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Presence of laminin-binding proteins in trichomonads and their role in adhesion

(laminin receptors/cell adhesion/cell–parasite interaction/*Trichomonas vaginalis*/*Tritrichomonas foetus*)

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ABSTRACT **Adhesion is regarded as an important feature in the pathogenesis of various microorganisms. Ability to recognize extracellular matrix proteins, such as laminin or fibronectin, has been correlated with invasiveness. We report that laminin enhances the adhesion of the parasitic protozoa *Trichomonas vaginalis* and *Tritrichomonas foetus* to a polystyrene substrate and to the surface of epithelial cells (Madin–Darby canine kidney cell line) *in vitro*. The enhancement was higher for *T. vaginalis* than for *T. foetus*. Addition of anti-laminin antibodies to medium significantly inhibited the adhesion of parasites to polystyrene substrate. Indirect immunofluorescence and transmission electron microscopy of replicas of the parasite's surface labeled with antibody–gold complexes showed laminin-binding sites distributed over the parasite surface. Iodinated P_1 fragment of laminin, which retains the laminin-binding site, binds saturably to the parasite surface with a K_d of 19.5 nM, for about 3×10^5 binding sites per cell. Immunoblotting analysis of whole parasite extracts showed that a protein of 118 kDa is responsible for laminin binding.**

Trichomonas vaginalis is a flagellate protozoan of the urogenital tract of humans, while *Tritrichomonas foetus* is found in cattle (1, 2). Few data are available concerning the basic aspects of trichomonad–host cell interaction. It is known that trichomonads spontaneously adhere to inert surfaces (3, 4) as well as to cells in culture (5).

The epithelial barrier formed by the cells lining the urogenital cavities represents an important defense of the host against microbes (6). Thus, epithelial monolayers have been used *in vitro* for studying parasite invasiveness. The Madin–Darby canine kidney (MDCK) cell is one of the most studied epithelial cell lines; *in vitro* it shows some basic properties of a true epithelium such as (*i*) functional polarity (7), (*ii*) tight junctions (8), (*iii*) some dome formation (9), (*iv*) a highly active Na^+,K^+-ATPase located in the basolateral portion of the cell (10), and (*v*) a transepithelial electric potential (11). It is therefore considered a good model for studies on basic aspects of parasite–epithelium interaction (12, 13).

Laminin is a noncollagenous glycoprotein of 800 kDa, found in all basement membranes (14), with the ability to promote cell adhesion, differentiation, shape, and motility (15, 16). These functions seem to be related to specific receptors present on the surface of normal (17–19) and tumor cells (20) as well as in some pathogenic trypanosomatids (21). Receptors have also been identified in various species of bacteria (22), including *Staphylococcus aureus*, in which a 52-kDa surface protein was characterized as laminin binding (23). In the present paper we report the presence of a laminin-binding protein on the surface of trichomonads and show that laminin mediates the attachment of these parasites to epithelial cells.

MATERIALS AND METHODS

Parasites. The K strain of *T. foetus* and the Jt strain of *T. vaginalis* (24) were used in the present study. Both were cultivated in TYM (25) or medium 199 (GIBCO) supplemented with 10% bovine serum, either containing laminin or depleted of laminin by affinity chromatography on heparin-Sepharose (26), and incubated in an atmosphere of 95% air/5% CO_2 at 37°C. Only parasites in the exponential phase of growth were used. Parasites were harvested and washed twice by centrifugation at 1400 × *g* for 15 min in 0.01 M sodium phosphate buffer/0.15 M NaCl, pH 7.2 (PBS), and used for adhesion experiments.

Cells. The MDCK cells were cultivated in medium 199 supplemented with 10% bovine serum at 37°C in an atmosphere of 95% air/5% CO_2 until they reached confluence. Cultures under study were washed once with PBS, and fresh culture medium with or without laminin was added. $B_{16}F_{10}$ mouse melanoma cells (a kind gift from Isaiah Fidler, Houston, TX) were similarly cultivated as previously described (27).

Adhesion of Parasites to Plastic and MDCK Cell Surfaces. Parasites were washed twice with PBS, counted, and adjusted to 10^5 per ml, and 2 ml was poured into plastic Petri dishes coated or not with laminin (28). After 1 hr of incubation nonadherent parasites were aspirated with a Pasteur pipette, and 2 ml of PBS containing 2.5% (vol/vol) glutaraldehyde was added. Adherent parasites were counted in an inverted microscope by using a transparent grid with 4-mm^2 squares placed below the plastic dishes (29). For MDCK cells two types of experiments were performed during the interaction. First, medium 199 was supplemented with different amounts of laminin and after 1 day of parasite–cell interaction the culture vessels were washed twice with PBS and then fixed with 2.5% glutaraldehyde. In other experiments either the parasites or the MDCK cells were incubated with laminin for 30 min at room temperature, and then the two were allowed to interact. Parasites adhering to the surface of the epithelial monolayers were counted with a graticulated eyepiece (Zeiss, Kpl 12.5×) (30).

Laminin and Related Reagents. Laminin was purified from Engelbreth-Holm–Swarm mouse tumor (14). Laminin P_1 fragment was prepared according to Rohde *et al.* (31). Polyclonal anti-laminin antibodies were obtained as described previously (23). Laminin P_1 fragment was labeled with ^{125}I (Amersham) by the Bolton and Hunter method (32) to a specific activity of 8.50 $\mu Ci \cdot \mu g^{-1}$ (1 Ci = 37 GBq).

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Binding of ^{125}I-Labeled P_1 (^{125}I-P_1) to the Parasite's Surface. Parasites (10^6 per ml) were suspended in 0.1 M Tris·HCl buffer, pH 8.0, and washed once in the same buffer, and 50-μl aliquots were used for incubation with ^{125}I-P_1 in the presence or absence of a 100-fold excess of unlabeled P_1. All reactions were performed in triplicate with equal final volumes independent of the amount of protein added. After 90 min of incubation at room temperature, cells were washed twice by centrifugation and bound radioactivity was determined in an LKB MiniGamma counter.

Indirect Immunofluorescence. Washed parasites were allowed to adhere for 15 min to the surface of glass coverslips previously cleaned with methanol and coated with 0.1% poly(L-lysine) (M_r 70,000; Sigma). Coverslips were rinsed with PBS/albumin (PBSA), fixed for 10 min at 4°C with methanol, and then twice washed with PBS. In some experiments laminin or its P_1 fragment was also added, each at 30 $\mu g \cdot ml^{-1}$. After incubation, coverslips were washed with Tris·HCl buffer, pH 8.0. Parasites previously treated with laminin or its P_1 fragment were sequentially incubated with rabbit anti-laminin serum (1:100) for 15 min, and fluorescein-labeled goat anti-rabbit IgG (1:50) for 60 min. Coverslips were thoroughly rinsed with Tris·HCl buffer between each step and finally mounted with a 9:1 (vol/vol) glycerol/PBS mixture containing 0.2 M *n*-propyl gallate. Reactions were analyzed in an epifluorescence Zeiss Universal microscope.

Scanning Electron Microscopy. Cells adhering to glass coverslips were fixed in 2.5% glutaraldehyde in PBS, post-fixed with 1% OsO_4, dehydrated in ethanol, dried at the critical point with CO_2, coated with gold, and observed in a JEOL 25SII scanning electron microscope.

Immunoelectron Microscopy. Immunogold staining procedure for replicas of parasites and $B_{16}F_{10}$ cells was carried out with both fixed in 0.1% glutaraldehyde plus 3% paraformaldehyde for 30 min at 4°C. After careful washing with cold PBS-A, they were allowed to adhere to poly(L-lysine)-coated glass coverslips and incubated for 30 min with whole laminin or its P_1 fragment. Coverslips were again washed and incubated for 30 min with polyclonal anti-laminin antibodies (1:50) followed by goat anti-rabbit IgG or staphylococcal protein A complexed to colloidal gold particles (mean diameter of 20 nm; Janssen Pharmaceutica, Beerse, Belgium; 1:20 dilution). Subsequently, cells and parasites were postfixed for 15 min with 1% OsO_4, dehydrated in ethanol, and dried at the critical point with CO_2. Replicas were obtained by evaporation of platinum–carbon at 45°C and carbon at 90°C. Replicas were released in 20% hydrofluoric acid, cleaned in sulfuric acid and distilled water, collected on 200-mesh copper grids, and observed in a JEOL 100 CX electron microscope.

Sodium Dodecyl Sulfate/Polyacrylamide Gel Electrophoresis (SDS/PAGE) and Immunoblotting. Parasites were harvested from cultures, washed by centrifugation with PBS, pH 7.4, pelleted, and lysed in the same volume of PBS containing 100 mM octyl β-D-glucopyranoside (Calbiochem) for 30 min at 37°C. Protein in the supernatant was measured by the Lowry method and used for electrophoretic analysis. SDS/PAGE and immunoblotting were performed as described elsewhere (23).

Cell Electrophoresis. The electrophoretic mobility of polystyrene beads (mean diameter of 4.43 μm) previously incubated or not for 60 min at room temperature in the presence of laminin (10 $\mu g \cdot ml^{-1}$) was determined (24). Briefly, laminin-coated or uncoated polystyrene beads were suspended in a saline solution whose pH and ionic strength were maintained at 7.2 and 0.145 $mol \cdot dm^{-3}$, respectively. Their electrophoretic mobilities were recorded in a rectangular chamber immersed in a water bath at 25°C. A potential gradient of 5.5 $V \cdot cm^{-1}$ was applied to the chamber and the electrophoretic mobility of the beads was recorded by using a Zeiss cytopherometer. The mean values of electrophoretic mobility were converted to zeta potentials by the equation $\zeta = 12.85 \mu$ (33), in which μ is the mean mobility value.

Table 1. Effect of incubation of polystyrene latex beads in the presence of poly(L-lysine) or laminin on the electrophoretic mobility and zeta potential

System	Electrophoretic mobility, $\mu m \cdot s^{-1} \cdot V^{-1} \cdot cm$	Zeta potential, mV	Change in zeta potential, %*
Beads	-0.77 ± 0.012	-9.99	—
Beads + poly(L-lysine)	$+1.022 \pm 0.008$	$+12.87$	$+228$
Beads + laminin	-2.062 ± 0.020	-26.49	-165

The latex beads had a mean diameter of 4.43 μm. Poly(L-lysine) was added at 0.1% and laminin was 10 $\mu g \cdot ml^{-1}$. Mobilities are given as mean ± SD.

*The values were obtained by using $\Delta\zeta = [(\zeta_1 - \zeta_2)/\zeta_1] \times 100$, in which ζ_1 is the zeta potential of untreated beads and ζ_2 is that of poly(L-lysine)- or laminin-treated beads.

RESULTS

Adhesion of Trichomonads to a Polystyrene Surface. In contrast to *T. vaginalis*, few *T. foetus* adhered to a polystyrene surface; *T. foetus* cells were easily removed by washing the dishes with PBS. Incubation of a polystyrene surface with laminin leads to the formation of a thin protein film on the surface (34). To determine how laminin treatment alters a plastic surface, the electrophoretic mobilities of control and laminin-treated polystyrene beads were measured and their zeta potentials were calculated. Laminin rendered them more negative than the control beads. Beads incubated with poly(L-lysine), which rendered their surfaces positive, were used as a positive control (Table 1).

Prior treatment of polystyrene dishes with laminin greatly increased the adhesion of the trichomonads to the substrate (Table 2). The effect was dependent on protein concentration, with greater adhesion at 20 $\mu g \cdot ml^{-1}$. Addition of anti-laminin polyclonal antibodies to the medium significantly inhibited the enhancement produced by laminin.

Adhesion of *T. vaginalis* or *T. foetus* on MDCK Monolayers. Both *T. foetus* and *T. vaginalis* adhered to MDCK monolayers either through their anterior flagella or through other regions of the cell body (Fig. 1*A*). Adhesion of *T. vaginalis* was greater than that of *T. foetus*. After extensive washing of the culture vessels, 39% of *T. vaginalis* but only 15% of *T. foetus* remained adhering to the monolayers. Addition of laminin to the interaction medium at 30 $\mu g \cdot ml^{-1}$ led to an increase in the adherence of both flagellates (Fig. 2). The effect was less pronounced for *T. foetus* than for *T. vaginalis*,

Table 2. Adhesion of trichomonads to polystyrene substrata treated with laminin at various concentrations

Laminin coating, $\mu g \cdot ml^{-1}$	Anti-laminin polyclonal antibody (1:10)	Increase in adhesion, %	
		T. foetus	*T. vaginalis*
0	−	—	—
0	+	14.2 ± 13.1	23.4 ± 10.3
10	−	65.4 ± 16.3	85.3 ± 12.4
20	−	80.5 ± 14.6	92.4 ± 16.3
30	−	78.4 ± 10.3	93.0 ± 10.8
30	+	ND	41.5 ± 12.5

The number of parasites adhering per substrate area was determined after 60 min of interaction at 37°C and converted to percent values. Results are mean ± SD. Absolute values of adhering parasites on uncoated substrata (controls) were 6.6×10^3 per mm^2 for *T. foetus* and 8.3×10^5 per mm^2 for *T. vaginalis*. ND, not determined.

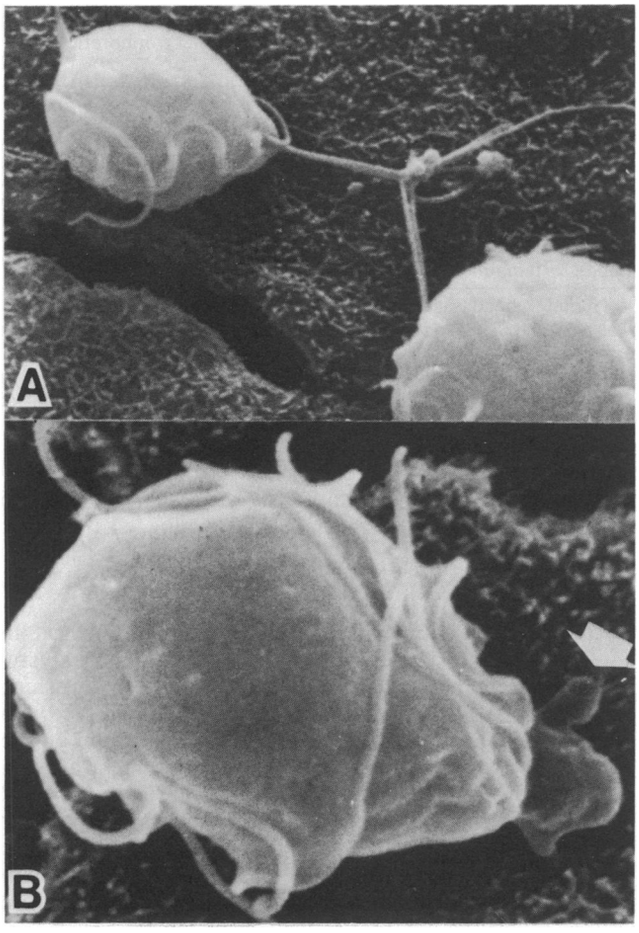

FIG. 1. Scanning electron micrographs showing the adhesion of *T. vaginalis* to MDCK cells in the absence (*A*) or presence (*B*) of laminin at 10 $\mu g \cdot ml^{-1}$. Note the presence of parasite's cytoplasmic projection (arrow) when laminin is present. (*A*, ×6500; *B*, ×12,600.)

which showed cytoplasmic projections (Fig. 1*B*). To investigate whether such effect was due to interaction of laminin with parasite or MDCK cell surface components, only one of the cells was incubated with laminin before interaction. Adhesion of parasites to the epithelium was significantly enhanced in both cases (Table 3), being more pronounced when the parasites were treated with the protein. Enhancement of adhesion to the monolayer was always higher with *T. vaginalis* than with *T. foetus*. Parasites from cultures supplemented with laminin-depleted serum produced similar results.

Table 3. Influence of laminin on the adhesion of trichomonads to MDCK cell monolayers

Cells treated with laminin*	Increase in cell adhesion, %†		
	T. foetus	*T. vaginalis*	MDCK
MDCK	37.6 ± 13.1	46.3 ± 15.4	—
T. foetus	—	—	75.4 ± 23.0
T. vaginalis	—	—	102.1 ± 15.6

*Either MDCK monolayers or parasites were treated with laminin at 30 $\mu g \cdot ml^{-1}$ for 30 min at room temperature before the parasites were added to the monolayer.

†These values (mean ± SD) were normalized as previously described (29). The following control values were considered as zero increase: 21.3 ± 9.2 *T. foetus* and 37.1 ± 10.2 *T. vaginalis* adhered per mm^2 of MDCK cell monolayers after 60 min of interaction in a medium without laminin.

Localization of the Receptors. Parasites from laminin-depleted medium were studied by two approaches. Fluorescein microscopy showed intense reactions on the surface of virtually 100% of *T. foetus* and *T. vaginalis* (Fig. 3 *A* and *B*). Second antibodies complexed with colloidal gold were used for transmission electron microscopy, which showed similar results (Fig. 3*C*). Labeling was not observed in cells initially incubated in the absence of the protein. Mouse melanoma cells ($B_{16}F_{10}$), which are known to possess a high number of cell surface laminin receptors, were used as positive controls and exhibited numerous gold particles on their surfaces (not shown).

Binding of Whole Laminin or Its P_1 Fragment to *T. vaginalis*. Binding of intact laminin (not shown) or its pepsin-derived P_1 fragment (Fig. 4) to *T. vaginalis* was specific and saturable. The K_d for the P_1 fragment was 19.5 nM, with approximately 3×10^5 binding sites per cell.

Characterization of Laminin Receptors from *T. vaginalis*. Indirect immunoblot analysis of *T. vaginalis* whole detergent extracts showed that laminin strongly binds to a 118-kDa protein, as revealed with anti-laminin rabbit serum (Fig. 5). No protein band was observed when laminin was omitted in the assay.

DISCUSSION

Several lines of evidence suggest that trichomonad parasitism can be correlated with the ability of the parasites to adhere to

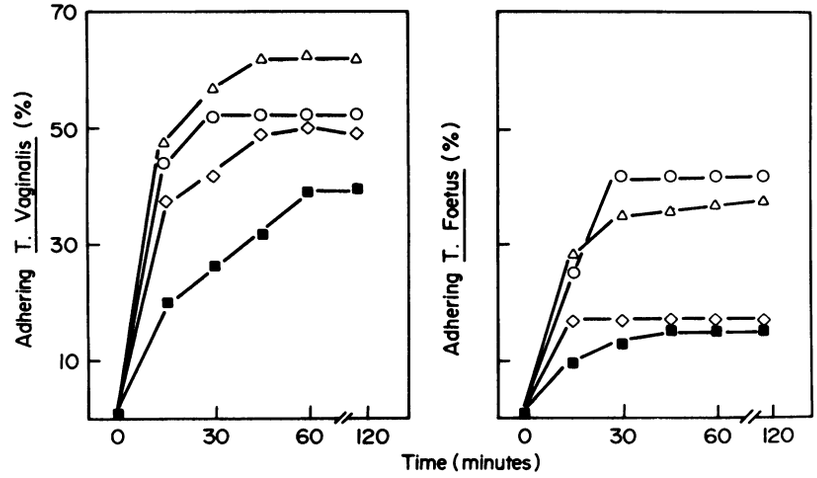

FIG. 2. Adhesion of *T. vaginalis* (*Left*) or *T. foetus* (*Right*) to confluent epithelial cultures that had been incubated with laminin at the following concentrations: ■, control without laminin; ◇, 10 $\mu g \cdot ml^{-1}$; ○, 20 $\mu g \cdot ml^{-1}$; △, 30 $\mu g \cdot ml^{-1}$.

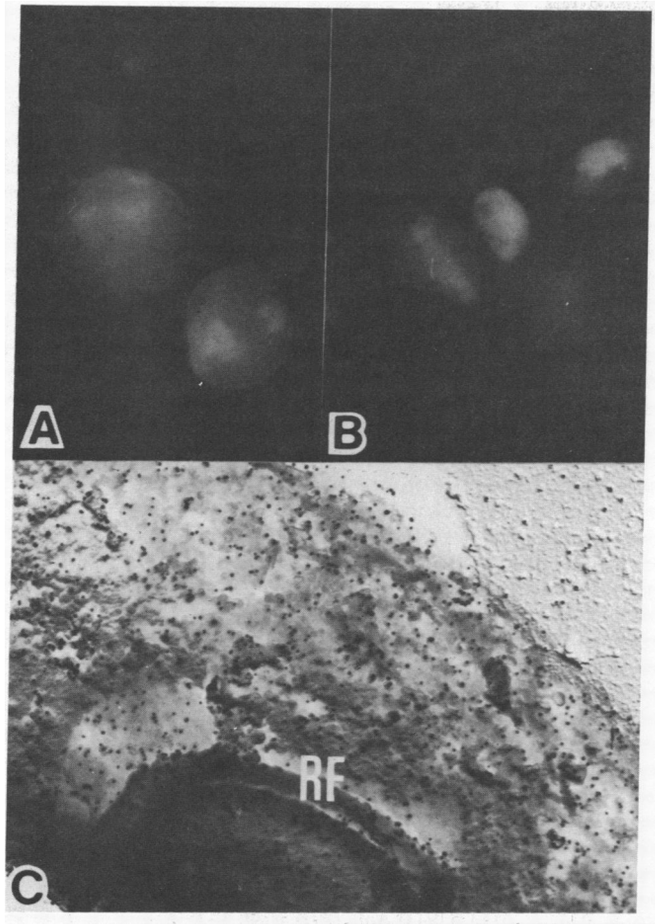

FIG. 3. Localization of laminin receptors on the surface of *T. vaginalis* (*A* and *C*) and *T. foetus* (*B*) by immunofluorescence microscopy (*A* and *B*) and by transmission electron microscopy of a replica of the surface of *T. vaginalis* labeled with antibody–gold complex (*C*). RF, recurrent flagellum. (*A* and *B*, ×790; *C*, ×21,000.)

various cultured cells (35–43), including MDCK epithelial cells (5, 30). Attachment to cells leads to monolayer disruption and cytotoxicity (39–42), a phenomenon not observed for nonpathogenic trichomonads (37, 41). Few, if any, molecules involved in this process have been characterized so far (44). We herein report the presence of laminin-binding proteins on the cell surface of the pathogenic *T. vaginalis* and *T. foetus*.

Affinity of both parasites for laminin could be demonstrated by the enhancement of adhesion observed by coating polystyrene with the protein. Since both polystyrene and the cell surface of trichomonads are negatively charged (24, 29, 45), laminin could act by rendering the plastic surface less negative or even positive. Our results showed otherwise. Bronner-Fraser (34) has shown that laminin spontaneously binds to polystyrene surfaces, and here we observed that these polystyrene surfaces became more negatively charged than beads not coated with laminin. These findings suggest that adhesion enhancement produced by laminin is not dependent on electrostatic interaction. More probably, specific recognition of laminin by molecules on the trichomonad surfaces seems to be correlated with protein hydropathicity, as we have recently shown for the fibronectin–fibronectin receptor system (46).

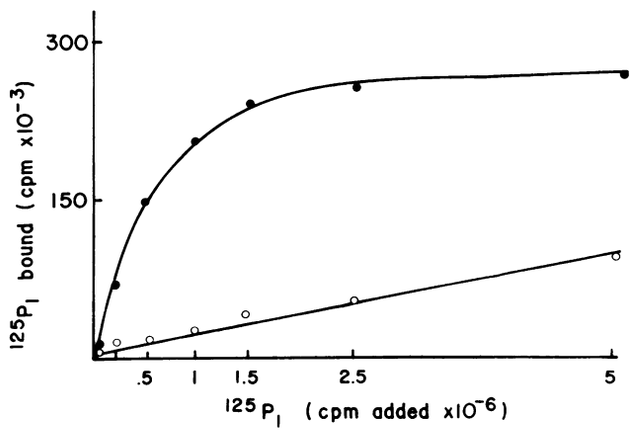

FIG. 4. Binding of ^{125}I-P_1 fragment to whole *T. vaginalis* in the absence (●) or presence (○) of an excess of P_1.

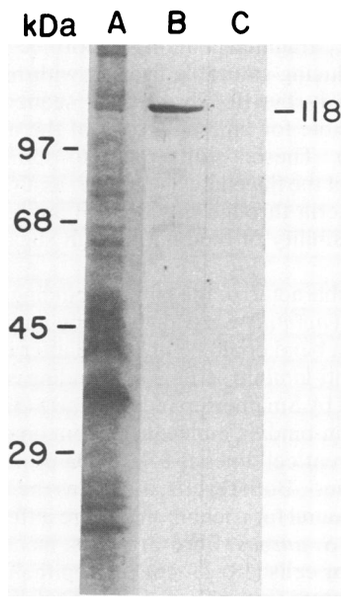

FIG. 5. SDS/PAGE analysis of the whole homogenate of *T. vaginalis*. Lane A was stained with Coomassie blue. A major band of 118 kDa was identified in an electrophoretic transfer of the extract to nitrocellulose that was sequentially allowed to react with laminin, anti-laminin antibody, and peroxidase-conjugated goat anti-rabbit IgG (lane B). No bands were detected when incubation with laminin was omitted (lane C).

Attachment of parasites from laminin-depleted medium to an epithelial monolayer was also enhanced by the addition of laminin. The finding that prior incubation of the parasites with laminin produced higher adhesion than did prior incubation with the epithelial cells could be due either to larger number of receptors expressed on the parasite's surface or to higher affinity of the surface proteins for laminin. The same rationale could be used when *T. vaginalis* and *T. foetus* were compared, since the former showed higher binding than the latter. Laminin also induced faster and more frequent protusion of cytoplasmic extensions from the flagellate's body. Laminin is known to have chemotactic and spread-inducing properties (47), and those extensions might represent higher spreading capacity besides higher adhesion.

Uniform parasitism of cell monolayers by trichomonads has been considered to be a consequence of adherence occurring only on sides opposite to the active undulating membrane (41). Our results obtained by immunofluorescence and by transmission electron microscopy of replicas showed, however, that one-side adherence seems not to be the case, as far as laminin-binding proteins are concerned. Laminin receptors were localized on the recurrent flagellum as well as on the whole parasite surface, and these observations could be compared with gold-labelling of $B_{16}F_{10}$ cells, known to

express large numbers of laminin receptors (48). Data on radiolabeled P_1 fragment binding confirmed these assumptions, by producing saturable ligation with high affinity. P_1 contains the Gly-Tyr-Ile-Gly-Ser-Arg sequence (49), putatively responsible for binding to one of the mammalian cell receptors (50). These results point to some evolutionary conservation of the flagellate's receptor, as already suggested for some bacteria through monoclonal antibodies (27), and make the possibility of binding through sugar moieties (51) less probable.

To further characterize the laminin-binding protein, whole extracts of *T. vaginalis* were analyzed by immunoblotting. A major 118-kDa band could be observed after incubation with laminin and anti-laminin serum. This finding is very similar to that described by Smalheiser and Schwartz (52), who showed that the laminin-binding molecule present on cell membranes of three different cell lines is a 120-kDa protein, which they termed "cranin." Both results, as well as the large number of binding sites found for trichomonads, are different from those described for *S. aureus*, fibrosarcomas, macrophages, polymorphonuclear cells (18, 23, 46), and epithelial cancers (20). Laminin receptors size in *S. aureus* and eukaryotic cells were in the range of 50–70 kDa. In addition, the affinity of the cell surface of *T. vaginalis* to laminin seems to be higher.

It has been shown that *T. vaginalis* may show an invasive behavior (53–57). On the other hand, it has been suggested that there is a correlation between expression of surface laminin receptors and metastasizing capacity of cancer cells (58). No correlation has so far been shown between strains of trichomonads of various degrees of pathogenicity and the expression of laminin-binding proteins. Our results, however, strongly suggest that laminin can mediate the parasite's attachment to cells and that receptors may be involved in the microorganism's pathogenesis.

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