

## Murine laminin binds to *Histoplasma capsulatum*. A possible mechanism of dissemination.

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### Research Article

Histoplasmosis, an increasingly important opportunistic infection in immunosuppressed subjects, is characterized by hematogenous dissemination of the yeast from the lung. The mechanism of this dissemination is not fully understood. Laminin, the major glycoprotein of the extracellular matrix, is known to mediate the attachment of various invasive pathogens to host tissues. In the current study, laminin is demonstrated to bind to *Histoplasma capsulatum* in a rapid, specific, and saturable manner. Scatchard analysis with <sup>125</sup>I-labeled laminin revealed an estimated  $3.0 \times 10^4$  binding sites per yeast with an apparent  $K_d$  for laminin binding of  $1.6 \times 10^{-9}$  M. Laminin binding to *H. capsulatum* was decreased from  $62 \pm 1$  to  $17 \pm 1$  ng ( $P < 0.001$ ) in the presence of 3,000 nM of Ile-Lys-Val-Ala-Val, a pentapeptide within one major cell attachment site of laminin. A 50-kD *H. capsulatum* laminin-binding protein was demonstrated using an <sup>125</sup>I-Ln blot of *H. capsulatum* cell wall proteins. The 50-kD protein is also recognized by antibodies directed at the 67-kD laminin receptor, suggesting they are related. This study proposes a possible mechanism for *H. capsulatum* attachment to laminin, an important first step required for the yeast to recognize and traverse the basement membrane.

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# Murine Laminin Binds to *Histoplasma capsulatum*

## A Possible Mechanism of Dissemination

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### Abstract

**Histoplasmosis, an increasingly important opportunistic infection in immunosuppressed subjects, is characterized by hematogenous dissemination of the yeast from the lung. The mechanism of this dissemination is not fully understood. Laminin, the major glycoprotein of the extracellular matrix, is known to mediate the attachment of various invasive pathogens to host tissues. In the current study, laminin is demonstrated to bind to *Histoplasma capsulatum* in a rapid, specific, and saturable manner. Scatchard analysis with <sup>125</sup>I-labeled laminin revealed an estimated  $3.0 \times 10^4$  binding sites per yeast with an apparent  $K_d$  for laminin binding of  $1.6 \times 10^{-9}$  M. Laminin binding to *H. capsulatum* was decreased from  $62 \pm 1$  to  $17 \pm 1$  ng ( $P < 0.001$ ) in the presence of 3,000 nM of Ile-Lys-Val-Ala-Val, a pentapeptide within one major cell attachment site of laminin. A 50-kD *H. capsulatum* laminin-binding protein was demonstrated using an <sup>125</sup>I-Ln blot of *H. capsulatum* cell wall proteins. The 50-kD protein is also recognized by antibodies directed at the 67-kD laminin receptor, suggesting they are related. This study proposes a possible mechanism for *H. capsulatum* attachment to laminin, an important first step required for the yeast to recognize and traverse the basement membrane. (*J. Clin. Invest.* 1995. 96:1010–1017.)** Key words: attachment sites • yeasts • extracellular matrix • glycoproteins • immunosuppression

### Introduction

*Histoplasma capsulatum*, a dimorphic fungal pathogen with worldwide distribution, is endemic to the United States. Disseminated histoplasmosis has emerged as an important opportunistic infection in immunosuppressed subjects, especially those with AIDS. Infection is acquired by inhaling airborne microconidia that elude pulmonary defense mechanisms and lodge in the alveoli and interstitium (1). Transformation of the infectious microconidia to the pathogenic yeast is an absolute requirement for progression to histoplasmosis (2).

The initial events following microconidia deposition in human alveoli are unknown. Experimental evidence with cultured

human alveolar macrophages suggest that the microconidia are rapidly ingested by alveolar macrophages (3). Experimental infections in animals demonstrated transformation of the microconidia phase to the yeast phase, which proliferate within the parasitized macrophages. The host macrophage ruptures and the released yeast parasitize other macrophages, producing a local exudate composed of infected macrophages and lymphocytes (1). Evidence from a human lung biopsy specimen demonstrated that extracellular yeast forms existed within the alveolar exudate and throughout the pulmonary interstitium (4). Current concepts of histoplasmosis in the normal host suggest there is spread of intracellular yeast to adjacent alveoli, to regional lymph nodes, and to distant metastatic sites. The mechanism of metastasis to distant sites is based on indirect evidence. The process of tissue invasion and dissemination is virtually unstudied, particularly during severe immune suppression, when extracellular *H. capsulatum* organisms may be involved in dissemination and pathogenesis of the disease.

The lung contains an alveolar basement membrane that lies between type I alveolar epithelial cells and capillary endothelial cells as well as an endothelial basement membrane that separates endothelial cells from interstitial connective tissue cells along the thick portion of the alveolar wall (5). Laminin, an 850-kD glycoprotein, is the most abundant glycoprotein of the basement membrane and is composed of three polypeptide chains designated A, B1, B2, each containing six domains (6, 7). The multiple domains confer upon laminin many biological properties, including promoting cellular adhesion, growth, differentiation, migration, and matrix assembly. Laminin receptors have been demonstrated on metastatic tumor cells, granulocytes, lymphocytes, and macrophages (8). Recent studies have demonstrated that the invasive pathogenic organisms *Candida albicans* (8, 9), *Aspergillus fumigatus* (10), *Paracoccidioides brasiliensis* (11), staphylococci (12–14), *Helicobacter pylori* (15, 16), *Treponema palladium* (17), streptococci (18–20), *Toxoplasma gondii* (21), and *Escherichia coli* (22, 23) possess surface proteins with strong laminin-binding activity. The mechanism of laminin binding to the microorganism's laminin receptor has only recently been investigated in *A. fumigatus*, enterobacteria, and *H. pylori* (10, 24, 25).

In this study, laminin is demonstrated to bind *H. capsulatum* in a rapid, specific, and saturable fashion. The major cell-binding region of the laminin A chain appears to be the molecular site of laminin binding to *H. capsulatum*. <sup>125</sup>I-labeled laminin (<sup>125</sup>I-Ln)<sup>1</sup> blotting with size-fractionated *H. capsulatum* cell wall proteins demonstrates a 50-kD protein that binds laminin.

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1. Abbreviations used in this paper: <sup>51</sup>Cr-*H. capsulatum*, <sup>51</sup>Cr-labeled *H. capsulatum*; ECM, extracellular matrix; <sup>125</sup>I-Ln, <sup>125</sup>I-labeled laminin; IKVAV, isoleucine-lysine-valine-alanine-valine; YIGSR, tyrosine-isoleucine-glycine-serine-arginine.

Further, antibodies directed at a known 67-kD laminin receptor recognize the 50-kD protein on *H. capsulatum*. These studies provide a biochemical mechanism for the interaction of *H. capsulatum* with a key basement membrane protein and suggest a specific protein on the surface of *H. capsulatum* as the site of laminin binding.

## Methods

***H. capsulatum* isolation.** The yeast phase of *H. capsulatum* strain G217b was maintained using brain–heart infusion agar (Difco Laboratories Inc., Detroit, MI) supplemented with 0.1% cysteine (Sigma Chemical Co., St. Louis, MO). Yeasts were inoculated onto brain–heart infusion agar slants, incubated for 24 h at 37°C, streaked over the remainder of the slant, and incubated for an additional 24–48 h at 37°C. The yeasts were then harvested from the slant, washed four times in PBS and centrifuged at 1,200 g. *H. capsulatum* were counted using a hemacytometer and adjusted to proper concentrations. A fresh preparation of *H. capsulatum* was used for each experiment.

***In vivo* pulmonary *H. capsulatum* infection.** In selected studies, *H. capsulatum* organisms ( $10^7/100 \mu\text{l}$ ) were transtracheally inoculated into CD4 lymphocyte-depleted mice using a minor adaptation of a previously described method (26). Mice were sacrificed 10 d later using Beuthanasia-D (0.1 ml, injected intraperitoneally) and exsanguination. The thoracic cavity was opened, the lungs were removed, and impression smears were made from the cut surface of the lung tissue and stained with Wright Geimsa stain. The lung tissue was paraffin embedded, sectioned (6  $\mu\text{m}$ ), and stained with hematoxylin-eosin.

***In vitro* *H. capsulatum* attachment assay.**  $3 \times 10^7$  *H. capsulatum* were incubated in 2 ml of RPMI 1640 (Biofluids, Rockville, MD) supplemented with glutamine (0.6  $\mu\text{g}/\text{ml}$ ), penicillin (100 U/ml), streptomycin (0.5  $\mu\text{g}/\text{ml}$ ), and 300  $\mu\text{Ci}$   $^{51}\text{Cr}$ -sodium chromate (specific activity 607.74 mCi/mg; New England Nuclear, Boston, MA) for 90 min at 37°C. After incubation, the  $^{51}\text{Cr}$ -labeled *H. capsulatum* ( $^{51}\text{Cr}$ -*H. capsulatum*) suspension was centrifuged (1,200 g for 10 min), the supernatant was saved, and the pellet was resuspended in RPMI 1640. The *H. capsulatum* suspension was washed four times in RPMI 1640 to remove unincorporated  $^{51}\text{Cr}$ . The pellet was resuspended in RPMI 1640 at a concentration of  $1 \times 10^8/\text{ml}$ .

***H. capsulatum* attachment to immobilized basement-membrane proteins** was performed using laminin or Matrigel<sup>®</sup> (Becton Dickinson, Bedford, MA) coated wells (Costar Corp., Cambridge, MA). Laminin-coated wells were prepared according to the manufacturer's recommendation. Briefly, laminin, isolated from murine Engelbreth-Holm-Swarm tumor (Becton Dickinson, Bedford, MD) at a concentration of 10  $\mu\text{g}$  in 150  $\mu\text{l}$  of phosphate-buffered saline, was added to each well for 18 h at room temperature. Subsequently, the wells were washed with PBS and incubated with 2% BSA in PBS for 3 h at room temperature. The wells were washed with PBS. Matrigel<sup>®</sup>-coated wells were prepared using the "thin gel method" provided by the manufacturer. Briefly, 50  $\mu\text{l}$  of Matrigel<sup>®</sup> was added per square centimeter of well surface area and allowed to adhere at 37°C for 30 min. The wells were washed with PBS prior to use.  $1 \times 10^6$   $^{51}\text{Cr}$ -*H. capsulatum* in 500  $\mu\text{l}$  RPMI 1640 were added to each extracellular matrix (ECM) coated well, in the presence or absence of polyclonal anti-mouse laminin IgG (1:5); the plates were then centrifuged (50 g for 5 min) to settle the yeast and incubated at 37°C for 30 min. After incubation, the media containing nonattached  $^{51}\text{Cr}$ -*H. capsulatum* were removed and the wells washed three times with PBS. The attached  $^{51}\text{Cr}$ -*H. capsulatum* were disrupted with 10% Triton X-100 (Sigma Chemical Co.) and the wells washed with PBS.  $^{51}\text{Cr}$ -*H. capsulatum* was quantified in each fraction (5500 gamma counter; Beckman Instruments, Inc., Fullerton, CA) with the percentage of attachment expressed as follows: percentage of attachment =  $(A / (A + B)) \times 100$ , where A =  $^{51}\text{Cr}$ -*H. capsulatum* bound to the ECM and B =  $^{51}\text{Cr}$ -*H. capsulatum* free in the media.

**Radioiodination of laminin.** Laminin was labeled with 1  $\mu\text{Ci}$  of  $^{125}\text{I}$  (specific activity 17.4 mCi/ $\mu\text{g}$ ; Amersham, Arlington Heights, IL) for

15 min at room temperature using Iodo-Beads (Pierce, Rockford, IL) (27).  $^{125}\text{I}$ -Ln was separated from free  $^{125}\text{I}$  by gel filtration on a Sephadex G-25 M PD-10 column (Pharmacia, Uppsala, Sweden) previously washed with equilibration buffer (0.05 M Tris Cl, 0.15 M NaCl, 0.02% sodium azide, pH 7.4) and saturated with 2 ml of 1% BSA in equilibration buffer (8). The specific activity of the  $^{125}\text{I}$ -Ln was 8.7  $\mu\text{Ci}/\mu\text{g}$ .

**Production of polyclonal antibody to the *H. capsulatum* 50-kD protein.** Polyclonal antibodies to the 50-kD *H. capsulatum* protein were developed according to previously described protocols (28) with a few modifications. Briefly, *H. capsulatum* cell wall proteins were separated by SDS-PAGE as outlined later. The gel was rinsed with ddH<sub>2</sub>O and stained for 15 min with ice-cold 0.25 M KCl and 1 mM DTT. The 50-kD band was identified and separated from the gel and destined for 10 min with cold ddH<sub>2</sub>O containing 1 mM DTT. The gel band was homogenized with a minimal amount of 1 mM DTT. Four volumes of cold acetone was added to the gel elute, and the samples were allowed to precipitate at  $-70^\circ\text{C}$ . The samples were centrifuged at 1,200 g for 10 min, the acetone supernatants removed, and the protein was reconstituted in a minimal volume of 1 mM DTT. The presence of cell wall protein was confirmed by the presence of a single band on SDS-PAGE. 50–100  $\mu\text{g}$  of the antigen was mixed with an equal volume of Hunter's TiterMax adjuvant (CytRx Corp., Norcross, GA) (1:1, vol/vol) and injected intraperitoneally into BALB/c mice. The primary and secondary injections were performed on days 1 and 14. The booster injection, without adjuvant, was performed on day 28. After the booster injection, the mice were exsanguinated, the serum was collected, and murine polyclonal antibodies to the 50-kD protein (PAb 50) were assayed. PAb 50 identified the 50-kD protein band in a Western blot of *H. capsulatum* cell wall proteins (Fig. 9, lane C).

**Immunofluorescence.** To confirm that the *H. capsulatum* 50-kD cell wall protein, identified in this study as the putative laminin-binding site, is a cell surface protein, the following immunofluorescence assay was employed.  $2 \times 10^7$  yeast were suspended in 1 ml of attachment buffer (50 mM Hepes, 5 mM CaCl<sub>2</sub>, 0.1% BSA, pH 7.4) and incubated for 60 min at 37°C with murine polyclonal antibodies to the 50-kD cell wall protein at a dilution of 1:100. The yeast were then washed two times in 1 ml of attachment buffer, resuspended, and incubated with fluorescein-conjugated goat anti-mouse IgG (Calbiochem-Novabiochem Corp., La Jolla, CA) at a dilution of 1:40. The yeast were washed in attachment buffer, and cytopreparation smears were prepared by centrifugation at 1,200 g (Cytospin 2; Shandon Inc., Pittsburgh, PA). The yeast were viewed using a Zeiss Axioplan microscope equipped with epifluorescence (Carl Zeiss Instruments Inc., Thornwood, NY). Control experiments were performed substituting preimmune serum for PAb 50.

**Binding of  $^{125}\text{I}$ -Ln to *H. capsulatum*.**  $^{125}\text{I}$ -Ln binding to *H. capsulatum* was quantified using minor modifications of a previously described method (29). Reaction mixtures containing  $2 \times 10^7$  *H. capsulatum*,  $^{125}\text{I}$ -Ln (concentration from 125 ng/ml to 3,000 ng/ml), and attachment buffer (50 mM Hepes, 5 mM CaCl<sub>2</sub>, 0.1% BSA, pH 7.4) in a volume of 1 ml were incubated and rotated at 37°C for 0.5–120 min. The mixture was centrifuged at 1,200 g for 5 min, the supernatant was removed and saved, and the pellet was resuspended in 1 ml of attachment buffer. After a second centrifugation, the supernatant was saved and added to the previous supernatant. The *H. capsulatum* pellet was resuspended in 1 ml of attachment buffer and was removed from the incubation tube and placed in a 12  $\times$  75 mm tube for counting. The pellet with the bound  $^{125}\text{I}$ -Ln and the remaining supernatant in the incubation tube containing unbound  $^{125}\text{I}$ -Ln were quantified. Specific binding was determined by the addition of 40  $\mu\text{g}$  of unlabeled laminin to the reaction mixture. Bound  $^{125}\text{I}$ -Ln was calculated as follows: Bound  $^{125}\text{I}$ -Ln =  $(\text{CPM}_{\text{pellet}} / \text{CPM}_{\text{pellet} + \text{supernatant}}) \times ^{125}\text{I}\text{-Ln added}$ .

There are high-affinity laminin-binding proteins which bind to oligosaccharide-binding sites within laminin. These sites include galactose and poly-*N*-acetyl-lactosamine oligosaccharides of laminin (30) as well as the oligomannoside chains of laminin (24). To determine if the carbohydrate structures of laminin were involved in the binding of laminin to *H. capsulatum*, the binding of  $^{125}\text{I}$ -Ln to *H. capsulatum* was conducted in the presence or absence of  $\alpha$ -methyl-D-mannoside and *N*-

acetyl-lactosamine. Binding assays were performed using  $2 \times 10^7$  *H. capsulatum* in attachment buffer, 250 ng  $^{125}\text{I}$ -Ln (0.3 nM), and increasing concentrations of  $\alpha$ -methyl-mannopyranoside or *N*-acetyl-lactosamine (3–3,000 nM) (Sigma Chemical Co) in a 1-ml volume. After 30 min, the amount of bound  $^{125}\text{I}$ -Ln was calculated as earlier.

Major cell attachment sites on laminin include the peptide sequences Tyr-Ile-Gly-Ser-Arg (YIGSR) present on the B1 chain (31) and Ile-Lys-Val-Ala-Val (IKVAV) on the A chain (32). To determine if defined laminin cell-binding domains were involved in laminin binding to *H. capsulatum*, the binding of  $^{125}\text{I}$ -Ln to *H. capsulatum* was conducted in the presence or absence of the peptide sequences YIGSR and IKVAV. Binding assays were performed using  $2 \times 10^7$  *H. capsulatum* in sample buffer, 250 ng  $^{125}\text{I}$ -Ln (0.3 nM), and increasing concentrations (750–3,000 nM) of amino acid sequence 925–933 of domain III of the B1 chain (includes YIGSR) and amino acid sequence 2,091–2,108 of domain I of the A chain (includes IKVAV) (Sigma Chemical Co.). The final volume of the mixture was 1 ml, which was incubated for 30 min, and the amount of bound  $^{125}\text{I}$ -Ln was calculated as discussed previously.

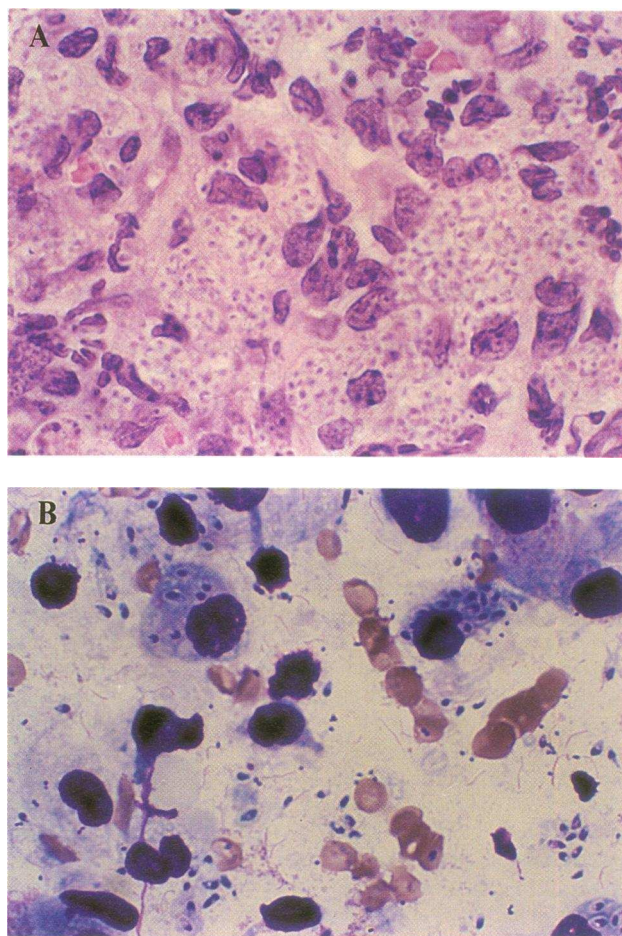
To confirm that the 50-kD *H. capsulatum* surface protein served as a receptor for laminin binding, the binding of  $^{125}\text{I}$ -Ln to *H. capsulatum* was performed in the presence or absence of PAb 50. Binding assays were performed using  $2 \times 10^7$  *H. capsulatum* in sample buffer, 250 ng  $^{125}\text{I}$ -Ln, and increasing concentrations of PAb 50 (1:500, 1:50) in a 1-ml volume. After 30 min, the amount of bound  $^{125}\text{I}$ -Ln was calculated as earlier.

**Binding of  $^{125}\text{I}$ -Ln to *H. capsulatum* cell wall proteins.** A cell wall preparation of *H. capsulatum* was prepared as previously described with minor modifications (33). The yeasts were harvested from BHI broth (Difco Laboratories, Detroit, MI), washed in PBS, and centrifuged at 1,200 g for 10 min. The yeasts were resuspended in PBS and were disrupted with glass beads in a homogenizer (B. Braun Biotech Intl. GmbH, Melsungen, Germany) at 4°C using 30-s alternating cycles of homogenizing and cooling for a total of 4–6 min. Disruption of cells was verified by light microscopy. The homogenate was centrifuged at 20,000 g for 20 min (Sorvall RC 28S; DuPont, Wilmington, DE), and the pellet was reconstituted in PBS at a concentration of 2 mg/ml as determined by bicinchoninic acid protein assay (Pierce).

To separate cell wall proteins, the cell wall preparation was solubilized in SDS-PAGE sample buffer (0.375 M Tris, 20% glycerol, 4% SDS, 0.002% bromophenol blue, and 4% 2-mercaptoethanol) and placed on a discontinuous 7.5–20% gradient sodium dodecyl sulfate polyacrylamide gel. The gel was electrophoresed at 20 mA for 4 h and stained with Coomassie blue. Alternatively, the proteins on the gels were transferred to polyvinylidene difluoride (PVDF) transfer membrane (Immobilon-P; Millipore Corp., Bedford, MA) in transfer buffer at 200 mA for 2 h (34). The PVDF membrane was incubated with blocking solution (5% dried milk in 150 mM NaCl, 50 mM Tris-Cl, pH 7.6, Tris-buffered saline [TBS]) for 1 h, washed with TBS-Tween-20 (TBS-T) three times and then blotted with 1 nM  $^{125}\text{I}$ -Ln in TBS-T for 90 min. The membrane was washed in TBS-T and air dried. Autoradiography was performed at  $-70^\circ\text{C}$  for 24 h with Kodak XAR-5 film (Eastman Kodak Co., Rochester, NY).

**Western blot with antibodies to laminin receptor proteins.** The *H. capsulatum* cell wall preparation was separated by SDS-PAGE (10% gel) and transferred to a PVDF membrane as described earlier. The PVDF membrane was incubated with blocking solution for 1 h, washed with TBS-T three times, and blotted with PAb 50 (1:1,000) and PAb 4099 (1:100) in TBS-T for 1 h. The membranes were washed and then incubated with anti-murine (PAb 50) and anti-rabbit (PAb 4099) alkaline phosphatase conjugate (BioRad, Hercules, CA) for 1 h in TBS-T. The membranes were washed and developed with a buffer (AP Conjugate Buffer Kit; BioRad), according to the manufacturer's specifications.

PAb 50 was described earlier; PAb 4099 recognizes the 67-kD laminin receptor of murine and human endothelial cells and has been described previously (35–37). PAb 4099 is affinity purified and diluted into PBS containing carrier ovalbumin. Its optimal dilution was empirically established to be specific and sufficiently sensitive at 1:100.



**Figure 1.** In vivo pulmonary *H. capsulatum* infection in CD4 lymphocyte-depleted mice. (A) The lung tissue section demonstrates an exuberant infection. The interstitium is swollen with both intracellular and extracellular *H. capsulatum* (hematoxylin and eosin stain,  $\times 1,000$ ). (B) Impression smears made from the cut surface of the infected lung tissue. Multiple free extracellular *H. capsulatum* are present as well as alveolar macrophages with intracellular organisms (Wright Giemsa stain,  $\times 1,000$ ).

**Statistical analysis.** All results are expressed as mean  $\pm$  SEM. Statistical comparisons were performed using Student's *t* test for unpaired data or the one-way analysis of variance with paired comparisons performed using Fisher's least significant difference method. Statistical significance was accepted for  $P < 0.05$  (38).

## Results

An exuberant pulmonary infection developed in the CD4 lymphocyte-depleted mice (Fig. 1). Free *H. capsulatum* organisms were easily demonstrated in the lung tissue and in lung impression smears of the infected mice. In profoundly immunosuppressed animals, *H. capsulatum* pulmonary infection develops rapidly, with evidence for both intracellular and extracellular organisms present in the lung tissue.

$^{51}\text{Cr}$ -*H. capsulatum* attached to immobilized laminin and the basement membrane preparation Matrigel<sup>®</sup> (Fig. 2). The percentage of attachment of  $^{51}\text{Cr}$ -*H. capsulatum* was in direct proportion to the amount of immobilized laminin adherent to the well (data not shown). The percentage of attachment of

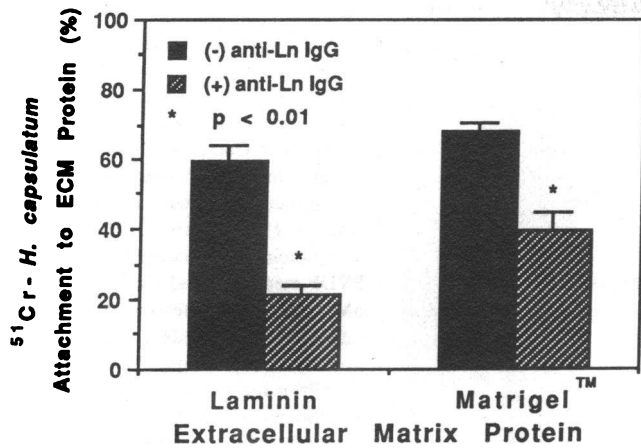


Figure 2. <sup>51</sup>Cr-*H. capsulatum* attachment to extracellular matrix proteins.  $1 \times 10^6$  <sup>51</sup>Cr-*H. capsulatum* were incubated with wells coated with laminin or the reconstituted basement-membrane preparation Matrigel™ for 30 min. The assay was performed in the presence or absence of anti-laminin IgG (1:5). The presence of anti-laminin IgG significantly reduced the percentage of attachment of <sup>51</sup>Cr-*H. capsulatum* to laminin and Matrigel™ ( $59 \pm 4$  to  $21 \pm 3$  and  $67 \pm 2$  to  $39 \pm 5$ , respectively). Values are mean  $\pm$  SEM;  $n = 9$ .

<sup>51</sup>Cr-*H. capsulatum* to laminin and Matrigel™ was significantly diminished in the presence of polyclonal anti-laminin IgG ( $59 \pm 4$  to  $21 \pm 3$  and  $67 \pm 2$  to  $39 \pm 5$ , respectively;  $P < 0.01$ , both comparisons). Matrigel™ is a solubilized basement-membrane preparation extracted from the Engelbreth-Holm-Swarm mouse sarcoma tumor. Its major component is laminin, followed by collagen IV, heparan sulfate proteoglycans, enactin, and nidogen.

Laminin binds *H. capsulatum* in vitro in a time-dependent manner and achieves apparent equilibrium by 10 min (Fig. 3). At 0.5 min,  $21 \pm 2$  ng <sup>125</sup>I-Ln per  $2 \times 10^7$  yeast is bound, whereas beyond 10 min, binding of <sup>125</sup>I-Ln reaches a plateau at approximately  $73 \pm 4$  ng per  $2 \times 10^7$  yeast.

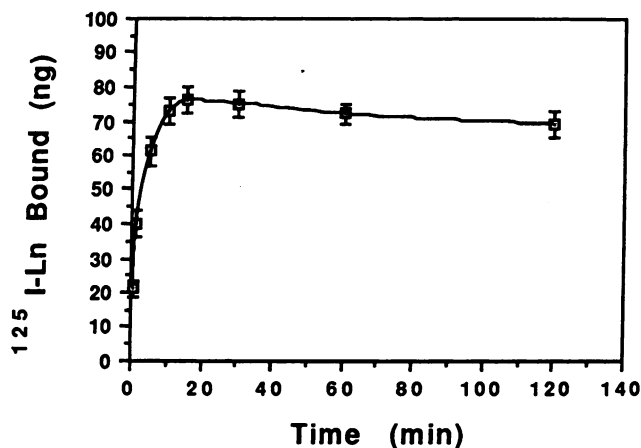


Figure 3. <sup>125</sup>I-Ln binds *H. capsulatum* in a time-dependent fashion. Binding assays were performed using  $2 \times 10^7$  *H. capsulatum* incubated with 250 ng <sup>125</sup>I-Ln in 50 mM Hepes, 5 mM CaCl<sub>2</sub>, 0.1% BSA, pH 7.4, for the indicated times. Binding equilibrium is evident at 10 min, with  $73 \pm 4$  ng <sup>125</sup>I-Ln bound per  $2 \times 10^7$  yeast. Values are mean  $\pm$  SEM;  $n = 9$ .

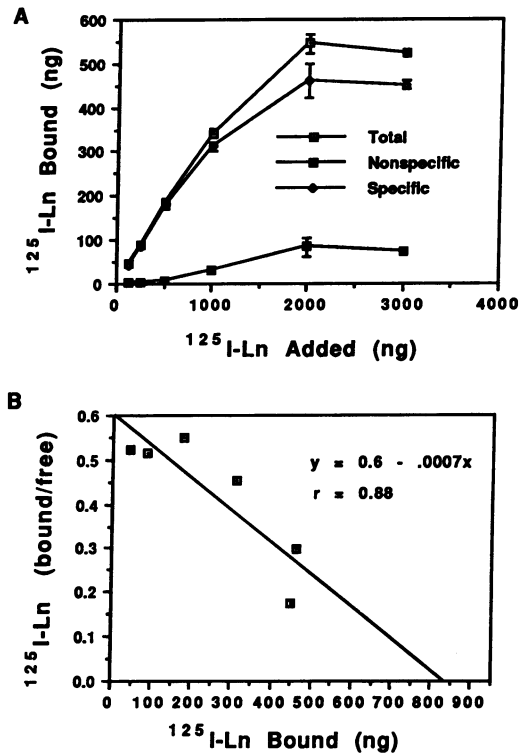
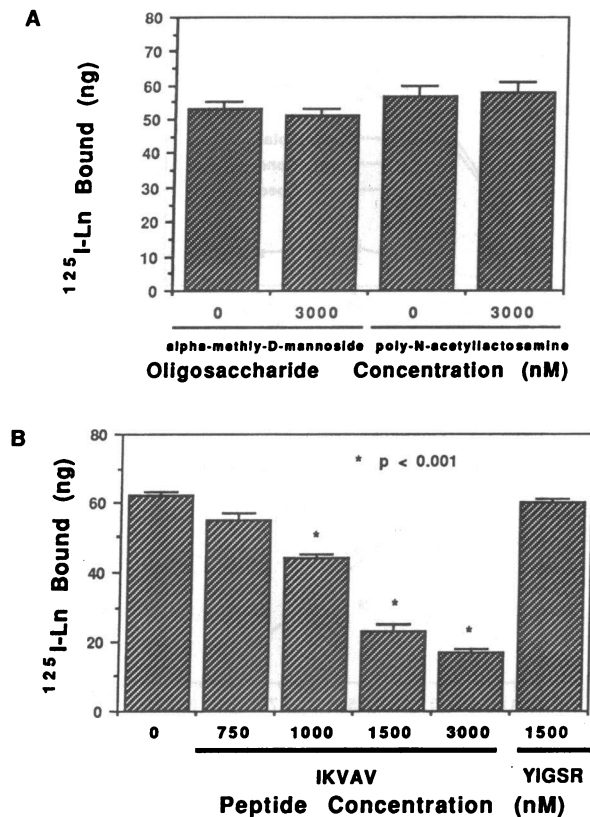


Figure 4. Saturation and specific binding of <sup>125</sup>I-Ln to *H. capsulatum*. The binding assay was performed using  $2 \times 10^7$  *H. capsulatum*, 125–3,000 ng of <sup>125</sup>I-Ln with and without 40- $\mu$ g excess unlabeled laminin. (A) Saturation of specific laminin binding to *H. capsulatum* is demonstrated in the presence of  $> 2,000$  ng <sup>125</sup>I-Ln added. The data expressed are the mean  $\pm$  SEM. (B) Scatchard plot of specific <sup>125</sup>I-Ln binding to *H. capsulatum*. The slope of the line represents a  $K_d$  of  $1.6 \times 10^{-9}$  M for the binding interaction of laminin with *H. capsulatum*. The number of laminin binding sites per organism was calculated from the x-intercept to be  $3.0 \times 10^4$ .

The specificity and saturability of <sup>125</sup>I-Ln binding to *H. capsulatum* were demonstrated using <sup>125</sup>I-Ln (125–3,000 ng) in the presence or absence of 40  $\mu$ g excess unlabeled laminin (Fig. 4 A). The addition of increasing amounts of <sup>125</sup>I-Ln to the binding assay resulted in an increase in both total and nonspecific binding, with saturation of specific binding evident at concentrations in excess of 2,000 ng <sup>125</sup>I-Ln added. The Scatchard plot using the specific binding data is linear ( $r = 0.88$ ), indicating a probable homogenous population of binding sites for laminin on *H. capsulatum* organisms (Fig. 4 B). The binding dissociation constant ( $K_d$ ), obtained from the slope of the linear plot, is  $1.6 \times 10^{-9}$  M. Each *H. capsulatum* yeast is estimated to have  $3.0 \times 10^4$  binding sites for laminin, as calculated from the abscissa intercept.

To determine if the carbohydrate structures of laminin were involved in the binding of laminin to *H. capsulatum*, <sup>125</sup>I-Ln binding to *H. capsulatum* was conducted in the presence or absence of  $\alpha$ -methyl-D-mannoside or *N*-acetyl-lactosamine. The amount of <sup>125</sup>I-Ln bound (ng) to *H. capsulatum* in the presence of 0 nM and 3,000 nM of  $\alpha$ -methyl-D-mannoside was  $53 \pm 2$  ng and  $51 \pm 2$  ng, respectively ( $P > 0.05$ ); similarly, the binding in the presence of 0 nM and 3,000 nM of *N*-acetyl-lactosamine was  $57 \pm 3$  ng and  $58 \pm 3$  ng, respectively ( $P > 0.05$ ). Therefore,

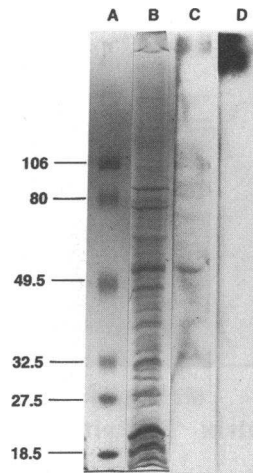


**Figure 5.** The effect of defined laminin domains on  $^{125}\text{I}$ -Ln binding to *H. capsulatum*. Binding assays were performed in the presence or absence of laminin-binding oligosaccharides or peptides. (A)  $^{125}\text{I}$ -Ln binding in the presence of  $\alpha$ -methyl-D-mannoside or N-acetyl-lactosamine (0–3,000 nM). There is no inhibition in the presence of either oligosaccharide. (B)  $^{125}\text{I}$ -Ln binding in the presence of peptides IKVAV or YIGSR (0–3,000 nM). In the presence of 3,000 nM IKVAV,  $17 \pm 1$  ng of  $^{125}\text{I}$ -Ln binds to *H. capsulatum* compared with  $62 \pm 1$  ng in the absence of IKVAV. There is no inhibition in the presence of YIGSR. Values are mean  $\pm$  SEM;  $n = 9$ .

there was no evidence that laminin oligosaccharide side chains were involved in laminin binding to *H. capsulatum* (Fig. 5 A).

The major epithelial, neuronal, and malignant cell attachment sites of laminin have been localized to domain III of the B1 chain and domain I of the A chain which contain the peptide sequences YIGSR and IKVAV, respectively (31, 32). Therefore, to determine if these laminin cell-binding domains were involved in laminin binding to *H. capsulatum*, the binding of  $^{125}\text{I}$ -Ln to *H. capsulatum* was conducted in the presence or absence of YIGSR or IKVAV containing peptide sequences. The peptide sequence containing YIGSR had no significant effect on  $^{125}\text{I}$ -Ln binding to *H. capsulatum* (Fig. 5 B); whereas, the A chain peptide containing IKVAV demonstrated a concentration-dependent inhibition of  $^{125}\text{I}$ -Ln binding to *H. capsulatum* (Fig. 5 B). As an example, laminin binding to *H. capsulatum* in the absence of IKVAV was  $62 \pm 1$  ng, compared with  $17 \pm 1$  ng in the presence of 3,000 nM of IKVAV ( $P < 0.001$ ). This suggests that laminin binds to *H. capsulatum* via the major cell attachment site located within domain I of the laminin A chain.

The *H. capsulatum* cell wall preparation, separated by SDS-PAGE and stained with Coomassie blue, revealed numerous protein bands whose sizes ranged from 18 kD to greater than



**Figure 6.** *H. capsulatum* laminin-binding protein. (A and B) Low molecular weight markers and *H. capsulatum* cell wall proteins were separated by SDS-PAGE on a 7.5–20% gradient gel and stained with Coomassie blue. Staining revealed multiple *H. capsulatum* cell wall protein bands with a molecular range of 18 to > 116 kD. *H. capsulatum* proteins were transferred to a PVDF membrane and incubated with 1 nM  $^{125}\text{I}$ -Ln. (C) Autoradiography demonstrates  $^{125}\text{I}$ -Ln binding to a *H. capsulatum* protein of approximately 50-kD. (D) Prior incubation of the PVDF membrane with unlabeled laminin completely blocked  $^{125}\text{I}$ -Ln binding to the 50-kD protein.

116 kD (Fig. 6). The *H. capsulatum* laminin-binding protein was demonstrated following the transfer of *H. capsulatum* proteins to a PVDF membrane and the subsequent incubation of these proteins with  $^{125}\text{I}$ -Ln. Autoradiography of the  $^{125}\text{I}$ -Ln blot reveals that laminin binds to an *H. capsulatum* protein with an estimated mass of 50 kD. There is no visible band when the *H. capsulatum* cell wall proteins are blotted with  $^{125}\text{I}$ -Ln in the presence of excess unlabeled laminin, indicating that the band is the result of laminin specifically binding to the *H. capsulatum* cell wall protein.

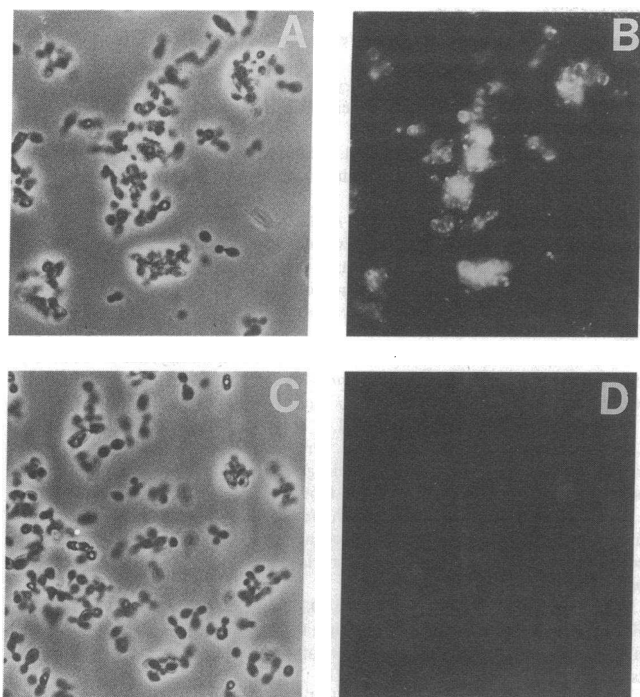
Evidence that the 50-kD *H. capsulatum* protein is a cell surface protein is provided using immunofluorescence of *H. capsulatum* with Pab 50.  $2 \times 10^7$  *H. capsulatum* were incubated with Pab 50 or preimmune serum (1:40), washed, and subsequently incubated with fluorescein-conjugated goat anti-mouse IgG (1:100). The immunofluorescent pattern (Fig. 7) by the yeast was prominent and was relatively similar for all yeast with respect to distribution and intensity. The yeast incubated with preimmune serum did not demonstrate immunofluorescence. These data indicate that the 50-kD protein is a cell surface protein.

To define further the role of the 50-kD protein as the *H. capsulatum* laminin-binding protein, the  $^{125}\text{I}$ -Ln binding studies were performed in the presence or absence of Pab 50.  $2 \times 10^7$  yeast were incubated with 250 ng of  $^{125}\text{I}$ -Ln in the presence or absence of Pab 50. Pab 50 demonstrated a significant inhibition of  $^{125}\text{I}$ -Ln binding to *H. capsulatum* (Fig. 8). The amount of  $^{125}\text{I}$ -Ln bound to *H. capsulatum* was  $93 \pm 9$  ng and decreased to  $40 \pm 5$  ng ( $P < 0.05$ ) in the presence of Pab 50 (1:50).

Western blot analysis of *H. capsulatum* cell wall proteins used Pab 4099, which recognizes the 67-kD laminin receptor. The *H. capsulatum* cell wall proteins were separated by SDS-PAGE, transferred to a PVDF membrane, and incubated with Pab 4099. This antibody recognized the 50-kD *H. capsulatum* cell wall protein (Fig. 9), indicating that the protein cross-reacts with an antibody that recognizes a known laminin receptor, suggesting that they are related.

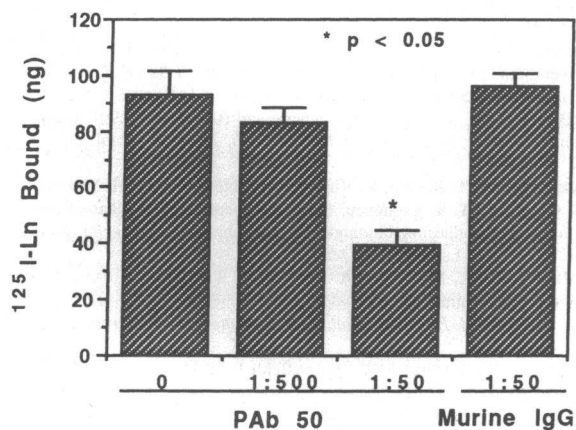
## Discussion

Histoplasmosis is characterized by early hematogenous dissemination of the yeast form from the initial site of infection. The mechanism of this dissemination is not completely understood

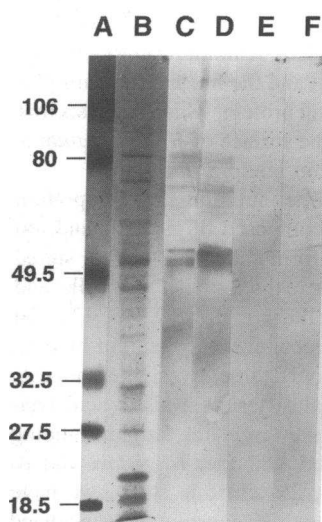


**Figure 7.** Immunofluorescence pattern demonstrating the *H. capsulatum* 50-kD cell surface protein.  $2 \times 10^7$  *H. capsulatum* were incubated with PAb 50 or with preimmune serum (1:100), followed by incubation with FITC-labeled goat anti-murine IgG (1:100). Phase and epifluorescence microscopy (A and B) of *H. capsulatum* incubated with PAb 50 demonstrate immunofluorescence, suggesting that the 50-kD protein is a surface protein. There was no immunofluorescence noted in the control experiment using preimmune serum (C and D).

and has been speculated to occur within blood-borne macrophages. The documentation of a patient with disseminated disease and interstitial and alveolar extracellular *H. capsulatum* yeast suggests that not all *H. capsulatum* are intracellular and, therefore, that alternative methods of dissemination are possible (4). In the current study, *H. capsulatum* yeast in CD4 lympho-



**Figure 8.** The effect of polyclonal antibody to *H. capsulatum* 50-kD surface protein on <sup>125</sup>I-Ln binding to *H. capsulatum*.  $2 \times 10^7$  *H. capsulatum* were incubated with 250 ng of <sup>125</sup>I-Ln in the presence of PAb 50 or murine IgG. PAb 50 demonstrated significant inhibition of <sup>125</sup>I-Ln binding to *H. capsulatum*. Values are mean  $\pm$  SEM;  $n = 9$ .



**Figure 9.** Western blot analysis of *H. capsulatum* cell wall proteins with PAb 4099. The cell wall proteins were separated by means of SDS-PAGE (10% gel), transferred to a PVDF membrane, and incubated with PAb 4099 (1:100), an antibody directed toward the 67-kD laminin receptor. Lane A demonstrates the molecular weight markers. Lane B is a Coomassie blue stain of the *H. capsulatum* cell wall proteins. Lane C demonstrates the 50-kD cell wall protein recognized by PAb 50. Lane D demonstrates the 50-kD cell wall protein recognized by PAb 4099. This suggests that the 50-kD cell protein of *H. capsulatum* shares epitopes with the known 67-kD laminin receptor. Lanes E and F are control lanes. In lane E, rabbit IgG is the primary antibody and in lane F there is no primary antibody, only the conjugate antiserum.

cyte-depleted mice exhibited exuberant growth, with both intracellular and extracellular organisms easily detected. Because dissemination during immunosuppression invariably occurs (39), these findings suggest that *H. capsulatum*, as an extracellular organism, may play a role in the pathogenesis of the disease in the immune-compromised host. Thus, the ability of *H. capsulatum* to interact with key constituents of the alveolar capillary barrier was examined.

*H. capsulatum* is capable of attaching to the basement-membrane preparation Matrigel<sup>®</sup>, and this attachment is significantly reduced by blocking laminin, the major glycoprotein of the basement membrane. This study demonstrates that laminin binds *H. capsulatum* in a rapid, specific, and saturable fashion. In addition, this study suggests that an *H. capsulatum* 50-kD surface protein is the laminin-binding protein and that antibodies to this 50-kD surface protein inhibit laminin binding to the yeast. Western blot analysis demonstrates the recognition of this surface receptor by 67-kD laminin receptor antibodies. The Scatchard plot estimates the number of laminin binding sites per *H. capsulatum* organism to be  $3.0 \times 10^4$ . Furthermore, the linearity of the plot suggests that a homogenous group of binding sites exists with a similar affinity for laminin.

Several classes of laminin-binding proteins exist, the function of which remains to be fully defined (40). However, the expression of laminin-binding proteins by pathogenic microorganisms has been demonstrated to enhance the organism's ability to attach to host cells and invade host tissues. These pathogenic microorganisms possess laminin-binding proteins which vary in molecular size from 32 (*T. gonodii*) to 145 kD (*Streptococcus gordonii*), with the majority of pathogens possessing laminin-binding proteins from 44 to 67 kD (8, 11, 14, 18, 21, 23). Invasive *S. aureus* has been demonstrated to possess a 52-kD surface protein which functions as a laminin-binding protein. Monoclonal antibodies to this 52-kD protein were demonstrated to bind to the 67-kD laminin receptor of murine melanoma cells, suggesting that conservation or convergence among laminin receptors exists (41). In this study, we demonstrate that the *H. capsulatum* 50-kD cell surface protein is involved in laminin binding and that this protein is recognized by antibody to the

67-kD laminin receptor, lending further support to the concept of conservation or convergence among laminin receptors. Western blot analysis, autoradiography, and the linear Scatchard plot all indicate a single laminin-binding protein, which suggests that a predominant receptor class on the surface of *H. capsulatum* is involved in the majority of laminin binding.

Laminin exists in vivo as a large multidomain glycoprotein of the extracellular matrix. It is composed of globular and rod like domains arranged in a cruciform shape and is well suited for mediating attachment between distant sites on cells and other components of the extracellular matrix (7, 40, 42). To date, the domain within laminin recognized by laminin-binding proteins has been described for only three invasive pathogens. The laminin-oligomannoside chains, terminal sialic acid residues, and the P1 fragment are the target for laminin-binding proteins of type I enterobacterial fimbriae, *H. pylori* and *A. fumigatus*, respectively (10, 24, 25). In this study, we have demonstrated that laminin binding to *H. capsulatum* is localized to domain I of the A chain and that this binding is dependent upon the IKVAV peptide sequence. The peptide sequence IKVAV has been demonstrated to mimic some of the biological activity of the intact laminin molecule (32). The IKVAV sequence is located in the carboxyl region of the  $\alpha$ -helical domain of the laminin A chain, which is one of the regions involved in promoting cell adhesion, spreading, and migration. The A chain serves as a ligand for the 67-kD laminin receptor group, among others (40).

In summary, we present evidence that the glycoprotein laminin binds to *H. capsulatum* yeast in a rapid, specific, and saturable fashion. The binding is inhibited by IKVAV, a peptide sequence from the major cell-binding region of laminin, and by antibodies to the cell surface 50-kD protein. The *H. capsulatum* laminin-binding protein appears to be the 50-kD cell surface protein, which may be a member of the family of 67-kD laminin receptors. This study provides strong evidence for the ability of *H. capsulatum* to interact with the important basement-membrane component laminin. This type of critical interaction has been demonstrated to be vital for establishing infection by other pathogenic organisms and for disseminating tumor cells. This study provides credible support for *H. capsulatum*-laminin interaction as a possible mechanism of dissemination. This model may have particular relevance for pulmonary histoplasmosis that occurs during severe immune suppression.

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