Infection and Immunity

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Binding of *Paracoccidioides brasiliensis* to Laminin through Surface Glycoprotein gp43 Leads to Enhancement of Fungal Pathogenesis

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Extracellular matrix protein laminin binds specifically to yeast forms of *Paracoccidioides brasiliensis* and enhances adhesion of the fungus to the surface of epithelial Madin-Darby canine kidney cells in vitro. Immunoblotting of fungal extracts showed that the gp43 glycoprotein is responsible for adhesion. This was confirmed by binding assays using purified gp43, with a K_d of 3.7 nM. The coating of *P. brasiliensis* yeast forms with laminin before injection into hamster testicles enhanced the fungus virulence, resulting in a faster and more severe granulomatous disease. These results indicate that interaction of fungi with extracellular matrix elements may constitute a basis for the evolution of fungal infection toward regional spreading and dissemination.

Paracoccidioidomycosis is a systemic mycosis caused by the dimorphic fungus *Paracoccidioides brasiliensis* with a spectrum of clinical forms. They can be severe, with an important mortality rate in the absence of specific therapy. It is assumed from experimental data that inhaled fungal propagules reach the lung, transform into yeast cells, and ultimately disseminate to distant organs (20). Also, overt disease usually occurs in the immunologically compromised host (8). Few other factors that could influence pathogenesis (24), besides the host immunological response, have so far been addressed.

Interaction with extracellular matrix proteins has been correlated with the invasive ability of different cells (15, 31). It has been shown that recognition of laminin may influence the pathogenesis of several microorganisms (17, 25, 26, 29). Laminin is a glycoprotein of 850 kDa, present in basement membranes (12, 31), with the capacity to promote cell adhesion, differentiation, shape, and motility (9, 10, 12, 19). Specific receptors for laminin present on the surface of normal (5, 11) as well as tumor (1, 16) cells or pathogenic microorganisms (17, 25, 29) have been held responsible for these functions.

To check the possible effect of laminin on the in vivo and in vitro behavior of *P. brasiliensis*, mouse laminin was used to promote adhesion to a Madin-Darby canine kidney cells (MDCK) monolayer and to enhance the pathogenesis of the fungus in the hamster model. We herein report that the specific binding of the gp43 surface protein of *P. brasiliensis* to laminin is correlated with the fungus's adhesiveness in vitro as well as to the enhancement of its pathogenesis, using the hamster testicle model.

P. brasiliensis B-339 (kindly provided by A. Restrepo, Corporación para Investigaciones Biologicas, Medellin, Colombia) and 18 (a kind gift from V. Calich, São Paulo University) were maintained by frequent subculturing on Sabouraud glucose

agar (Difco Laboratories). Yeast forms of the fungus were obtained in the same medium at 35°C and subcultured every third day. All experiments described below were performed with both fungal strains. MDCK cells were cultivated in RPMI 1640 medium supplemented with 10% bovine serum at 37°C in an atmosphere of 95% air–5% CO_2 until confluence. Cultures were prepared either on 13-mm-diameter glass coverslips, for electronic microscopy (EM), or in wells of 96-well polystyrene plates (Costar), for binding assays.

P. brasiliensis cell-free antigen was prepared as described previously (2). Protein concentration of the cell-free antigen was measured by the Bradford method (3). The gp43 secreted by the fungus yeast cells was purified as previously described (22). Laminin was purified from Engelbrecht-Holm-Swarm mouse tumor cells (13). Polyclonal antilaminin antibodies were obtained by intramuscular injection of rabbits with purified laminin incorporated in complete Freund's adjuvant, for the first injection, and in incomplete Freund's adjuvant, for the subsequent injections, at monthly intervals. Rabbit sera were tested and titrated by conventional enzyme immunoassay. Purified laminin was labeled with ^{125}I (Amersham) by the IodoGen method (7) to a specific activity of 14.7 μCi (1 Ci = 37 GBq) μg^{-1}.

To measure adhesion of fungi to MDCK cell surfaces, yeast forms of *P. brasiliensis* were washed twice by centrifugation with phosphate-buffered saline (PBS), adjusted to 10^6 forms per ml, and preincubated with different concentrations of laminin in PBS for 30 min at room temperature. For the binding assays, whole yeast forms were previously labeled with ^{125}I by the IodoGen method. The fungi were then added to the MDCK cultures and incubated overnight. Cultures were washed three times with PBS to remove nonadherent fungi. Plastic wells were cut and counted in a Packard Gamma Counter, and the glass coverslips were prepared for EM by fixation with 2.5% (vol/vol) glutaraldehyde in PBS. For scanning EM, cells adhering to glass coverslips were fixed in 2.5% glutaraldehyde in PBS, postfixed with 1% OsO_2, dehydrated in ethanol, dried at the critical point with CO_2, coated with gold, and observed in a JEOL 25SII scanning electron microscope.

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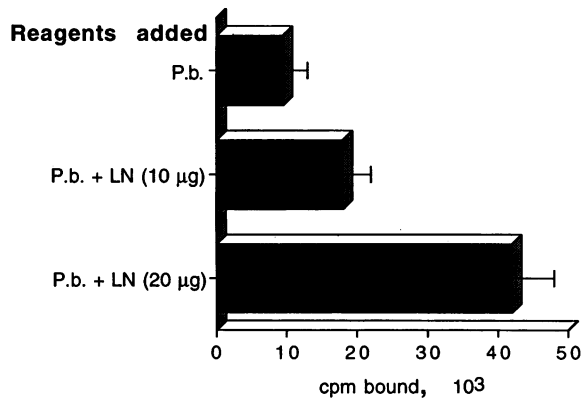
FIG. 1. Binding of ^{125}I-labeled yeast forms of *P. brasiliensis* (P.b.) to MDCK cell monolayers. Yeast forms of *P. brasiliensis* were iodine labeled by the IodoGen method and added to MDCK cell monolayers grown in wells of polyvinyl plates. After incubation and washings, wells were cut and counted in a Packard Gamma Counter. Binding was increased by the addition of mouse laminin (LN) at 10 and 20 μg/ml in PBS.

FIG. 2. Binding of ^{125}I-labeled mouse laminin to purified gp43 of *P. brasiliensis*. Binding was saturable (□), total binding; ○, nonspecific binding; ■, specific binding), with a K_d of 3.7 nM and n = 1.09, as measured by Hill analysis (insert). Results refer to the b phase of the curve.

A dot blot apparatus (Bio-Rad) was used to evaluate the binding of ^{125}I-labeled laminin to purified gp43. Fifty microliters of a 1-μg/ml solution of the protein was applied onto the wells with a nitrocellulose membrane at the bottom. The assay was performed according to the manufacturer's instructions, by adding different concentrations of ^{125}I-labeled laminin, in the presence or absence of 100-fold unlabeled laminin. After washings with PBS containing 0.05% (vol/vol) Tween 20 (Sigma Chemical Co.), the membrane was cut and the piece corresponding to each well was counted in a Packard Gamma Counter.

Cell-free antigen was used for electrophoretic analysis. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting were performed as described elsewhere (17, 32).

To investigate the influence of laminin in the hamster infections, 3-month-old male hamsters, obtained from the Animal Farm, UNESP Botucatu, Brazil, were divided in two groups of eight animals each and injected in the left testicle with 10^6 viable forms of *P. brasiliensis* in the presence (30-min preincubation in PBS) or absence of 20 μg of laminin. The animals were sacrificed after 2 and 4 weeks of infection, and testicles were removed, fixed in 10% formalin in PBS, embedded in paraffin, cut in 4-mm sections, and stained with hematoxylin-eosin. Extension of infection was evaluated by measuring the testicular area occupied by *P. brasiliensis* granulomas in two 200× microscopic fields and then measuring the area occupied by four granulomas per field by using the Mini-mopp system (Kontron Bildanalyse; Image Analysis Systems, Eching, Germany) coupled to a standard Zeiss microscope (Germany). Statistical evaluation of the area occupied by *P. brasiliensis* granulomas in the hamster testicles for both groups of animals was done by the Kruskal-Wallis test.

Previous treatment of fungal yeast cells with laminin greatly increase their adhesion to MDCK cells. This adhesion was shown to be dose dependent in binding assays (Fig. 1). These results were confirmed by scanning EM, which showed that very few fungal forms adhered to the MDCK cells in the absence of laminin, whereas large clusters of yeast tightly bound to the MDCK cells were observed when laminin was added to the culture (not shown).
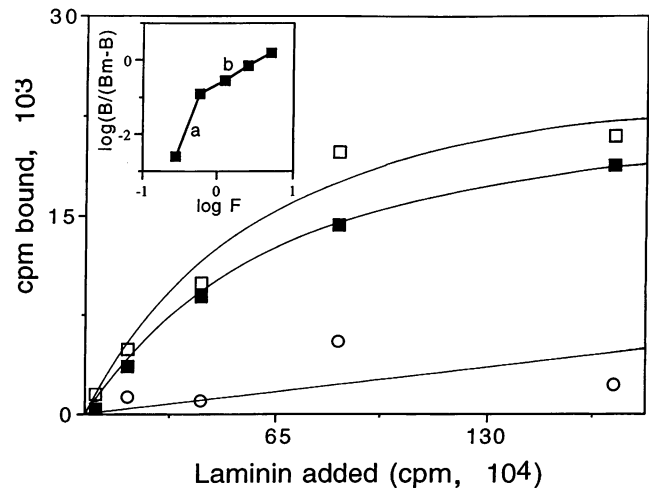
Binding of laminin to purified gp43 was specific and saturable (Fig. 2). When the data obtained were plotted according to Scatchard (not shown), a bell-shaped curve was observed, suggesting positive cooperativity (14). Hill analysis, which foresees that possibility (18) and in which results are plotted as log [B/(Bm − B)] versus log F, where Bm is the maximal binding capacity and F is the amount of free ligand at equilibrium, was then used. An estimated K_d of 3.7 nM, with n = 1.09, was obtained. The a phase of the curve is nonanalyzable and could represent an artifact. The b phase of the curve shows a correlation of r = 0.98 (Fig. 2, b, insert). Binding of laminin to whole yeast forms of *P. brasiliensis* showed specific binding in the same nanomolar range (not shown), but the number of binding sites found per cell (15×10^4) may not be representative since large amounts of gp43 are secreted into
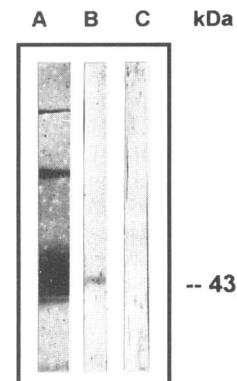

FIG. 3. SDS-PAGE and immunoblotting of cell-free antigen from *P. brasiliensis*. Whole cell-free antigen (lane A; SDS-PAGE and silver staining) was transferred electrophoretically to nitrocellulose membranes and sequentially treated with purified laminin, rabbit anti-laminin antibody, and peroxidase-conjugated anti-rabbit immunoglobulin G (lane B). Reactions were developed with 4-chloro-1-naphthol. In the control reaction (lane C), laminin was omitted and no protein band was revealed.
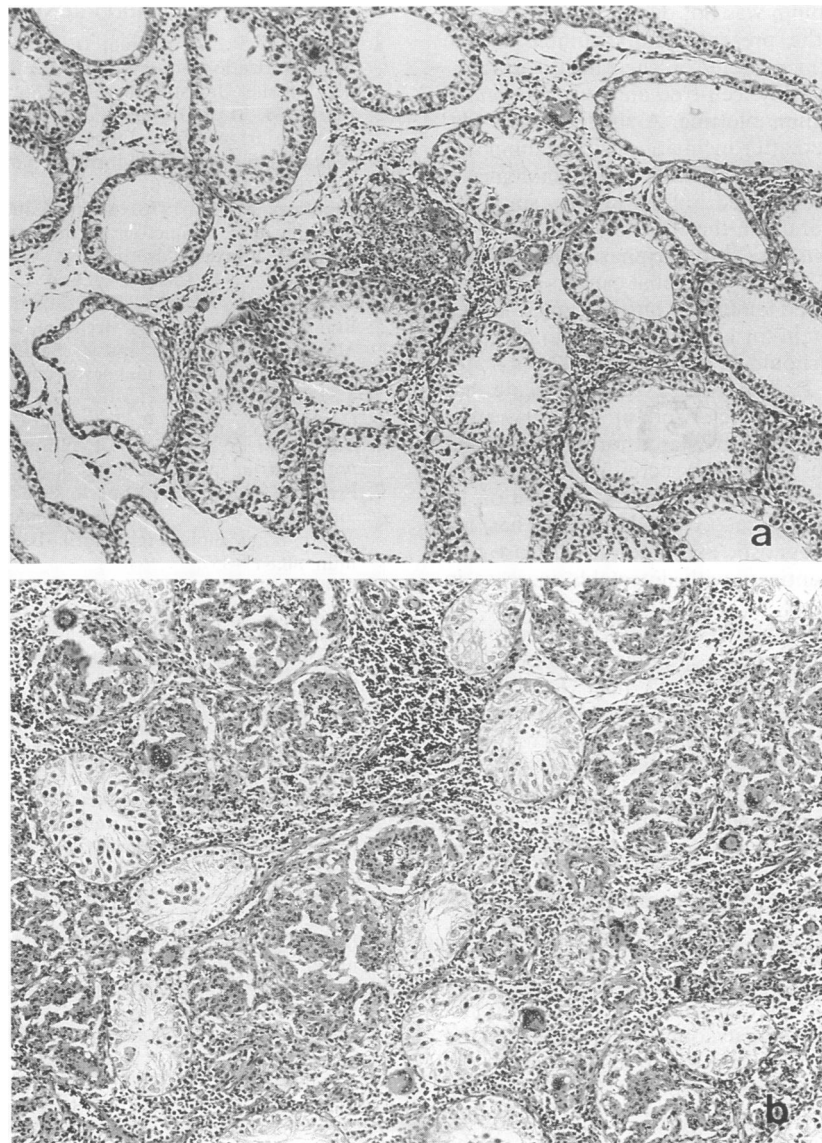
FIG. 4. Histology of hamster testicles after injection of *P. brasiliensis*. Hamster testicles were injected with 10^6 viable yeast forms of *P. brasiliensis* in the presence or absence of 20 µg of mouse laminin. Animals were sacrificed after 4 weeks of infection, and testicles were removed, fixed, cut, and stained with hematoxylin-eosin. The number and extension of granulomas were greatly increased by the addition of laminin (b) compared with the infection seen in the absence of laminin (a). Magnification, ×40.

the culture medium. Indirect immunoblot analysis of *P. brasiliensis* total cell-free antigen showed that laminin strongly and specifically bound to gp43, as revealed with antilaminin rabbit serum (Fig. 3).

Histological analysis of infected hamster testicles showed that tissue destruction and replacement by *P. brasiliensis* granulomas, after 2 weeks of injection, was much more intense in the group infected with yeast cells coated with laminin (Fig. 4b) than in the group injected with the fungus only (Fig. 4a). Similar results were observed after 4 weeks of injection (not shown). Evaluation of the testicular area occupied by granulomas in both animal groups (four hamsters per group) for infections of 2 and 4 weeks demonstrated that infection with fungi pretreated with laminin was significantly more extensive than that with untreated fungus ($P < 0.05$; not shown).

The mechanisms involving the pathogenesis of *P. brasiliensis* in humans are not well understood. Since the infection may disseminate to different organs and tissues, the ability of the fungus to adhere to and escape the compartment lined by the basal membrane is admitted. Experiments were done to determine whether interaction of the fungus with laminin could influence the pathogenesis of *P. brasiliensis*.

Affinity of the fungus for laminin could be demonstrated by the enhancement of yeast adhesion to a monolayer of MDCK cells, as observed by quantitative binding assays and scanning EM. These results are similar to those previously described for *Trichomonas vaginalis* and *Trichomonas foetus* (25). Their adhesion to and subsequent disruption of the epithelial monolayer were greatly enhanced by the addition of laminin to the system. Since previous findings suggested that the increased

adhesion produced by laminin was not dependent on electrostatic interactions (25), the present results pointed to the existence of laminin-binding proteins on the fungal surface.

To investigate this possibility, cell-free antigen of *P. brasiliensis* was analyzed by immunoblotting. A single 43-kDa glycoprotein could be detected after incubation with laminin and antilaminin serum. This receptor protein, although biologically similar to others already described, differs in size, since the laminin receptors are generally in the 50- to 70-kDa range, as shown in *Staphylococcus aureus*, fibrosarcomas, macrophages, polymorphonuclear cells (17), and epithelial cancers (31). Our data on the binding of labeled laminin to purified gp43 showed that the affinity of the protein for laminin appears to be in the same nanomolar range. It should be noted that gp43 is a major antigenic component of *P. brasiliensis*. This molecule has already been purified and characterized in our laboratory (21–23, 27) in its native and deglycosylated forms. It is found on the surface of the fungus and is also secreted in measurable amounts into the culture medium (2, 27). Its recognition by sera of most patients with paracoccidioidomycosis has led some groups to develop diagnostic assays for the disease that use the gp43 as the sole antigen, with detectable increase in specificity and sensitivity (2, 6, 23, 30). The spontaneous secretion of gp43 also occurs in vivo and could act as a fungal evasion mechanism against the host's immune response. Thus, the secreted antigen could bind to the host's antibodies, leaving the cell-bound gp43 free to bind to extracellular matrix laminin, therefore increasing the ability of the fungus to invade and disseminate to distant organs and tissues.

Laminin is known to change the metastatic behavior of cancer cells, increasing their invasiveness after binding to their specific surface receptors (15, 28). The same seems to happen with some parasites like *T. vaginalis* and *T. foetus*, whose adherence and virulence to the subjacent epithelial cells is greatly increased in the presence of laminin (25). To determine whether laminin could influence *P. brasiliensis* behavior in a similar way, we injected the fungus into hamster testicles in the presence and in the absence of laminin. In this in vivo model for the study of fungal pathogenicity, our results confirmed the assumption that addition of laminin to the system causes the infection to be much more intense and severe, with much larger areas of the testicles being occupied by fungal granulomas. To explain the failure of macrophages in arresting the infection (4, 8), one can assume that because these cells express laminin receptors (11), as well as complement receptors, which are themselves activated by *P. brasiliensis*, ingestion of laminin-coated fungi could be enhanced in such a way as to override their ability of fungal intracellular destruction. Since gp43 was the only detectable *P. brasiliensis* protein specifically binding laminin, it probably can act as a receptor for the extracellular matrix protein, thus inducing intracellular changes after the binding. The nature of these changes are not yet defined, but they seem sufficient to greatly increase fungal pathogenicity in the hamster model and could be one of the factors responsible for the ability of the fungus to disseminate in the human host.

REFERENCES

1. **Barsky, S. R., C. N. Rao, D. Hyams, and L. A. Liotta.** 1984. Characterization of a laminin receptor from human breast carcinoma tissue. Breast Cancer Res. Treat. **4:**181–188.
2. **Blotta, M. M. S. L., and Z. P. Camargo.** 1993. Immunological response to cell-free antigens of *Paracoccidioides brasiliensis*: relationship with clinical forms of paracoccidiodomycosis. J. Clin. Microbiol. **31:**671–676.
3. **Bradford, M. M.** 1976. A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. **72:**248–254.
4. **Brummer, E., E. Castaneda, and A. Restrepo.** 1993. Paracoccidioidomycosis: an update. Clin. Microbiol. Rev. **6:**89–117.
5. **Bryant, G., C. N. Rao, M. Brentani, W. Martins, J. D. Lopes, S. E. Martin, L. A. Liotta, and E. Schiffman.** 1987. A role for the laminin receptor in leukocyte chemotaxis. J. Leukocyte Biol. **41:**220–227.
6. **Camargo, Z. P., C. P. Taborda, E. G. Rodrigues, and L. R. Travassos.** 1991. The use of cell-free antigen of *Paracoccidioides brasiliensis* in serological tests. J. Med. Vet. Mycol. **29:**31–38.
7. **Fraker, J. P., and J. C. Speck.** 1978. Protein and cell membrane iodinations with a sparingly soluble chloroamide 1,3,4,6-tetrachloro 3α,6α-diphenylglycoluril. Biochem. Biophys. Res. Commun. **80:**849–857.
8. **Franco, M.** 1986. Host-parasite relationships in paracoccidioidomycosis. J. Med. Vet. Mycol. **25:**5–18.
9. **Gold, L. I., and E. Pearlstein.** 1980. Fibronectin collagen binding and requirement during cellular adhesion. Biochem. J. **186:**551–559.
10. **Grover, A., G. Andrews, and E. D. Adamson.** 1983. Role of laminin in epithelium formation by F9 aggregates. J. Cell Biol. **97:**137–144.
11. **Huard, T. K., H. L. Malinoff, and M. S. Wicha.** 1986. Macrophages express a plasma membrane receptor for basement membrane laminin. Am. J. Pathol. **123:**365–370.
12. **Kleinman, H. K., F. B. Cannon, G. W. Laurie, J. R. Hassel, M. Aumailley, V. P. Terranova, G. R. Martin, and M. Dubois-Dalcq.** 1985. Biological activities of laminin. J. Cell. Biochem. **27:**317–325.
13. **Kleinman, H. K., M. L. McGarvey, L. A. Liotta, P. G. Robey, K. Tryggvason, and G. R. Martin.** 1982. Isolation and characterization of type IV procollagen, laminin and heparan sulfate proteoglycan from the EHS sarcoma. Biochemistry **21:**6188–6193.
14. **Levitzki, A.** 1984. Receptors—a quantitative approach. The Benjamin/Cummings Publishing Co. Inc., Menlo Park, Calif.
15. **Liotta, L. A.** 1986. Tumor invasion and metastases—role of the extracellular matrix: Rhoads Memorial Award Lecture. Cancer Res. **46:**1–7.
16. **Liotta, L. A., C. N. Rao, and S. H. Barski.** 1983. Tumor invasion and the extracellular matrix. Lab. Invest. **49:**636–649.
17. **Lopes, J. D., M. dos Reis, and R. R. Brentani.** 1985. Presence of laminin receptors in *Staphylococcus aureus*. Science **229:**275–277.
18. **Marquezini, M. W., R. Chammas, S. Sonohara, and M. M. Brentani.** 1991. Multiple laminin receptors—a radioligand binding approach. Mem. Inst. Oswaldo Cruz **86(Suppl. III):**119–120.
19. **McCarthy, J. B., S. L. Palm, and L. T. Furcht.** 1983. Migration of heptotaxis of a Schwann cell tumor line to the basement membrane glycoprotein laminin. J. Cell Biol. **97:**772–777.
20. **McEween, J. G., V. Bedoya, M. M. Patino, M. E. Salazar, and A. Restrepo.** 1987. Experimental murine paracoccidiodomycosis induced by the inhalation of conidia. J. Med. Vet. Mycol. **25:**165–175.
21. **Puccia, R., S. Schenkman, P. A. J. Gorin, and L. R. Travassos.** 1986. Exocellular components of *Paracoccidioides brasiliensis*: identification of a specific antigen. Infect. Immun. **53:**199–206.
22. **Puccia, R., and L. R. Travassos.** 1991. The 43-kDa glycoprotein from the human pathogen *Paracoccidioides brasiliensis* and its deglycosylated form: excretion and susceptibility to proteolysis. Arch. Biochem. Biophys. **289:**298–302.
23. **Puccia, R., and L. R. Travassos.** 1991. 43-kilodalton glycoprotein from *Paracoccidioides brasiliensis*: immunochemical reactions with sera from patients with paracoccidiodomycosis, histoplasmosis or Jorge Lobo's disease. J. Clin. Microbiol. **29:**1610–1615.
24. **San-Blas, G., A. Restrepo, K. Clemons, D. A. Stevens, F. San-Blas, R. Puccia, L. R. Travassos, J. I. Figueroa, A. J. Hamilton, M. A.**

Bartholomew, T. Harada, L. Fenelon, et al. 1992. Paracoccidiodo-mycosis. J. Med. Vet. Mycol. 30(Suppl. 1):59–71.

25. Silva Filho, F., W. Souza, and J. D. Lopes. 1988. Presence of laminin-binding proteins in trichomonads and their role in adhesion. Proc. Natl. Acad. Sci. USA 85:8042–8046.

26. Speziale, P., M. Hook, T. Wadstrom, and R. Timpl. 1982. Binding of the basement membrane protein laminin to Escherichia coli. FEBS Lett. 146:55–58.

27. Stambuck, B. V., R. Puccia, M. L. C. de Almeida, L. R. Travassos, and S. Schenkman. 1988. Secretion of the 43-kDa glycoprotein antigen by Paracoccidioides brasiliensis. J. Med. Vet. Mycol. 26:367–373.

28. Switalski, L. M., H. Murchison, R. Timpl, R. Curtiss III, and M. Hook. 1987. Binding of laminin to oral and endocarditis strains of viridans streptococci. J. Bacteriol. 169:1095–1101.

29. Switalski, L. M., P. Speziale, M. Hook, T. Waldstrom, and R. Timpl. 1984. Binding of Streptococcus pyogenes to laminin. J. Biol. Chem. 259:3734–3738.

30. Taborda, C. P., and Z. P. Camargo. 1993. Diagnosis of paracoccidioidomycosis by passive haemagglutination assay of antibody using a purified and specific antigen-gp43. J. Med. Vet. Mycol. 31:155–160.

31. Terranova, J. P., C. N. Rao, T. Kalebic, I. M. Margulies, and L. A. Liotta. 1983. Laminin receptor on human breast carcinoma cells. Proc. Natl. Acad. Sci. USA 80:444–448.

32. Towbin, H., T. Staehelin, and J. Gordon. 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc. Natl. Acad. Sci. USA 76:4350–4354.