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Vitronectin Binds to *Pneumocystis carinii* and Mediates Organism Attachment to Cultured Lung Epithelial Cells

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***Pneumocystis carinii* attaches to alveolar epithelial cells during the development of pneumonia. Adhesive proteins found within the alveolar space have been proposed to mediate *P. carinii* adherence to lung cells. Vitronectin (Vn), a 75-kDa glycoprotein present in the lower respiratory tract, has substantial cell-adhesive properties and might participate in the host-parasite interaction during *P. carinii* pneumonia. To address whether Vn binds to *P. carinii*, we studied the interaction of radiolabeled Vn with purified *P. carinii* organisms. Vn bound to *P. carinii*, occupying an estimated 5.47×10^5 binding sites per organism, with an affinity constant, K_d, of 4.24×10^{-7} M. Interestingly, the interaction of Vn with *P. carinii* was not mediated through the Arg-Gly-Asp cell-adhesive domain of Vn. Addition of Arg-Gly-Asp-Ser (RGDS) peptides did not inhibit binding. In contrast, Vn binding to *P. carinii* was substantially inhibited by the addition of heparin or by digesting the organisms with heparitinase, suggesting that *P. carinii* may interact with the glycoaminoglycan-binding domain of Vn. To determine whether Vn might enhance *P. carinii* attachment to lung epithelial cells, we studied the binding of ^{51}Cr-labeled *P. carinii* to cultured A549 lung cells. Addition of Vn resulted in significantly increased binding of *P. carinii* to A549 cells, whereas a neutralizing anti-Vn serum substantially reduced the binding of *P. carinii* to A549 cells. These data suggest that Vn binds to *P. carinii* and that Vn might provide an additional means by which *P. carinii* attaches to respiratory epithelial cells.**

Pneumocystis carinii pneumonia is a serious complication of immunosuppression occurring in patients with hematologic and solid malignancies, a transplanted organ, or AIDS (7, 18, 22, 27, 37). Ultrastructural studies reveal that *P. carinii* attaches to lung epithelial cells during the development of pneumonia (6, 17, 20, 25). The adhesive protein fibronectin has been implicated in the binding of *P. carinii* to cultured A549 lung cells through its interaction with gp120, a mannose-rich surface glycoprotein present on *P. carinii* (32, 33). The corresponding receptor for fibronectin-coated *P. carinii* on intact respiratory epithelium has not been determined. Cultured A549 lung cells possess integrin fibronectin receptors. However, mature bronchial epithelial cells do not exhibit substantial numbers of fibronectin receptors in vivo (1). Lower respiratory tract epithelial cells instead possess receptors for the adhesive protein vitronectin (Vn) (1). In view of the substantial cell-adhesive properties of Vn and the presence of Vn receptors in the lower respiratory tract, we hypothesized that Vn might bind to *P. carinii* and mediate its attachment to epithelial cells.

Vn is a 75-kDa glycoprotein present in serum and produced locally in the lung by alveolar macrophages. Vn has multiple biological functions which promote cell adhesion, migration, and proliferation. These effects are mediated predominantly through the Arg-Gly-Asp (RGD) sequence of the Vn molecule (15, 35, 40). Immunoreactive Vn has been detected in bronchoalveolar lavage samples obtained from normal volunteers and from patients with inflammatory lung disease (31). Previously, Vn has been shown to interact with a number of pathogenic microorganisms, including *Staphylococcus aureus*, streptococci, and *Escherichia coli* (8). The significance of Vn-microbe interactions in the pathogenesis of infection has not yet been established.

Although *P. carinii* organisms encounter substantial concentrations of Vn in both normal and diseased lung, the role of Vn in *P. carinii* pneumonia has not been fully studied. Accordingly, this investigation was undertaken to determine whether Vn interacts with purified *P. carinii* and to determine which regions of Vn interact with the organism. To also determine the potential role of Vn in mediating *P. carinii* attachment to host epithelial cells, we assessed the impact of Vn in mediating *P. carinii* attachment to cultured A549 lung cells.

MATERIALS AND METHODS

Materials. All organic chemicals were of analytical grade and obtained from Sigma Chemical Co. (St. Louis, Mo.) or Fischer Scientific Co. (Pittsburgh, Pa.) unless otherwise specified. Iodo-Beads were obtained from Pierce Chemical Co., Rockford, Ill.; fetal bovine serum was from GIBCO, Grand Island, N.Y.; and carrier-free $Na^{125}I$ and Rainbow molecular weight standards were from Amersham, Arlington Heights, Ill. Outdated human plasma was obtained from the Mayo Medical Center blood products laboratory (Rochester, Minn.). A polyclonal rabbit antiserum generated against human Vn was the generous gift of Deane Mosher (Department of Medicine, University of Wisconsin, Madison). Monoclonal antibody 5E12, which recognizes the major surface antigen gp120 of *P. carinii*, was kindly provided by Francis Gigliotti, Department of Pediatrics, University of Rochester, Rochester, N.Y. (12). Heparitinase was purchased from Seikagaku Kogyo Co. Ltd., Tokyo, Japan.

Preparation of *P. carinii*. *P. carinii* pneumonia was induced in Harlan Sprague-Dawley rats by immunosuppression with dexamethasone as previously reported (2). Specif-

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ic-pathogen-free rats (Harlan Sprague-Dawley, Inc., Indianapolis, Ind.) were provided with drinking water containing dexamethasone (2 mg/liter), tetracycline hydrochloride (500 mg/liter), and nystatin (200,000 U/liter) ad libitum. After 5 days of immunosuppression, rats were inoculated intratracheally with *P. carinii* (500,000 organisms). The *P. carinii* inocula were prepared by aseptically grinding lung tissue from previously immunosuppressed rats with *P. carinii* pneumonia through a stainless steel screen (150 mesh). After 4 to 6 weeks of immunosuppression, inoculated rats exhibiting respiratory distress were killed and exsanguinated, and whole-lung lavage was performed with 50 ml of Hanks' balanced salt solution (HBSS) in sequential 10-ml aliquots. *P. carinii* organisms were purified by differential centrifugation as previously described (21). Lavage fluid was centrifuged (400 × *g*, 10 min), and *P. carinii* in the cell pellet was identified with a modified Wright-Giemsa stain (Diff-Quick; Harleco, Dade Diagnostics). The supernatant containing predominantly *P. carinii* organisms was recentrifuged (1,400 × *g*, 30 min), the pellet was resuspended in 1 ml of HBSS, and duplicate 10-μl aliquots of suspension were spotted onto glass slides and stained with Diff-Quick.

The *P. carinii* isolates were quantified by counting *P. carinii* nuclei as described previously (9). The number of *P. carinii* nuclei in at least five oil immersion fields was determined for each spot as follows: no. of nuclei per ml [(no. of nuclei/field) × (no. of fields/drop)] × 10^2. Typically, repeat-determination values for *P. carinii* number ranged within 5 and 15%, depending on the organism burden. All *P. carinii* organism numbers reported in the text represent total *P. carinii* nuclei. The average yield per rat was approximately 1.5 × 10^7 organisms. The *P. carinii* isolates contained both cyst and trophozoite forms. The trophozoites are substantially smaller (1 to 2 μm) than the cysts (8 μm). The ratio of trophozoites to cysts was typically 9:1 in our preparations. *P. carinii* generally represented greater than 97% of the cellular differential on Diff-Quik-stained smears, with the remainder representing fragmented (and hence nonviable) host cells. Viable host cells were not detected in *P. carinii* isolates by trypan blue staining. If other microorganisms were noted in the lavage smear, the material was discarded. Whole-lung lavage samples from control rats without *P. carinii* failed to yield any material after the second centrifugation (21). Complete microbiologic cultures of selected *P. carinii* isolates failed to reveal growth of bacteria or fungi over 72 h.

Binding of radiolabeled Vn to *P. carinii*. Plasma Vn was isolated from outdated human plasma by heparin affinity chromatography as described by Yatohgo and coworkers (44). Native Vn is a 75-kDa molecule, but most preparations contain various amounts of a 65-kDa protein resulting from proteolytic loss of a 10-kDa fragment from the carboxy terminus (41). The function of this 10-kDa region is unclear, but it has not been shown to participate in cell adhesion or in the interaction of Vn with glycosaminoglycans (31). Our preparations were assessed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Coomassie blue protein staining and found to contain roughly equivalent amounts of 65-kDa and 75-kDa Vn without other contaminants. The identities of both bands were confirmed by amino-terminal sequence analysis with the initial 12 amino-terminal residues matching those published for human Vn (DQESCKGRCTEG) (40). Purified 65- and 75-kDa Vn (1.0 mg in 0.5 ml of phosphate-buffered saline [PBS]) was iodinated with 1 mCi of sodium iodide-125 in a siliconized glass tube for 10 min at 20°C with four Iodo-Beads to a specific activity of approximately 200,000 cpm/μg.

The binding of radiolabeled Vn to *P. carinii* was assessed by suspension binding in polypropylene tubes in the presence of bovine serum albumin as described previously for other ligands (23, 32, 45). Two million *P. carinii* were resuspended in 500 μl of Dulbecco's modified Eagle's medium (DMEM) containing bovine serum albumin (BSA; 1 mg/ml). ^{125}I-Vn was added at various concentrations and incubated with shaking at different temperatures for various times, as indicated below and in the figures. Following incubation, the *P. carinii* were washed three times with DMEM containing BSA by short centrifugations (1,400 × *g*, 1 min each), and the pellets were transferred to clean tubes. Levels of bound and nonbound ^{125}I-Vn were determined by gamma counting. To determine the extent of nonspecific binding, parallel Vn binding assays were performed in the presence of a 100-fold excess of unlabeled Vn.

The specific binding data were used to estimate the binding affinity of Vn for the *P. carinii* isolates and the number of binding sites on the organism by the method of Scatchard (36). Plotting the ratio of bound Vn to free Vn against the amount of bound Vn results in a straight line whose slope is the dissociation constant (K_d) and whose intercept gives the number of Vn binding sites per *P. carinii* organism.

Regions of Vn which interact with *P. carinii*. Vn contains several distinct domains capable of interacting with macromolecules and cells. These include Vn's cell-adhesive domain, which contains an Arg-Gly-Asp sequence, necessary for attachment and spreading of mammalian cells on Vn substrates, and a glycosaminoglycan-binding region, which interacts with heparin-containing molecules (41). To determine whether the cell-adhesive domain of Vn mediated the binding of Vn to *P. carinii*, the binding of radiolabeled Vn was assayed in the presence of peptides RGDS and RGES (1 and 2 mM). RGDS has been reported to inhibit mammalian cell adhesion to Vn, whereas RGES does not (35).

Two approaches were taken to determine whether the glycosaminoglycan-binding region of Vn mediated binding to *P. carinii*. First, Vn binding to *P. carinii* in the presence and absence of heparin (1 mg/ml, porcine intestinal mucosa heparin; Sigma) was studied. Second, we digested freshly isolated *P. carinii* with heparitinase (0.05 U/ml) in PBS containing 10^{-5} M calcium for 1 h at 37°C. Following digestion, the specific binding of Vn was assayed as described above and compared with that of undigested *P. carinii* maintained for 1 h in PBS containing 10^{-5} M calcium without heparitinase.

Ligand blotting and immunoblotting. The interaction of Vn with purified *P. carinii* components was assessed by studying the binding of ^{125}I-Vn to solubilized *P. carinii* separated by SDS-PAGE and immobilized on nitrocellulose. *P. carinii* (20 × 10^6 organisms) were dissolved in 2% SDS containing 50 mM dithiothreitol. This *P. carinii* extract was separated by discontinuous SDS-PAGE with 3% acrylamide stacking and 7% acrylamide resolving gels. Following separation, *P. carinii* components were electrophoretically transfered to nitrocellulose with a Hoeffer Transphor apparatus (60 V for 6 h) as described previously (13). The nitrocellulose membranes were washed with PBS containing 0.05% Tween 20. Nonspecific binding sites were blocked by overnight incubation with PBS containing 1% Tween 20 at 4°C. The nitrocellulose membranes were incubated overnight with ^{125}I-Vn (20 μg/ml) in PBS with 0.05% Tween 20, washed four times, and examined by autoradiography. In some experiments, an

identical number of *P. carinii* (20×10^6 organisms) were digested with heparitinase prior to extraction, SDS-PAGE, and blotting with ^{125}I-Vn.

The presence of gp120 in the *P. carinii* extracts was evaluated by immunoblotting with monoclonal antibody (MAb) 5E12, a mouse immunoglobulin M (IgM) antibody developed against rat *P. carinii* (12). Although antigenic differences have been noted between *P. carinii* isolates from different host species (4, 19, 38, 42, 43), at present all of these organisms are believed to represent different strains of *P. carinii*. Furthermore, antigenic similarity exists between *P. carinii* strains (12). Specifically, MAb 5E12 recognizes the major surface antigen (gp120) found on *P. carinii* from rats, ferrets, and humans (12). Therefore, to confirm the presence of gp120 in our isolates, *P. carinii* components separated by electrophoresis were transferred to nitrocellulose membranes, washed with PBS containing 0.05% Tween 20, and blocked by incubation with PBS containing 1% Tween 20 at 4°C. The nitrocellulose membranes were incubated with MAb 5E12 (1:50 dilution of 5E12 ascites) in PBS containing 1% Tween 20, washed, and reincubated with a secondary peroxidase-conjugated goat anti-mouse IgM antibody (Sigma). After a wash, bound antibodies were visualized by the reaction of peroxidase with diaminobenzidine in the presence of H_2O_2 (DAB substrate; Pierce Chemical Co.).

Role of Vn in mediating *P. carinii* adherence to cultured lung cells. Electron microscopy reveals that *P. carinii* attach preferentially to type I pneumocytes but occasionally to type II cells (6, 17, 25). The A549 cell line has been used extensively to study the biology of *P. carinii* in culture (9, 10, 21, 32). This cell line, derived from a bronchoalveolar carcinoma, has been characterized as presumptive type II pneumocytes that do not secrete phosphatidylcholine (14). A549 cells have been reported to support *P. carinii* viability in tissue culture (9, 10) and have been used as a model to study *P. carinii*-lung cell interactions in vitro (10, 21, 32). In addition, preliminary studies in our laboratory demonstrate that A549 cells can bind ^{125}I-Vn in a saturable manner which is inhibitable by EDTA, confirming the presence of Vn receptors on A549 lung cells.

Vn has well-documented cell-adhesive properties. Therefore, in order to assess the potential role of Vn in the attachment of *P. carinii* to respiratory epithelial cells, we quantified the adherence of radiolabeled *P. carinii* to cultured A549 cells in the presence and absence of Vn and in the presence of a neutralizing Vn antiserum. The adherence of radiolabeled *P. carinii* to A549 cells was assayed as described previously (21, 23, 32, 33). Briefly, purified *P. carinii* were incubated for 18 h in 1 ml of DMEM (Whittaker M. A. Bioproducts, Walkersville, Md.) containing penicillin (10 μg/ml), gentamicin (4 μg/ml), amphotericin (0.5 μg/ml), and 50 μCi of ^{51}Cr (679 mCi/mg; New England Nuclear) per ml. This suspension was washed four times to remove unincorporated ^{51}Cr. Uptake of ^{51}Cr label was generally within the range of ± 20% of the total radioactivity for any individual *P. carinii* isolate. Two million ^{51}Cr-labeled *P. carinii* were incubated with confluent monolayers of A549 cells (American Type Culture Collection, Rockville, Md.) grown in 24-well plates in Dulbecco's modified Eagle's medium containing 10% heat-inactivated fetal calf serum, penicillin, gentamicin, and amphotericin for 8 h. The cells were washed three times with DMEM containing BSA to remove medium containing free serum. After incubation, organisms appeared to be adherent to the A549 cell monolayers by phase-contrast microscopy. Cultures of *P. carinii* on A549 cells which showed any evidence of growth of other microorganisms were discarded. Subsequently, the medium was removed, and the A549 cell monolayers were washed three times with HBSS to remove any unattached *P. carinii*. Adherence of *P. carinii* was defined as follows: % adherence = $(A/A + B) \times 100$, where A is ^{51}Cr disintegrations per minute (dpm) associated with the A549 cell monolayer and B is dpm of ^{51}Cr-labeled *P. carinii* not adherent to the cellular monolayer. To assess the effect of Vn on the attachment of *P. carinii* to A549 lung cells in culture, *P. carinii* adherence assays were conducted in the presence and absence of purified Vn (100 μg/ml) or in the presence of nonimmune rabbit serum or a polyclonal rabbit antiserum generated against Vn (20% by volume in DMEM containing BSA [1 mg/ml]).

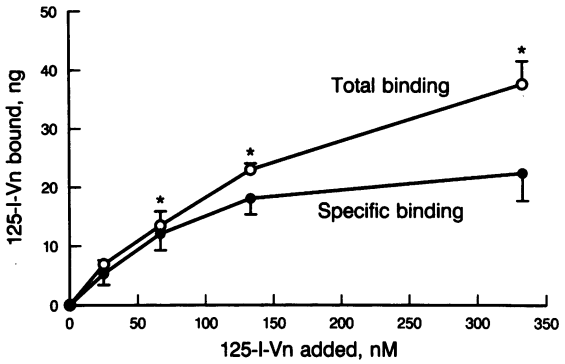

FIG. 1. Total and specific binding of Vn to *P. carinii*. *P. carinii* (10^6) were incubated in 0.5 ml of DMEM containing BSA (1 mg/ml) in the presence of ^{125}I-Vn at the concentrations noted at 37°C for 1 h. Nonspecific binding was assessed in parallel binding assays performed in the presence of a 100-fold excess of unlabeled Vn. Specific binding was defined as total minus nonspecific binding. Vn exhibited significant specific binding to *P. carinii* at all concentrations greater than or equal to 5 μg/ml. *, $P < 0.05$ for total versus nonspecific binding. Each point represents the mean ± SEM for four experiments.

Statistical methods. Data are expressed as mean ± standard error of the mean (SEM). Differences between control and experimental data groups were assessed by analysis of variance for multiple treatments. Data from paired experiments were analyzed with the two-sample Student's *t* test. Correlation coefficients were determined by the linear regression method. Statistical testing was performed with the Statview II statistical package (Abacus Concepts, Inc., Berkeley, Calif.) on a Macintosh IIci personal computer. A value of $P < 0.05$ was defined as a statistically significant difference for two-sided alternatives.

RESULTS

Vn binds specifically to *P. carinii*. To assess the interaction of *P. carinii* with the adhesive protein Vn, we studied the specific binding of radiolabeled Vn to purified *P. carinii* organisms (Fig. 1). The interaction of Vn with *P. carinii* demonstrated concentration dependence of binding and saturation at higher concentrations. All concentrations of ^{125}I-Vn greater than or equal to 5 μg/ml resulted in significant specific binding of Vn to *P. carinii*, comparing total and nonspecific binding. Specific binding of ^{125}I-Vn to the *P. carinii* isolates containing trophozoites and cyst forms was used to construct an overall Scatchard plot (Fig. 2). The data were analyzed by linear regression consistent with a homogeneous population of Vn binding sites on *P. carinii*. The

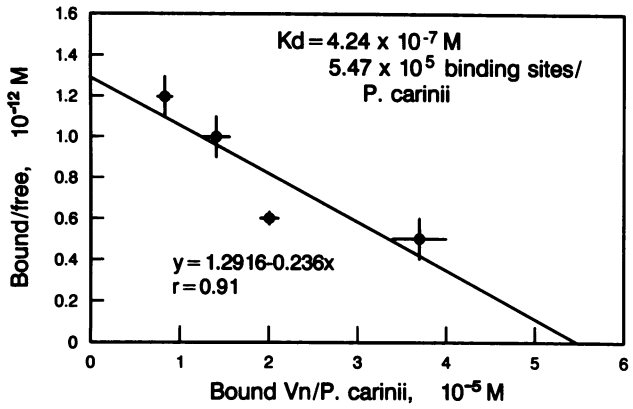

FIG. 2. Scatchard binding analysis of interaction of Vn with *P. carinii*. The specific binding data from a representative set of experiments were used to construct a Scatchard plot of Vn binding to *P. carinii*. Plotting bound/free versus free Vn resulted in a straight line whose negative slope is equal to the K_d and whose intercept revealed the number of Vn binding sites per *P. carinii* organism. The linear nature of the regression line ($r = 0.91$) suggests that a single population of Vn receptors is present on *P. carinii* with an affinity constant (K_d) of 4.24×10^{-7} M and with 5.47×10^5 specific Vn binding sites on each *P. carinii* organism.

linear model of specific binding can be described as follows: $y = 1.2916 - 0.236x$ ($r = 0.91$), where y represents the ratio of the number of molecules of Vn bound to *P. carinii* versus the molar concentration of free Vn and x represents the number of molecules of Vn bound to each *P. carinii* organism. The slope of this equation yields an estimated affinity constant (K_d) of 4.24×10^{-7} M, and the x intercept suggests the presence of roughly 5.47×10^5 specific Vn binding sites per *P. carinii* organism.

We evaluated the time and temperature dependence of Vn binding to purified *P. carinii* organisms (Fig. 3). Specific binding of Vn to *P. carinii* was optimal at 37°C and occurred within 15 min. Binding at 37°C plateaued between 30 and 60

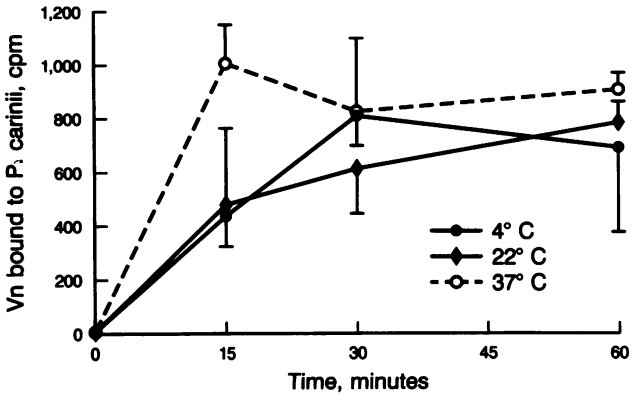

FIG. 3. Time and temperature dependence of Vn binding to *P. carinii*. *P. carinii* (10^6) were incubated in 0.5 ml of DMEM containing BSA (1 mg/ml) in the presence of ^{125}I-Vn (10 μg/ml) at the indicated times and temperatures. Nonspecific binding was assessed in parallel binding assays performed in the presence of a 100-fold excess of unlabeled Vn. Shown is specific binding, defined as total binding minus nonspecific binding under each condition. Optimal binding occurred within 15 min at 37°C. The data represent the mean ± SEM for three experiments.

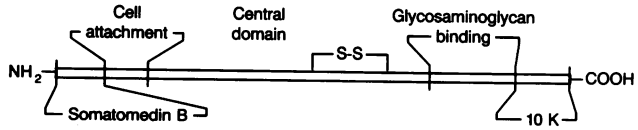

FIG. 4. Domain structure of Vn. This map of Vn demonstrates the amino-terminal region, homologous to somatomedin B; the Arg-Gly-Asp-containing domain, which mediates adhesion to mammalian cells; the central domain, which contains one intrachain disulfide bond (S-S); the glycosaminoglycan-binding domain, which interacts with heparin; and the 10-kDa carboxy-terminal protease-sensitive region. (Adapted from Suzuki et al. [41].)

min. Longer time intervals did not result in significantly greater binding. Lower temperature (4 and 22°C) resulted in a slower onset of binding, which attained roughly the same maximal binding after 60 min as observed at 37°C. Autoclaving of *P. carinii* (125°C for 20 min) resulted in a substantial (83.2% ± 6.0%) reduction in specific binding of Vn to the organism. This likely resulted from denaturation of the Vn-binding molecule present on *P. carinii* under these conditions.

Vn binding to *P. carinii* is not mediated by simple binding of the Arg-Gly-Asp region. Vn contains multiple regions capable of interacting with macromolecules and eukaryotic cells. Proteolytic studies by Suzuki and coworkers have mapped several specific biochemical domains on Vn (Fig. 4) (41). The binding of mammalian cells to Vn is mediated by the interaction of integrin receptors with Vn's Arg-Gly-Asp sequence, contained within the cell-adhesive domain of the molecule. Exogenous RGDS peptide has been shown to inhibit the binding of mammalian cells to Vn, whereas the tetrapeptide RGES does not impair cell binding to Vn substrates (35).

To test whether Vn binds to *P. carinii* through its Arg-Gly-Asp region, Vn-binding assays were conducted in the presence of RGDS and RGES tetrapeptides. Surprisingly, RGDS peptides did not inhibit binding but instead increased the binding of Vn to *P. carinii*. Binding in the presence of 2 mM RGDS resulted in a 3.5-fold increase in Vn binding to *P. carinii* ($P = 0.007$ versus control). Furthermore, RGES, a peptide which does not inhibit mammalian cell binding to Vn, also caused a significant 2.2-fold increase in the specific binding of Vn to *P. carinii* ($P = 0.0004$ versus control). Lower concentrations of RGDS (1 mM) resulted in a 1.3-fold increase in Vn binding to *P. carinii*. These data suggested that the binding of soluble Vn to *P. carinii* was not mediated through a simple interaction of the cell-adhesive RGD domain of Vn with *P. carinii* organisms. The mechanism of enhanced Vn binding to *P. carinii* in the presence of RGD peptides is not clear but may involve the activation of other receptors on *P. carinii* which recognize an alternative site on the Vn molecule.

Vn binding to *P. carinii* is inhibited by heparin and heparitinase treatment. Since the binding of Vn to *P. carinii* was not inhibited by RGD peptide, we hypothesized that the organism might be interacting with the glycosaminoglycan-binding domain of Vn. To test this, two approaches were undertaken. First, *P. carinii* binding of ^{125}I-Vn was conducted in the presence of heparin. Vn binding to the organism was inhibited 64.5% ± 13.7% in the presence of heparin ($P = 0.036$ versus control) (Fig. 5). Additionally, *P. carinii* isolates were digested with heparitinase, an enzyme which cleaves α-*N*-acetyl-D-glucosaminidine linkages in glycosaminoglycans. Heparitinase treatment of *P. carinii* similarly

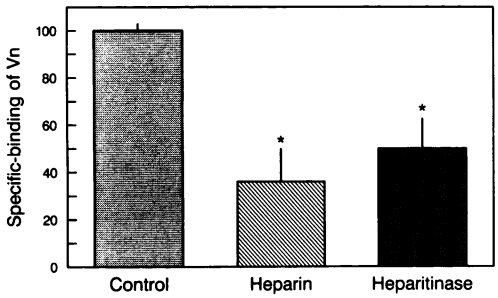

FIG. 5. Vn binding to *P. carinii* inhibited by heparin or by heparitinase digestion. *P. carinii* (10^6) were incubated in 0.5 ml of DMEM containing BSA (1 mg/ml) in the presence of ^{125}I-Vn (10 μg/ml) with either no added heparin (control), heparin (1 mg/ml), or after 1 h of digestion with heparitinase. The addition of heparin or digestion of the organisms with heparitinase resulted in a substantial decrease in specific binding of ^{125}I-Vn to *P. carinii*. *, $P < 0.05$ versus control. These data strongly suggest that *P. carinii* interacts with the glycosaminoglycan-binding region of Vn. Each bar represents the mean ± SEM for three experiments.

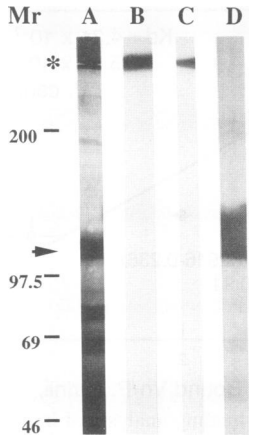

FIG. 6. Vn interacts with a high-molecular-weight component of *P. carinii*. Purified *P. carinii* were extracted in 2% SDS containing 50 mM dithiothreitol, and the extracted components were separated by SDS-PAGE and transferred to nitrocellulose. Lane A, Coomassie blue staining of the separated *P. carinii* extract demonstrates a prominent band at 120 kDa (arrow). Material of >250 kDa (*) was present at the stacking gel-resolving gel interface. Additional components of between 40 and 70 kDa are also visible. Lane B, radiolabeled Vn binds to the component of >250 kDa but not to any of the smaller components, including gp120. Lane C, digestion of an identical number of *P. carinii* with heparitinase resulted in reduced binding of Vn to this >250-kDa component. Lane D, immunoblotting with MAb 5E12 demonstrates reactivity with the 120-kDa material, confirming the presence of gp120 in the extract. However, the component of *P. carinii* which bound Vn did not react with MAb 5E12.

decreased Vn binding to the organism by 50.1% ± 12.9% ($P < 0.05$ versus control). Together, these observations suggest that the glycosaminoglycan-binding domain of Vn interacts with *P. carinii*.

Vn interacts with a high-molecular-weight component of *P. carinii*. The finding that RGD peptides did not inhibit the binding of Vn to *P. carinii* was somewhat surprising, since Vn is known to contain a cell-adhesive RGD sequence (40). Furthermore, recent work has shown that fibronectin, an immunologically distinct adhesive glycoprotein, binds to gp120 present on the surface of *P. carinii* in a manner which can be inhibited by RGDS (32, 33). Therefore, to investigate which components of *P. carinii* bound Vn, we performed ligand blotting of radiolabeled Vn on *P. carinii* components separated by SDS-PAGE and transferred to nitrocellulose (Fig. 6). These *P. carinii* extracts contained abundant amounts of 120-kDa protein as well as the usual components which migrate between 45 and 70 kDa. Additional material of higher molecular mass (greater than 250 kDa) failed to penetrate deeply in the resolving gel and was present mainly at the stacking gel-resolving gel interface. ^{125}I-Vn bound to the higher-molecular-weight component(s) at this interface. We further observed that digesting *P. carinii* with heparitinase prior to extraction and blotting substantially, although not completely, reduced binding of ^{125}I-Vn to this component. Together, these findings suggest that *P. carinii* contains a high-molecular-weight component(s) which interacts with the glycosaminoglycan-binding domain of Vn.

The presence of gp120 in our specimens was confirmed by immunoblotting with MAb 5E12, a mouse MAb which recognizes gp120 (9). *P. carinii* gp120 is a highly mannosylated glycoprotein which runs as a broad band on SDS-PAGE (12, 13). Abundant immunoreactive gp120 was detected in our extracts with MAb 5E12. Although gp120 has been reported to interact with fibronectin (33), ^{125}I-Vn did not bind appreciably to the gp120 present in our *P. carinii* extracts.

Vn enhances the attachment of *P. carinii* to cultured lung cells. To evaluate the potential significance of Vn's interaction with *P. carinii*, we assessed the role of Vn in the attachment of *P. carinii* to cultured A549 lung epithelial cells (Fig. 7A). After 8 h of incubation, 33.9% ± 1.8% of the ^{51}Cr-labeled *P. carinii* cultured were attached to the cultured A549 cells in the absence of Vn. In contrast, incubation of radiolabeled *P. carinii* in the presence of exogenous Vn (100 μg/ml) increased the binding of *P. carinii* to A549 cells to 42.4% ± 3.5% ($P = 0.001$ for presence versus absence of Vn).

Although addition of Vn resulted in significantly increased binding of *P. carinii* to cultured A549 lung cells, the degree of the observed increase was modest. We hypothesized that this might reflect the presence of Vn or other adhesive molecules on the surface of freshly isolated *P. carinii* organisms. To test this, we determined the effect of a polyclonal rabbit antiserum generated against Vn on the attachment of radiolabeled freshly isolated *P. carinii* to cultured A549 cells (Fig. 7B). Binding of *P. carinii* organisms to A549 cells in the presence of nonimmune rabbit serum (20% by volume in DMEM) was 44.3% ± 3.4%. The increased binding of *P. carinii* observed in the presence of nonimmune serum may reflect the presence of Vn and other adhesive proteins in the serum. In contrast, however, the rabbit antiserum to Vn (20% by volume in DMEM) reduced *P. carinii* adherence on A549 cells to 20.0% ± 1.9% ($P = 0.0003$, comparing *P. carinii* adherence in the presence of nonimmune serum versus immune antiserum). Organism viability was assessed by the release of ^{51}Cr label and was not significantly altered by either Vn or the polyclonal antiserum over the time course of these assays. Together, these data strongly support a role for Vn in the attachment of *P. carinii* to cultured A549 lung epithelial cells.

DISCUSSION

Our study indicates that Vn, a cell-adhesive glycoprotein present within the lower respiratory tract, interacts with *P.*

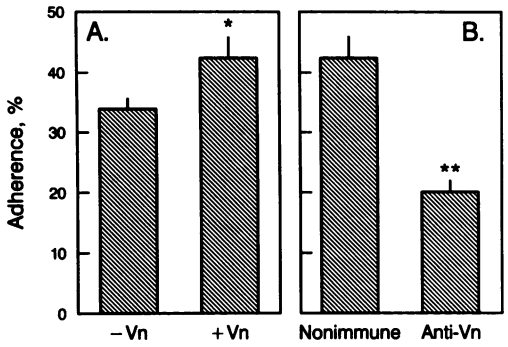

FIG. 7. Role of Vn in the adherence of *P. carinii* to cultured A549 lung cells. (A) ^{51}Cr-labeled *P. carinii* were permitted to bind to confluent monolayers of A549 cells for 8 h in DMEM containing BSA (1 mg/ml) without added Vn or in the presence of added Vn (100 μg/ml). Addition of purified Vn resulted in a modest but significant increase in attachment of radiolabeled *P. carinii* to cultured A549 lung cells. *, $P = 0.001$ for adherence of *P. carinii* to A549 cells in the presence versus the absence of added Vn). (B) To further assess the role of Vn in the attachment of *P. carinii* to lung cells, we determined the adherence of *P. carinii* organisms to cultured A549 cells in the presence of a nonimmune rabbit serum and a polyclonal rabbit antiserum generated against Vn. ^{51}Cr-labeled *P. carinii* were incubated on A549 lung cells for 8 h in the presence of either nonimmune serum or antiserum recognizing Vn (20% by volume in DMEM containing BSA [1 mg/ml]). The specific Vn antiserum resulted in substantial reduction in *P. carinii* attachment to A549 cells. **, $P = 0.0003$ for adherence of *P. carinii* to A549 cells in the presence of nonimmune serum versus antiserum recognizing Vn. Each point represents the mean ± SEM for three experiments.

carinii. Although Vn contains an Arg-Gly-Asp sequence which mediates the attachment of Vn to mammalian cells (35, 40), the adhesion of Vn to *P. carinii* does not appear to be mediated through this sequence. Instead, Vn binding to *P. carinii* can be inhibited by heparin or heparitinase treatment. We also demonstrated that Vn binds with a high-molecular-weight component of *P. carinii*. We have additionally shown that the binding of Vn to *P. carinii* mediates in part the adherence of *P. carinii* to cultured A549 lung epithelial cells.

P. carinii has been described previously as interacting with a number of molecules, including gangliosides (39), immunoglobulins (5), and fibronectins (32, 33). Nonetheless, the interaction of *P. carinii* with certain extracellular matrix molecules, including Vn, appears to be specific. Further evidence suggests that *P. carinii* does not bind randomly with all extracellular matrix proteins present in the lower respiratory tract. For instance, elastin, a prominent lung matrix molecule, does not interact significantly with *P. carinii*. Using similar methods of radiolabeling and suspension binding analysis, we did not observe any significant specific or saturable binding of radiolabeled κ-elastin (Elastin Products Co., Owensville, Mo.) to purified *P. carinii*. It is therefore likely that *P. carinii* binds selectively with specific proteins present within the lower respiratory tract.

The molecular nature of the receptor on *P. carinii* which recognizes Vn is not completely known. Although Vn contains an Arg-Gly-Asp sequence, our data show that Vn binding to *P. carinii* is not inhibited by RGD peptides, and radiolabeled Vn does not appear to bind to gp120, an Arg-Gly-Asp-binding glycoprotein present on *P. carinii*. Indeed, we observed increased binding of Vn to *P. carinii* in the presence of RGD peptides. Potentially, the occupancy of RGD receptors, such as gp120 on the surface of *P. carinii*, may act to potentiate the binding of a separate set of receptors with affinity for Vn.

We have shown that radiolabeled Vn interacts, at least in part, with a high-molecular-weight component of *P. carinii*, potentially representing a high-molecular-weight proteoglycan, glycoaminoglycan, or similar complex carbohydrate antigen on the surface of the organism. This is compatible with our observation that Vn binding to *P. carinii* is reduced by exogenous heparin and by digestion of *P. carinii* with heparitinase. *P. carinii* binding to the glycosaminoglycan-binding region of Vn renders Vn's Arg-Gly-Asp sequence free to interact with cell surface integrins. Thus, the attachment of Vn-coated *P. carinii* to respiratory epithelial cells involves both the interaction of *P. carinii* with the glycosaminoglycan-binding domain of Vn and the binding of epithelial integrin receptors with the Arg-Gly-Asp sequence of the Vn molecule.

In addition to the specific binding of Vn to *P. carinii*, which is inhibitable by excess unlabeled ligand, radiolabeled Vn also exhibited substantial nonspecific interactions with the organism. This nonspecific binding might represent binding of Vn to *P. carinii* through ionic or lectin-mediated binding mechanisms. Further, even Vn bound to the organism on a nonspecific basis may still possess biological activity within the host.

P. carinii attach to alveolar epithelial cells during development of infection (6, 10, 21, 22, 25). Furthermore, successful propagation of *P. carinii* in vitro generally requires the interaction of *P. carinii* with a feeder cell substrate (3, 9, 10). In view of this intimate interaction of *P. carinii* attachment with alveolar epithelial cells, it is not surprising that multiple receptor-ligand systems have evolved to augment attachment of the organism to the host. Extracellular matrix components, most notably fibronectins, have been proposed as potential mediators of *P. carinii* attachment to respiratory epithelial cells (11, 32, 33). Pottratz and colleagues have shown that fibronectin mediates the attachment of *P. carinii* to cultured A549 lung cells through its interaction with gp120 on the surface of *P. carinii* (33). In contrast, Fishman and associates were not able to demonstrate a role for either fibronectin or laminin in the attachment of freshly isolated *P. carinii* to cultured epithelial cell lines (11). The cell surface receptors which potentially mediate the attachment of fibronectin-coated *P. carinii* to epithelium have not been elucidated. Fibronectins frequently interact with dimeric cell surface receptors termed integrins (26). Integrin receptor expression varies in different populations of respiratory epithelial cells. Whereas cultured A549 cells possess fibronectin and Vn receptors, mature lower respiratory epithelial cells do not exhibit substantial numbers of integrin fibronectin receptors in vivo (1). Instead, bronchial epithelial cells possess receptors for Vn (1), suggesting that Vn may mediate the attachment of *P. carinii* to the lower respiratory tract of living hosts. The exact role of integrin receptors in effecting *P. carinii* attachment to alveolar epithelium in living hosts remains to be determined.

A number of lines of evidence document the presence of Vn in the lower respiratory tract during health and disease. Immunoreactive Vn has been detected in the bronchoalveolar lavage fluids of normal subjects and patients with interstitial lung disease (31). The source of Vn in the lung is not fully established. Vn circulates in the plasma and can penetrate the lung following disruption of alveolar-capillary membranes. Additionally, both monocytic cells and alveolar macrophages have been documented to synthesize and se-

crete Vn in vitro (16). Further studies suggest that Vn recovered in the bronchoalveolar lavage fluids of patients with respiratory tract inflammation may be due to enhanced local synthesis (31). Regardless of its source, *P. carinii* are undoubtedly exposed to substantial concentrations of Vn in the lung. Accordingly, freshly purified *P. carinii* may already be partially coated with Vn and other adhesive molecules when isolated (28, 29). This may explain why Vn antiserum substantially inhibited the attachment of *P. carinii* to A549 epithelial cells even in the absence of additional purified Vn.

We have recently shown that Vn levels are increased in the lower respiratory tract of patients with *P. carinii* pneumonia and detectable on the surface of *P. carinii* organisms by immunoblot analysis (28, 29). In addition, Vn has been shown to bind with other pathogens, including *S. aureus*, streptococci, and *E. coli* (8). Additional studies in our laboratory have documented binding of Vn with *Candida albicans* in vitro (24).

The interaction of Vn with *P. carinii* may represent an additional important component of the host-parasite relationship. Whether the interaction of Vn with *P. carinii* results in net advantage or detriment to the parasite remains to be concluded. Our data suggest a potential role for Vn in the attachment of *P. carinii* to cultured lung cells. However, in addition to mediating cellular adhesion events, Vn has a number of other functions which may affect infection. For instance, Vn has been documented to increase the uptake of opsonized particles by monocytic cells (30). Other studies demonstrate a role for Vn in neutralizing the attack complex of complement, thereby preventing further cell lysis (35). Clearly, further evaluations will be necessary to establish the net impact of these other functions of Vn during *P. carinii* pneumonia.

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