macrophage mannose receptors and mediates organism attachment to these phagocytes. To assess this, gpA was purified from *P. carinii* by continuous-elution gel electrophoresis. <sup>125</sup>I-labeled gpA bound to alveolar macrophages in a saturable fashion. In addition, gpA binding was substantially inhibited by both  $\alpha$ -mannan and EDTA, further suggesting that gpA interacts with macrophage mannose receptors. Macrophage membrane proteins capable of binding to gpA were isolated with a gpA-Sepharose column. A 165-kDa membrane-associated protein was specifically eluted from the gpA-Sepharose column with EDTA (20 mM). This protein was identified as the macrophage mannose receptor by immunoprecipitation with a polyclonal anti-mannose receptor antiserum. To further investigate the role of gpA in *P. carinii*-macrophage interactions, <sup>51</sup>Cr-labeled *P. carinii* cells were incubated with macrophages in the presence of increasing concentrations of soluble gpA, and organism attachment was quantified. Soluble gpA (2.5 mg/dl) competitively inhibited *P. carinii* attachment to alveolar macrophages by 51.3%  $\pm$  3.7% ( $P = 0.01$ ). Our findings demonstrate that gpA present on *P. carinii* interacts directly with mannose receptors, thereby mediating organism attachment to alveolar macrophages.

*Pneumocystis carinii* is an opportunistic protist which causes severe pneumonia in immunocompromised patients with AIDS, hematologic or solid malignancies, organ transplants, and inflammatory conditions treated with immunosuppressive agents (7, 26, 33, 34, 38, 45). Recent studies suggest that alveolar macrophages participate in host defense during *P. carinii* infection by binding to, internalizing, and degrading the organism (24, 32, 42, 52). Additional in vitro studies indicate that the interaction of *P. carinii* with macrophages causes the release of reactive oxidants, arachidonic acid metabolites, and tumor necrosis factor alpha from the phagocyte (5, 17, 18). Other investigations have provided insights into the mechanisms by which macrophages interact with *P. carinii*. In particular, Ezekowitz and colleagues have demonstrated that mannose receptors can mediate macrophage uptake of the organism (12, 23). The relative importance of these interactions of *P. carinii* with alveolar macrophages during pneumonia in the living host has not yet been fully characterized.

Mannose receptors are integral membrane glycoproteins of approximately 165 kDa expressed on the surface of mature macrophages in humans, rodents, and other animals (10, 11, 47, 48, 54). The presence of mannose receptors on the cell membrane permits binding and internalization of glycoconjugates containing mannose and fucose, *N*-acetylglucosamine, and, to much lesser extent, galactose residues (3, 10). Glycoconjugates interacting with mannose receptors bind optimally at pH 7 and require calcium, indicating that these receptors belong to the family of C-type lectins (47). Recent studies also

reveal that impairment of mannose receptor function significantly inhibits macrophage uptake of *P. carinii* (12, 23).

The potential ligands on *P. carinii* recognized by macrophage mannose receptors have not been formally determined. *P. carinii* possesses several carbohydrate-rich surface proteins capable of interacting with lectins and other glycoproteins (8, 28, 30, 31, 37, 44). In particular, *P. carinii* contains a major surface glycoprotein complex which has been variously termed gpA, gp95, gp120, or major surface glycoprotein (13, 16, 22, 51, 53, 55). Differences in the relative molecular mass of this complex are related to the host species from which the *P. carinii* strain is derived (30). Therefore, several investigators have recently adopted the nomenclature gpA for this glycoprotein complex (14, 16, 49, 55). Molecular studies indicate that gpA is encoded by a family of genes and contains relatively conserved cysteine-rich regions (22, 49, 53, 55). N-linked carbohydrate rich in mannose, glucose, and *N*-acetylglucosamine residues represents approximately 1/10 of gpA's mass (30, 31, 44). Additional studies reveal that gpA interacts with several glycoproteins, including concanavalin A, fibronectin, and surfactant protein A and likely represents a major ligand recognized during the interactions of *P. carinii* with alveolar epithelial cells (28, 41, 43, 56). The carbohydrate structure of gpA makes it a likely molecular target for interaction with mannose receptors. The following studies were therefore undertaken to examine the interaction of gpA with mannose receptors and determine the role of gpA in organism attachment to alveolar macrophages.

### MATERIALS AND METHODS

**Materials.** All organic chemicals were of analytical grade and obtained from Sigma Chemical Co. (St. Louis, Mo.) unless otherwise specified. <sup>125</sup>I-Di-Iodo Bolton-Hunter reagent was from Amersham (Arlington Heights, Ill.). A goat polyclonal anti-mannose receptor antiserum was the generous gift of Philip

\* Corresponding author. Mailing address: Thoracic Diseases Research Unit, 601A Guggenheim Building, Mayo Clinic, Rochester, MN 55905. Phone: (507) 284-2301. Fax: (507) 284-4521.

Stahl, Department of Cell Biology, Washington University, St. Louis, Mo. This antiserum was initially generated against mannose receptors purified from human placenta (21). Preliminary studies in our laboratory indicated that this polyclonal antiserum also reacted with rodent macrophage mannose receptors in immunostaining and immunoprecipitation applications. Monoclonal antibody 5E12, which recognizes gpA from rodent, ferret, and human sources, was a gift of Francis Gigliotti, Department of Pediatrics, University of Rochester, Rochester, N.Y. (15). 5E12 has previously been shown to react with rat gpA in immunofluorescence and immunoblotting analyses (15, 29).

**Preparation of *P. carinii*.** *P. carinii* pneumonia was induced in Harlan Sprague-Dawley rats by immunosuppression with dexamethasone as previously reported (2, 25, 29). Pathogen-free rats were provided with drinking water containing dexamethasone (2 mg/ml), tetracycline hydrochloride (500 mg/ml), and nystatin (200,000 U/liter) ad libitum. After 5 days, rats were inoculated intratracheally with *P. carinii* ( $3 \times 10^6$  to  $5 \times 10^6$  organisms) prepared by aseptically homogenizing lung from rats with *P. carinii* pneumonia with a Stomacher blender (Tekmar, Cincinnati, Ohio). All animals were inoculated from the same preparation of *P. carinii* in our isolated rat population. After 6 to 8 weeks of additional immunosuppression, the rats were killed, and whole-lung lavage was performed with 50 ml of Hanks' balanced salt solution (HBSS). *P. carinii* organisms were purified from this lavage by differential centrifugation as previously reported (25, 29). Lavage fluid was centrifuged ( $400 \times g$  for 10 min), and *P. carinii* cysts in the pellet were identified by Diff-Quik staining. The supernatant containing predominantly *P. carinii* organisms was recentrifuged ( $1,400 \times g$  for 30 min), and the pellet was resuspended in 1 ml of HBSS. Duplicate 10-ml aliquots of suspension were spotted onto glass slides and stained with Diff-Quik, and *P. carinii* was quantified as described before (29). All *P. carinii* numbers reported in the text represent total *P. carinii* nuclei (29). Prior studies indicate that the *P. carinii* isolates contained both trophozoite and cyst forms, typically in a ratio of 9:1 (18, 29). *P. carinii* generally represented greater than 97% of the cellular material on Diff-Quik-stained smears, with the remainder representing fragmented host cells (29). Complete microbiologic cultures of selected *P. carinii* isolates failed to reveal growth of bacteria or fungi over 72 h. If other microorganisms were noted in the lavage, the material was discarded. Whole lung lavage fluid from control rats without *P. carinii* failed to yield any appreciable material after the second centrifugation (25).

**Purification and characterization of gpA.** Purified *P. carinii* organisms were solubilized in 125 mM Tris-4% sodium dodecyl sulfate (SDS)-4% 2-mercaptoethanol-0.002% bromophenol blue-20% glycerol (pH 7.4). From this extract, gpA was purified by continuous-flow gel electrophoresis on a 10% polyacrylamide preparative tube gel as our laboratory has previously reported (PrepCell Apparatus; Bio-Rad, Hercules, Calif.) (36). The gel was resolved over 48 h at 25 mA current and continuously eluted with 25 mM Tris base-192 mM glycine-0.1% SDS. Fractions were analyzed by SDS-polyacrylamide gel electrophoresis (PAGE) and silver staining on 4 to 15% gradient resolving gels (Phast Gel System; Pharmacia, Piscataway, N.J.). Fractions containing the band of 120 kDa were pooled, dialyzed, and concentrated. Immunoblotting of the purified protein was performed to verify the identity of this 120-kDa protein as genuine *P. carinii* gpA. To accomplish this, the 120-kDa fraction was electrophoresed and transferred to nitrocellulose membranes at 60 V for 6 h. Nitrocellulose membranes were cut into strips, washed, and blocked overnight in 5% dried milk solution in Tris-buffered saline (TBS). The strips were then incubated with either antibody 5E12 (50  $\mu$ g/ml) or a similar concentration of nonimmune mouse immunoglobulin M (IgM) antibody for 1 h at room temperature. Monoclonal antibody 5E12 is a mouse anti-*P. carinii* antibody recognizing gpA from rodent, ferret, and human sources (15). Bound antibodies were visualized with a commercial murine antibody detection system (Amersham).

To further quantify whether small amounts of other *P. carinii* or host molecules might migrate at roughly 120 kDa and thereby copurify with the gpA, the purified 120 kDa gpA isolates were radiolabeled, and immunoprecipitation studies were performed with antibody 5E12. The 120-kDa gpA preparation was radiolabeled by the method of Bolton and Hunter (4).  $^{125}$ I-Di-Iodo Bolton-Hunter reagent was dried under nitrogen, and gpA (20 to 30  $\mu$ g) in 0.1 M borate buffer, pH 8.5, containing 1 mM CaCl<sub>2</sub> was added directly to the vial. The reaction mixture was incubated on ice for 2 h with occasional agitation, and unreacted iodination reagents were subsequently removed by dialysis against TBS. Aliquots of  $^{125}$ I-gpA isolates were preabsorbed with protein G-Sepharose, and parallel aliquots were incubated either with monoclonal antibody 5E12 (100  $\mu$ g/ml) or with an identical concentration of nonimmune mouse IgM. After 60 min of incubation, a goat anti-mouse polyvalent Ig was added (Sigma; 100  $\mu$ g/ml), and the samples were incubated for an additional hour. Radiolabeled protein-antibody complexes were precipitated with protein G-Sepharose, washed three times with TBS containing calcium, and eluted with 1% SDS containing 5%  $\beta$ -mercaptoethanol. The relative amounts of 120-kDa material precipitated by antibody 5E12 and remaining in the supernatants were determined by gamma counting.

**Binding of radiolabeled gpA to alveolar macrophages.** To investigate the binding of gpA to normal rat alveolar macrophages, purified gpA was radioiodinated by the Bolton-Hunter method as described above. Alveolar macrophages were obtained from the lungs of uninfected rats by lavage with HBSS. Cytologic smears showed the preparations to contain 95% macrophages on average. Macrophages ( $2 \times 10^5$ ) were incubated in suspension with  $^{125}$ I-gpA (0.5 to 12  $\mu$ g/ml)

for 1 h at 4°C and washed three times by brief ( $1,400 \times g$ , 1 min) centrifugations, and bound and free gpA amounts were determined by gamma counting. Yeast  $\alpha$ -mannans are known to inhibit the interactions of glycoconjugates with macrophage mannose receptors (12, 18, 48). Therefore, parallel binding assays were performed in the presence of yeast  $\alpha$ -mannan (1 mg/ml) to assess the degree to which gpA binds nonspecifically to sites other than mannose receptors. In other experiments, the role of divalent cations in gpA interaction was assessed by determining binding in the presence of EDTA (10 mM).

**Isolation of macrophage membrane proteins which interact with gpA.** To further investigate which macrophage membrane molecules bound to immobilized gp120, a gpA-Sepharose column (2 mg/ml in a 2-ml column) was prepared by coupling purified gpA protein to cyanogen bromide-activated Sepharose by the method of Porath et al. (40). Binding efficiency was estimated at approximately 65% by the  $A_{280}$  before and after coupling. Radiolabeled macrophage membrane proteins were prepared as follows. Macrophages ( $6 \times 10^6$  to  $10 \times 10^6$ ) were isolated from uninfected rats by lavage, starved in methionine-free RPMI medium containing 10% fetal bovine serum (FBS) for 1 h, and incubated with  $^{35}$ S-Trans label (250  $\mu$ Ci; New England Nuclear) in 5 ml of methionine-free RPMI containing 10% FBS overnight at 37°C. The cells were washed, disrupted by repeated freeze-thaw cycles, and sonicated in 50 mM Tris buffer (pH 7.4) containing 1 mM leupeptin and 1 mM pepstatin. Membrane-associated proteins were recovered by centrifugation at  $100,000 \times g$  for 30 min and solubilized in phosphate-buffered saline (PBS) containing 1% Triton X-100 and 1 mM each calcium and magnesium. Radiolabeled macrophage proteins (approximately 650  $\mu$ g, containing  $3.3 \times 10^8$  cpm) were chromatographed over the gpA-Sepharose, washed with 15 column volumes of PBS containing 1% Triton X-100 with 1 mM each calcium and magnesium, and then eluted with 10 column volumes of 20 mM EDTA. Fractions (1 ml) were collected, and recovered proteins were analyzed by SDS-PAGE and fluorography. To determine whether eluted proteins represented mannose receptors, recovered fractions were immunoprecipitated with a polyclonal goat anti-mannose receptor antiserum or with normal goat serum. Fractions were divided into equal aliquots and preabsorbed with protein G-Sepharose, and parallel aliquots were incubated with either mannose receptor antiserum or normal goat serum (1:50 dilution). Protein-antibody complexes were precipitated with protein G-Sepharose, eluted with 1% SDS containing 5%  $\beta$ -mercaptoethanol, and analyzed by SDS-PAGE.

**Role of gpA in mediating *P. carinii* attachment to alveolar macrophages.** It was further postulated that purified soluble gpA might compete with native gpA present on the surface of *P. carinii* for interaction with macrophage receptors which mediate attachment of the organism to the phagocyte. To test this, *P. carinii* adherence to cultured alveolar macrophages was quantified in the presence of purified gpA. *P. carinii* organisms were radiolabeled overnight at 37°C in 2 ml of Dulbecco's modified Eagle's medium (DMEM) containing 20% fetal calf serum and 200  $\mu$ Ci of  $^{51}$ Cr sodium chromate (New England Nuclear, Boston, Mass.) (25, 28). Subsequently, the *P. carinii* cells were washed to remove unincorporated radiolabel and resuspended in DMEM containing bovine serum albumin (BSA; 1 mg/ml). Macrophages from uninfected rats were plated on 96-well tissue culture dishes ( $10^5$  cells per well) in mixed medium (1:1 mixture of medium 199 and RPMI 1640 supplemented with 2 mM glutamine, 100 U of penicillin per ml, and 100 mg of streptomycin per ml) and then gently washed with warm HBSS to remove nonadherent cells. Previous studies in our laboratory have demonstrated that  $\approx 95\%$  of the macrophages are adherent after this initial incubation (5). In a manner analogous to that used in our prior studies (18), macrophages were incubated with gpA at the indicated concentrations for 20 min prior to the addition of  $^{51}$ Cr-*P. carinii* (*P. carinii*/macrophage ratio, 5:1) and throughout an additional 1-h incubation at 37°C. Subsequently, nonadherent *P. carinii* cells were removed by washing. The macrophage monolayers containing adherent *P. carinii* cells were solubilized in 1 N NaOH and quantified. Results are expressed as percent maximal adherence, where the adherence of  $^{51}$ Cr-*P. carinii* cells to alveolar macrophages in the absence of gpA or other inhibitors (control) was defined as 100% adherence.

**Statistical methods.** Data are expressed as the mean  $\pm$  standard error. Differences between experimental groups were assessed by an unpaired two-sample Student's *t* test. Statistical testing was accomplished with the Statview II statistical package (Abacus Concepts, Inc., Berkeley, Calif.).  $P < 0.05$  was taken as a statistically significant difference for two-sided alternatives.

## RESULTS

**Purification and characterization of gpA derived from *P. carinii*.** Detergent extracts of purified *P. carinii* organisms from rats contain a major band migrating with an apparent molecular mass of 120 kDa under reducing conditions on SDS-PAGE (Fig. 1). Accordingly, preparative SDS-PAGE was used to purify gpA from *P. carinii*. This purification scheme is possible because the gpA complex migrates at roughly 120 kDa and is therefore distinguishable from other major components of *P. carinii* which run between 45 and 75 kDa (28, 42). *P. carinii* also contains a number of host proteins bound to the

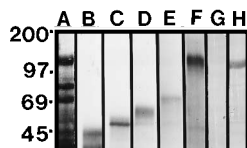


FIG. 1. Purification and characterization of gpA derived from *P. carinii*. *P. carinii* from six moribund rats was extracted in SDS containing  $\beta$ -mercaptoethanol as described in the text. The extract was electrophoresed on a 10% polyacrylamide matrix in a PrepCell apparatus (Bio-Rad). Eluted fractions were collected and dialyzed to remove SDS and analyzed by SDS-PAGE (4 to 15% gradient). Lane A, total *P. carinii* extract (500 ng of total *P. carinii* extract, Coomassie brilliant blue staining). Lanes B to E, selected fractions containing lower-molecular-weight components of *P. carinii* (20 ng each, sensitive silver staining). Lane F, fractions demonstrating the isolated 120-kDa protein (20 ng, sensitive silver staining). In lane G, the 120-kDa fraction was subjected to SDS-PAGE, transferred to nitrocellulose, and evaluated by immunoblot analysis. Shown is 10 ng reacted with nonimmune mouse IgM. The 120-kDa fraction showed no reactivity to nonimmune antibody. Lane H, the 120-kDa fraction bound antibody 5E12, a monoclonal antibody reacting with gpA derived from *P. carinii*, confirming the identity of the purified protein (10 ng).

surface, including fibronectin (220 kDa), vitronectin (65 kDa), surfactant protein A (30 kDa), surfactant protein D (45 kDa), and IgG (50 and 25 kDa under denaturing and reducing conditions, respectively). The Bio-Rad PrepCell apparatus was reliably able to resolve differences in molecular mass of roughly 10%. Therefore, this approach proved reasonable for initial purification of the gpA complex. Scanning densitometry of three silver-stained preparations revealed the preparations to contain  $97.5\% \pm 0.8\%$  of the 120-kDa material. Since the purification is based on a size exclusion strategy, the small amounts of lower-molecular-mass materials ( $\sim 55$  kDa) likely represented minor proteolytic fragments of the 120-kDa material. Attempts at protein sequencing revealed that the amino terminus of the protein was blocked, consistent with the observations of others (35). Using this procedure, approximately 500  $\mu$ g of gpA complex was isolated from  $5 \times 10^9$  *P. carinii* purified from six rats by differential centrifugation.

To verify that this material indeed represented gpA, immunoblot analysis was undertaken with monoclonal antibody 5E12, a specific antibody recognizing *P. carinii* gpA derived from the rat. The recovered protein of 120 kDa reacted with 5E12 but not with nonimmune IgM, verifying its identity as the major *P. carinii* surface antigen gpA (Fig. 1). However, it remained possible that small quantities of other *P. carinii* or host proteins may also migrate at 120 kDa and therefore co-purify with the gpA complex. To further quantify the extent to which this material was indeed gpA, the isolated 120-kDa material was radiolabeled, and immunoprecipitation was performed with monoclonal antibody 5E12. Quantitative immunoprecipitation studies indicate that the material collected in the 120-kDa fraction was  $91.6\% \pm 0.1\%$  reactive with monoclonal 5E12. Taken together, these data indicate that the 120-kDa gpA complex has been isolated to relatively high purity.

***P. carinii* gpA binds to alveolar macrophages in a saturable manner which can be inhibited by mannan and requires divalent cations.** To determine whether gel-purified gpA interacts with alveolar macrophages, gpA was radiolabeled, and suspension binding studies were performed with normal alveolar macrophages (Fig. 2).  $^{125}\text{I}$ -gpA bound to macrophages in a concentration-dependent and saturable manner. To evaluate specific binding of  $^{125}\text{I}$ -gpA to mannose receptors, ligand binding was evaluated in the presence of  $\alpha$ -mannan (1 mg/ml). Soluble  $\alpha$ -mannan effectively inhibits binding of glycoconjugates with macrophage mannose receptors (10, 11, 47, 48). Residual binding of  $^{125}\text{I}$ -gpA in the presence of  $\alpha$ -mannan

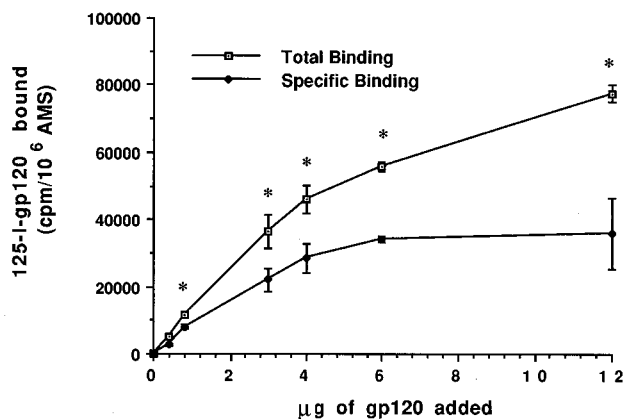


FIG. 2. *P. carinii* gpA binds to alveolar macrophages in a saturable manner which can be inhibited by  $\alpha$ -mannan. Alveolar macrophages (AMS;  $2 \times 10^6$ ) were incubated in suspension with  $^{125}\text{I}$ -gpA (0 to 12  $\mu\text{g}/\text{ml}$ ) for 1 h at  $4^\circ\text{C}$  and washed, and the bound  $^{125}\text{I}$ -gpA was quantified. Nonspecific binding of gpA to sites other than macrophage mannose receptors was assessed in parallel binding assays performed in the presence of unlabeled  $\alpha$ -mannan (1 mg/ml), a known antagonist of mannose receptors. Specific binding to mannose receptors was defined as total binding minus nonspecific binding. *P. carinii*-derived gpA exhibited significant specific binding to alveolar macrophages at all concentrations greater than or equal to 0.8  $\mu\text{g}/\text{ml}$ . Specific binding of gpA on macrophages plateaued at a concentration of 6  $\mu\text{g}/\text{ml}$ , indicating that binding of gpA to mannose receptors is a saturable process. The data represent the mean  $\pm$  standard error of the mean from four determinations. \*,  $P < 0.05$  comparing specific with total binding of  $^{125}\text{I}$ -gpA.

represented nonspecific binding of ligand to sites other than mannose receptors. Significant specific binding of  $^{125}\text{I}$ -gpA to alveolar macrophages was detected at concentrations as low as 0.8  $\mu\text{g}/\text{ml}$  ( $P = 0.002$  comparing total and nonspecific binding). Specific binding of  $^{125}\text{I}$ -gpA plateaued at a concentration of 6  $\mu\text{g}/\text{ml}$ , indicating saturability of this interaction. It was also observed that specific binding of  $^{125}\text{I}$ -gpA (5  $\mu\text{g}/\text{ml}$ ) to alveolar macrophages could be inhibited by  $90.1\% \pm 8.2\%$  in the presence or absence of EDTA (10 mM) ( $P = 0.001$  compared with control). As a further test of specificity, additional  $^{125}\text{I}$ -gpA binding assays were performed in the presence of soluble  $\beta$ -glucan (1 mg/ml; Sigma) (6).  $\beta$ -Glucans are homopolymers which are known to interact with macrophage receptors, distinct from mannose receptors (18). The addition of  $\beta$ -glucan did not significantly impair the binding of  $^{125}\text{I}$ -gpA to alveolar macrophages ( $P = 0.27$ , not significantly different in the presence and absence of  $\beta$ -glucan). Since gpA binding to macrophages could be inhibited by  $\alpha$ -mannan and EDTA but not by  $\beta$ -glucan, these investigations strongly support a direct interaction of gpA with macrophage mannose receptors.

**Immobilized gpA binds to a 165-kDa macrophage membrane protein identified as the mannose receptor.** To further define which macrophage membrane protein(s) interacts with *P. carinii* gpA, the binding of radiolabeled macrophage components to gpA immobilized on Sepharose was evaluated. Radiolabeled alveolar macrophage membrane proteins were isolated and chromatographed over a gpA-Sepharose column (Fig. 3). Elution of this column with EDTA (20 mM) yielded a 165-kDa radiolabeled protein. This molecule was subsequently confirmed to be the macrophage mannose receptor by immunoprecipitation, as the 165-kDa protein was specifically precipitated with antiserum to mannose receptor but not with nonimmune serum (Fig. 3, lanes C and D). These findings confirm that gpA derived from *P. carinii* binds with macrophage mannose receptors in a divalent cation-dependent fashion within a cell-free system.

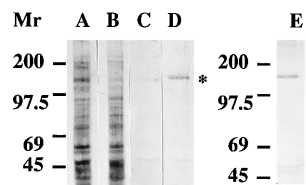


FIG. 3. Immobilized gpA binds to a 165-kDa macrophage membrane protein identified as the mannose receptor. (Lane A)  $^{35}\text{S}$ -labeled membrane-associated proteins isolated from alveolar macrophages. This extract was subsequently loaded over a gpA-Sepharose column (2 mg/ml in a 2-ml column) as described in the text. (Lane B) Flowthrough of  $^{35}\text{S}$ -labeled macrophage membrane proteins which did not adhere to the gpA-Sepharose column. Note the relative loss of the 165-kDa band. After washing, the column was eluted with EDTA (20 mM) and immunoprecipitated. Nonimmune goat serum revealed no specific precipitation product (lane C). Immunoprecipitation of the eluted fraction with a goat anti-mannose receptor antiserum specifically yielded a 165-kDa molecule (lane D), strongly suggesting direct interaction of gpA and macrophage mannose receptors in this cell-free system. (Lane E) Aliquot of the total material eluted from the column with EDTA before immunoprecipitation with the mannose receptor antiserum and protein G-Sepharose. Scanning densitometry reveals that the 165-kDa mannose receptor represented >85% of the protein eluted from the gpA-Sepharose column. Each lane contains 15 ng of loaded protein.

**Purified gpA competitively inhibits the adherence of *P. carinii* to cultured alveolar macrophages.** To determine the functional significance of gpA's interaction with mannose receptors, the effect of soluble purified gpA on attachment of radiolabeled *P. carinii* to cultured alveolar macrophages was quantified. If gpA is important for *P. carinii* attachment to alveolar macrophages, then the addition of purified gpA in soluble form should compete with gpA present on the surface of *P. carinii* for binding to corresponding macrophage receptors. Accordingly, the adherence of radiolabeled *P. carinii* to macrophages (*P. carinii*/macrophage ratio, 5:1) was quantified in the presence of increasing concentrations of purified soluble gpA. Addition of soluble gpA (0.1 to 2.5 mg/ml) resulted in a significant, concentration-dependent reduction in *P. carinii* binding to macrophages (Fig. 4). Significant inhibition of *P. carinii* interaction with alveolar macrophages occurred with as little as 0.1 mg of soluble gpA per ml, which resulted in a  $27.0\% \pm 3.1\%$  reduction in organism binding ( $P = 0.047$  com-

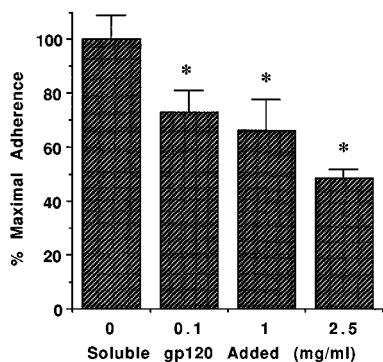


FIG. 4. Soluble gpA competitively inhibits *P. carinii* adherence to cultured alveolar macrophages.  $^{51}\text{Cr}$ -labeled *P. carinii* cells were permitted to bind to rat alveolar macrophages (*P. carinii*/macrophage ratio of 5:1) over 1 h in DMEM containing BSA (1 mg/ml) in the absence or presence of soluble gpA (0 to 2.5 mg/ml). Addition of gpA resulted in a concentration-dependent decrease in adherence of radiolabeled *P. carinii* to cultured alveolar macrophages. Maximal inhibition was observed at a gpA concentration of 2.5 mg/ml ( $P = 0.01$  compared with control). Larger concentrations did not result in greater reduction in *P. carinii* interaction with alveolar macrophages. The data represent mean  $\pm$  standard error of the mean from three experiments. \*,  $P < 0.05$ , comparing the presence and absence of gpA.

pared with control without gpA). Soluble gpA (2.5 mg/ml) maximally caused a  $51.5\% \pm 3.7\%$  reduction in *P. carinii* adherence to macrophages ( $P = 0.01$ ). Larger concentrations of gpA did not result in significantly greater reduction in *P. carinii* adherence to the phagocytes. Similar concentrations of purified soluble yeast mannans have been used previously to inhibit *P. carinii* interactions with macrophage mannose receptors (12, 18). Purified gpA itself caused no adverse effects on macrophage viability, as assessed by exclusion of trypan blue. To further ensure the specificity of the *P. carinii* adherence assay, additional studies were performed with an irrelevant glycoprotein, k-elasticin, a matrix protein which does not significantly interact with *P. carinii* (29). *P. carinii* adherence to macrophages in the presence of k-elasticin (2.5 mg/ml) was not significantly different from that in controls ( $P = 0.24$ ). Taken together, these results indicate that the interaction of gpA on the surface of *P. carinii* with macrophage mannose receptors mediates organism recognition and adherence to alveolar macrophages.

To further study whether glycosylation of gpA participated in the interaction of gpA with macrophages, purified gpA was also treated with sodium periodate (10 mM overnight, followed by gel filtration through a P-2 column [Pierce Chemical Co.]) and tested for its ability to inhibit the binding of *P. carinii* to alveolar macrophages. Periodate acts to destroy carbohydrates by cleaving C-C bonds between vicinal hydroxyl groups on sugars, forming aldehyde moieties. Periodate-treated gpA (2.5 mg/ml) maximally caused only  $26.8\% \pm 1.7\%$  inhibition of *P. carinii* adherence to alveolar macrophages ( $P = 0.0001$  compared with untreated gpA). These findings further suggest that glycosylated side chains of gpA promote the interaction of *P. carinii* with alveolar macrophages.

## DISCUSSION

This investigation provides the first direct evidence that gpA, a mannose-rich glycoprotein found on the surface of *P. carinii*, functions as a ligand for macrophage mannose receptors. Purified radiolabeled gpA bound to alveolar macrophages in a saturable manner which was inhibited by both mannan and EDTA. It was additionally observed that immobilized gpA bound a 165-kDa macrophage membrane protein, identified as the macrophage mannose receptor by immunoprecipitation analysis. Our study further demonstrates that *P. carinii* gpA facilitates the interaction of *P. carinii* with macrophages, as purified gpA acted as a soluble competitive inhibitor for organism attachment to cultured alveolar macrophages.

The specificity of gpA binding to macrophage mannose receptors was addressed by several approaches. Addition of  $\beta$ -glucan, a biopolymer which binds to macrophage receptors as distinct from mannose receptors, did not significantly impair binding of radiolabeled gpA to macrophages. Furthermore, addition of a 100-fold excess of unlabeled gpA resulted in significant inhibition in the binding of radiolabeled gpA, by approximately 50%. The binding of gpA which could not be inhibited by unlabeled gpA may represent additional binding of gpA through ionic, electrostatic, or alternate lectin-mediated processes.

In vitro studies indicate that alveolar macrophages phagocytize and degrade *P. carinii* and release reactive oxidants, eicosanoids, and cytokines, including tumor necrosis factor alpha (5, 17, 18, 24, 32, 42, 52). Recent investigations have begun to elucidate the mechanisms by which alveolar macrophages interact with this organism. In particular, several studies indicate that mannose receptors mediate macrophage recognition of *P. carinii* (12, 18, 23, 24). Competitive inhibitors of

mannose receptors, such as  $\alpha$ -mannan, or alteration in the distribution of mannose receptors via adherence of cells to mannan-coated surfaces impairs macrophage uptake of *P. carinii*. Furthermore, human immunodeficiency virus infection has been shown to impair macrophage mannose receptor function against *P. carinii* (23). The current investigation demonstrates that gpA on the surface of *P. carinii* functions as a ligand for mannose receptors and that this interaction participates in adherence of the organism to alveolar macrophages. Prior work has similarly shown that gpA also participates in the adherence of *P. carinii* to alveolar epithelial cells (28, 41, 43). The relative importance of the interactions of gpA on *P. carinii* with receptors on macrophages and epithelial cells in the living host has not yet been fully characterized.

Carbohydrate analysis reveals that 1/10 of gpA's molecular mass consists of N-linked carbohydrates rich in mannose, glucose, and N-acetylglucosamine (30, 31, 37, 44). Mannose-rich surface antigens have been shown to promote interaction of a number of microorganisms with host cells (1, 9, 19, 20, 39, 46, 50). For instance, *Candida albicans*  $\alpha$ -mannan mediates interaction of the organism with spleen and lymphatic tissues (20). Mannose-containing glycoconjugates are similarly expressed on the surfaces of a number of protozoal, bacterial, and fungal organisms (3, 19, 39, 50). These surface glycoconjugates are capable of mediating lectin-type interactions with receptors on phagocytic cells (1, 46).

It is noteworthy that soluble gpA did not completely inhibit the association of *P. carinii* with alveolar macrophages. The most likely explanation is that the binding of gpA with macrophage mannose receptors represents only one facet of the complex interaction between *P. carinii* and phagocytic cells. Additional studies suggest that multiple receptor-ligand pairs may facilitate this process. For example, the adhesive glycoproteins fibronectin and vitronectin have been implicated in *P. carinii* attachment to cultured lung epithelial cells and also to potentiate macrophage interactions with *P. carinii* (29, 36, 41–43). The macrophage receptors interacting with these extracellular adhesive glycoproteins which coat *P. carinii* organisms have not been defined but are likely members of the integrin family of receptors. It is also likely that other macrophage receptors, including complement and Fc receptors, further promote macrophage interactions with the organism. Additional studies will be required to determine the relative importance of various receptor types in mediating macrophage uptake of *P. carinii* during infection. It is additionally possible that the denaturing conditions used during purification may have somewhat reduced the ability of soluble gpA to compete with native gpA on the organisms for the cognate receptors on macrophages. Prior studies indicate that native gpA is likely present in multimeric form on the surface of the organism (31). At present, information about the relative affinities of purified soluble gpA and native gpA associated with the surface of *P. carinii* is not available.

In summary, our investigation demonstrates that gpA, a potent antigenic complex present on the surface of *P. carinii*, binds with mannose receptors present on alveolar macrophages. This interaction likely represents a significant component in the recognition of *P. carinii* by the host. Interventions aimed at modulating mannose receptor expression or enhancing the interaction of *P. carinii* gpA with phagocytes may provide further novel therapeutic insights for treatment of this infection.

#### ACKNOWLEDGMENTS

This work was supported by NIH grant AI 34336-02, a research grant from the American Lung Association Research, and funds from

the Mayo Foundation (to A.H.L.). These studies were performed during the tenure of an American Heart Association Clinician-Scientist Award (to A.H.L.).

We thank Philip Stahl, who provided the anti-mannose receptor antiserum; Francis Gigliotti for monoclonal antibody 5E12; and Daniel McCormick, who performed amino acid sequencing. We also appreciate Edward B. Leof and Ulrich Specks for their many helpful discussions and Kathy Streich for her assistance in the final preparation of this manuscript.

#### REFERENCES

1. Bar-Shavit, Z., R. Goldman, I. Ofek, N. Sharon, and D. Mirelman. 1980. Mannose-binding activity of *Escherichia coli*: a determinant of attachment and ingestion of the bacteria by macrophages. *Infect. Immun.* **29**:417–424.
2. Bartlett, M. S., J. A. Fishman, S. A. Queener, M. M. Durkin, M. A. Jay, and J. W. Smith. 1988. New rat model of *Pneumocystis carinii* pneumonia. *J. Clin. Microbiol.* **26**:1100–1102.
3. Blackwell, J. M., R. A. B. Ezekowitz, M. B. Roberts, J. Y. Channon, R. B. Sim, and S. Gordon. 1985. Macrophage complement and lectin-like receptors bind Leishmania in the absence of serum. *J. Exp. Med.* **162**:324–331.
4. Bolton, A. E., and W. M. Hunter. 1973. The labeling of proteins to high specific radioactivities by conjugation to a 125-I-containing acylating agent. *Biochem. J.* **133**:529–539.
5. Castro, M., T. I. Morgenthaler, O. A. Hoffman, J. E. Standing, M. S. Rohrbach, and A. H. Limper. 1993. *Pneumocystis carinii* induces the release of arachidonic acid and its metabolites from alveolar macrophages. *Am. J. Respir. Cell Mol. Biol.* **9**:73–81.
6. Castro, M., N. V. C. Ralston, T. I. Morgenthaler, M. S. Rohrbach, and A. H. Limper. 1994. *Candida albicans* stimulates arachidonic acid liberation from alveolar macrophages through  $\alpha$ -mannan and  $\beta$ -glucan cell wall components. *Infect. Immun.* **62**:3138–3145.
7. Centers for Disease Control. 1989. Guidelines for prophylaxis against *Pneumocystis carinii* pneumonia for persons with human immunodeficiency virus infection. *Morbidity and Mortality Weekly Report*. **38**:1–8.
8. Cushion, M. T., M. Kaselis, S. L. Stringer, and J. Stringer. 1993. Genetic stability and diversity of *Pneumocystis carinii* infecting rat colonies. *Infect. Immun.* **61**:4801–4813.
9. Drickamer, K. 1989. Demonstration of carbohydrate-recognition activity in diverse proteins which share a common primary structure motif. *Biochem. Soc. Trans.* **17**:13–15.
10. Ezekowitz, R. A. B., R. Sim, M. Hill, and S. Gordon. 1984. Local opsonisation of secreted macrophage components: role of receptors for complement in uptake of zymosan. *J. Exp. Med.* **159**:244–260.
11. Ezekowitz, R. A. B., and P. D. Stahl. 1988. The structure-function of vertebrate mannose lectin-like proteins. *J. Cell Sci. Suppl.* **9**:121–133.
12. Ezekowitz, R. A. B., D. J. Williams, H. Koziel, M. Y. K. Armstrong, A. Warner, F. F. Richards, and R. M. Rose. 1991. Uptake of *Pneumocystis carinii* mediated by the macrophage mannose receptor. *Nature (London)* **351**:155–158.
13. Gigliotti, F. 1992. Host species specific antigenic variation of mannoseylated surface glycoprotein of *Pneumocystis carinii*. *J. Infect. Dis.* **165**:329–337.
14. Gigliotti, F., P. J. Haidaris, C. G. Haidaris, T. W. Wright, and K. R. Van der Meid. 1993. Further evidence of host species-specific variation in antigens of *Pneumocystis carinii* using the polymerase chain reaction. *J. Infect. Dis.* **168**:191–194.
15. Gigliotti, F., D. C. Stokes, A. B. Cheatham, D. S. Davis, and W. T. Hughes. 1986. Development of murine monoclonal antibodies to *Pneumocystis carinii*. *J. Infect. Dis.* **154**:315–322.
16. Haidaris, P. J., T. W. Wright, F. Gigliotti, and C. G. Haidaris. 1992. Expression and characterization of a cDNA clone encoding an immunodominant surface glycoprotein of *Pneumocystis carinii*. *J. Infect. Dis.* **166**:1113–1123.
17. Hidalgo, H. A., R. J. Helmke, V. F. German, and J. A. Mangos. 1992. *Pneumocystis carinii* induces an oxidative burst in alveolar macrophages. *Infect. Immun.* **60**:1–7.
18. Hoffman, O. A., J. E. Standing, and A. H. Limper. 1993. *Pneumocystis carinii* stimulates tumor necrosis factor- $\alpha$  release from alveolar macrophages through a  $\beta$ -glucan-mediated mechanism. *J. Immunol.* **150**:3932–3940.
19. Hunter, S. W., H. Gaylord, and P. Brennan. 1986. Structure and antigenicity of the phosphorylated lipopolysaccharide antigens from the leprosy and tubercle bacilli. *J. Biol. Chem.* **261**:12345–12351.
20. Kanbe, T., Y. Han, B. Redgrave, M. H. Riesselman, and J. E. Cutler. 1993. Evidence that mannans of *Candida albicans* are responsible for adherence of yeast forms to spleen and lymph node tissue. *Infect. Immun.* **61**:2578–2584.
21. Kery, V., J. J. Krepinsky, C. D. Warren, P. Capek, and P. D. Stahl. 1992. Ligand recognition of purified human mannose receptor. *Arch. Biochem. Biophys.* **298**:49–55.
22. Kovacs, J. A., F. Powell, J. C. Edman, B. Lundgren, A. Martinez, B. Drew, and C. W. Angus. 1993. Multiple genes encode the major surface glycoprotein of *Pneumocystis carinii*. *J. Biol. Chem.* **268**:6034–6040.
23. Koziel, H., B. A. Kruskal, A. B. Ezekowitz, and R. M. Rose. 1993. HIV impairs alveolar macrophage mannose receptor function against *Pneumo-*

- cystis carinii*. Chest **103**:111S–112S.
24. **Koziel, H., D. J. Williams, M. Y. K. Armstrong, F. F. Richards, J. A. Fishman, R. A. B. Ezekowitz, A. Warner, J. Fugelstad, and R. M. Rose.** 1991. New rapid method for the study of *Pneumocystis carinii* interaction with alveolar macrophages. J. Protozool. **38**:173S–174S.
  25. **Limper, A. H., and W. J. Martin.** 1990. *Pneumocystis carinii*: inhibition of lung cell growth mediated by parasite attachment. J. Clin. Invest. **85**:391–396.
  26. **Limper, A. H., K. P. Offord, T. F. Smith, and W. J. Martin.** 1989. *Pneumocystis carinii* pneumonia: differences in lung parasite number and inflammation in patients with and without AIDS. Am. Rev. Respir. Dis. **140**:1204–1209.
  27. **Limper, A. H., D. M. O' Riordan, and J. E. Standing.** 1993. *Pneumocystis carinii* gp120 mediates organism attachment to alveolar macrophages but not release of tumor necrosis factor- $\alpha$  (abstract). Clin. Res. **41**:680A.
  28. **Limper, A. H., S. T. Pottratz, and W. J. Martin.** 1991. Modulation of *Pneumocystis carinii* adherence to cultured lung cells by a mannose-dependent mechanism. J. Lab. Clin. Med. **118**:492–499.
  29. **Limper, A. H., J. E. Standing, O. A. Hoffman, M. Castro, and L. W. Neese.** 1993. Vitronectin binds to *Pneumocystis carinii* and mediates organism attachment to cultured lung epithelial cells. Infect. Immun. **61**:4302–4309.
  30. **Linke, M. J., M. T. Cushion, and P. D. Walzer.** 1989. Properties of the major antigens of rat and human *Pneumocystis carinii*. Infect. Immun. **57**:1547–1555.
  31. **Lundgren, B., G. Y. Lipchik, and J. A. Kovacs.** 1991. Purification and characterization of a major human *Pneumocystis carinii* surface antigen. J. Clin. Invest. **87**:163–170.
  32. **Masur, H., and T. C. Jones.** 1978. The interaction *in vitro* of *Pneumocystis carinii* with macrophages and L-cells. J. Exp. Med. **147**:157–170.
  33. **Murray, J. F., S. M. Garay, P. C. Hopewell, J. Mills, G. L. Snider, and D. E. Stover.** 1989. NHLBI workshop summary: pulmonary complications of AIDS. Am. Rev. Respir. Dis. **135**:504–509.
  34. **Murray, J. F., and J. Mills.** 1990. Pulmonary infectious complications of human immunodeficiency virus infection. Am. Rev. Respir. Dis. **141**:1582–1598.
  35. **Nakamura, Y., K. Kazuhiro, M. Wada, and M. Saito.** 1991. Epitope study and cDNA screening of a major surface glycoprotein of *Pneumocystis carinii*. J. Protozool. **38**:3S–4S.
  36. **Neese, L. W., J. E. Standing, E. J. Olson, M. Castro, and A. H. Limper.** 1994. The role of vitronectin, fibronectin, and immune opsonization in augmenting tumor necrosis factor- $\alpha$  release during *Pneumocystis carinii* pneumonia. J. Immunol. **152**:4549–4556.
  37. **Pesanti, E. L., and J. D. Shanley.** 1988. Glycoproteins of *Pneumocystis carinii*: characterization by electrophoresis and microscopy. J. Infect. Dis. **158**:1353–1359.
  38. **Peters, S. G., and U. B. S. Prakash.** 1987. *Pneumocystis carinii* pneumonia. Am. J. Med. **82**:73–78.
  39. **Pistole, T. G.** 1981. Interaction of bacteria and fungi with lectins and lectin-like substances. Annu. Rev. Microbiol. **35**:85–112.
  40. **Porath, J., R. Axen, and S. Ernback.** 1967. Chemical coupling of peptides and proteins to polysaccharides by means of cyanogen halides. Nature (London) **214**:1302–1304.
  41. **Pottratz, S. T., and W. J. Martin.** 1990. Role of fibronectin in *Pneumocystis carinii* attachment to cultured lung cells. J. Clin. Invest. **85**:351–356.
  42. **Pottratz, S. T., and W. J. Martin.** 1990. Mechanism of *Pneumocystis carinii* attachment to cultured rat alveolar macrophages. J. Clin. Invest. **86**:1678–1683.
  43. **Pottratz, S. T., J. Paulsrud, J. Smith, and W. J. Martin.** 1991. *Pneumocystis carinii* attachment to cultured lung cells by gp120, a fibronectin binding protein. J. Clin. Invest. **88**:403–407.
  44. **Radding, J. A., M. Y. K. Armstrong, E. Ullu, and F. F. Richards.** 1989. Identification and isolation of a major cell surface glycoprotein of *Pneumocystis carinii*. Infect. Immun. **57**:2149–2157.
  45. **Sepkowitz, K. A., A. E. Brown, E. E. Telzak, S. Gottlieb, and D. Armstrong.** 1992. *Pneumocystis carinii* pneumonia among patients without AIDS in a cancer hospital. JAMA **267**:832–837.
  46. **Silverblatt, F. J., J. S. Dreyer, and S. Schauer.** 1979. Effect of pili on susceptibility of *Escherichia coli* to phagocytosis. Infect. Immun. **24**:218–223.
  47. **Stahl, R. D.** 1990. The macrophage mannose receptor: current status. Am. J. Respir. Cell Mol. Biol. **2**:317–318.
  48. **Stahl, R. D., T. Wileman, and V. L. Shepherd.** 1985. The mannose receptor of macrophages: a current perspective, p. 59–69. In R. van Furth (ed.), Mononuclear phagocytes: characteristics, physiology and function. Martinus Nijhoff, Dordrecht, The Netherlands.
  49. **Stringer, S. L., T. Garbe, S. M. Suskin, and J. R. Stringer.** 1993. Genes encoding antigenic surface glycoproteins in *Pneumocystis carinii* in humans. J. Eukaryot. Microbiol. **40**:821–826.
  50. **Sutherland, I. W.** 1985. Biosynthesis and composition of gram-negative bacterial extracellular cell wall polysaccharides. Annu. Rev. Microbiol. **39**:243–270.
  51. **Theus, S. A., M. J. Linke, R. P. Andrews, and P. D. Walzer.** 1993. Proliferative and cytokine responses to a major surface glycoprotein of *Pneumocystis carinii*. Infect. Immun. **61**:4703–4709.
  52. **Von Behren, L. A., and E. L. Pesanti.** 1978. Uptake and degradation of *Pneumocystis carinii* by macrophages *in vitro*. Rev. Respir. Dis. **118**:1051–1059.
  53. **Wada, M., K. Kitada, M. Saito, K. Egawa, and Y. Nakamura.** 1993. cDNA sequence diversity and genomic clusters of major surface glycoprotein genes of *Pneumocystis carinii*. J. Infect. Dis. **168**:979–985.
  54. **Wileman, T. E., M. R. Lennartz, and P. D. Stahl.** 1986. Identification of the macrophage mannose receptor as a 175 kDa membrane protein. Proc. Natl. Acad. Sci. USA **83**:2501–2505.
  55. **Wright, T. W., P. J. Simpson-Haidaris, F. Gigliotti, A. G. Harmsen, and C. G. Haidaris.** 1994. Conserved sequence homology of cysteine-rich regions in genes encoding glycoprotein A in *Pneumocystis carinii* derived from different host species. Infect. Immun. **62**:1513–1519.
  56. **Zimmerman, P. E., D. R. Voelker, F. X. McCormack, J. R. Paulsrud, and W. J. Martin.** 1992. 120-kD surface glycoprotein of *Pneumocystis carinii* is a ligand for surfactant protein A. J. Clin. Invest. **89**:143–149.