The binding of *Tritrichomonas foetus* to immobilized laminin-1 and its role in the cytotoxicity exerted by the parasite

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The recognition and binding of pathogens to extracellular matrix glycoproteins may determine the outcome of infective processes. The interaction between the bovine urogenital parasite *Tritrichomonas foetus* and the major basal membrane glycoprotein laminin-1 (LMN-1) was investigated. The chemical nature of parasite molecules involved in the attachment of *T. foetus* to immobilized LMN-1 and the influence of LMN-1 in the toxicity exerted by the parasite to HeLa cells was studied. Attachment of *T. foetus* to LMN-1 resulted in notable morphological alterations of the parasite, which became amoeboid. *T. foetus* recognized LMN-1 through specific amino acid sequences (AG73, C16, A208 and A13) in the LMN-1 molecule, and the protein nature of the parasite molecules involved in the recognition was demonstrated by dot-blot analyses. Such molecular recognition was cation-dependent and five LMN-1-binding molecules (220, 200, 130, 125 and 80 kDa) were identified in *T. foetus*. Binding of *T. foetus* to LMN-1 rendered the parasite toxic to HeLa cell monolayers. Thus, LMN-1 appears to provide signalling cues that mediate important cell functions in *T. foetus* concerning its interaction with host cells.

INTRODUCTION

Tritrichomonas foetus is a venereal pathogen of naturally bred cattle which may cause infertility and abortion (BonDurant, 2005). The related pathology, usually referred to as bovine trichomoniasis, includes different clinical manifestations between bulls and cows (BonDurant, 1997). In bulls the parasite can be detected at the preputial cavity to the urethra, and also in deeper parts of the urogenital tract (Parsonson *et al.*, 1974). Once infected, bulls may harbour *T. foetus* throughout their lives without exhibiting clinical symptoms. In contrast, among cows the effects of the disease range from asymptomatic to severe clinical manifestations including vaginitis, cervicitis, endometritis and pyometra, resulting in transient infertility or fetal loss. Such clinical characteristics indicate the ability of *T. foetus* to invade host tissues (Parsonson *et al.*, 1976; López *et al.*, 2000). Trichomonads possess a diversity of surface glycoconjugates including adhesins (Corbeil *et al.*, 1989; Singh *et al.*, 1999; Alderete & Garza, 1988) and extracellular matrix (ECM)-binding molecules (Silva-Filho *et al.*, 1988), which play important roles during the interaction of the parasites with their hosts.

In mammals, the ECM is composed of a complex assortment of glycoproteins and proteoglycans, which not only serve as

scaffolds to provide a structural framework for tissues but also regulate cell behaviour (Bissell & Barcellos-Hoff, 1987; Nelson & Bissell, 2006). Among the ECM components, laminin-1 (LMN-1) is a large (≈ 850 kDa) glycoprotein involved in various biological phenomena (Ekblom *et al.*, 2003). LMN-1 provides signalling cues mediating important cell functions, including cell adhesion (Arrighi & Hurd, 2002; Ghosh *et al.*, 1999; Gordon *et al.*, 1993; Silva-Filho *et al.*, 2002) and invasiveness by micro-organisms (Bandyopadhyay *et al.*, 2001; Li *et al.*, 1995).

Biochemical studies focusing on the binding of eukaryotic cells to LMN-1 and the chemical nature of the related cell surface receptors have shown that the binding of some eukaryotic cells to LMN-1 is modulated by integrins (Mercurio, 1995; Hynes, 2002) and requires the participation of certain cationic species. The functionality of integrins is indeed revealed only in the presence of minimum amounts of each one of Mn^{2+} , Ca^{2+} , Mg^{2+} , or both Ca^{2+} and Mg^{2+} (Plow *et al.*, 2000). In addition, it has been found that the oligosaccharide moieties of LMN-1 (Fujiwara *et al.*, 1988) and their lectin-like counterpart molecules residing on pathogen surfaces (Ferreira *et al.*, 2006; Saarela *et al.*, 1996) may mediate both recognition and binding of micro-organisms to LMN-1.

Since ECM binding by prokaryotic and eukaryotic pathogens may trigger pathogenesis and tissue invasion (Li *et al.*,

Abbreviations: ECM, extracellular matrix; LMN-1, laminin-1.

1995; Hernandez-Ramirez *et al.*, 2000; Meza, 2000), the aim of this study was to characterize the binding of *T. foetus* to immobilized LMN-1, including the molecular nature of the related receptor and the role played by parasite-LMN-1 binding in the interaction between *T. foetus* and cultured mammalian cells.

METHODS

Parasites. The K strain of *T. foetus*, isolated by Dr H. Guida (Embrapa, Rio de Janeiro, Brazil) from the urogenital tract of a bull (Silva-Filho *et al.*, 1986), was used throughout. The parasites were cultured in modified TYM medium (Diamond, 1957; Lockwood *et al.*, 1984) supplemented with 10% fetal calf serum at 37 °C. Parasite density and viability were estimated by counting in a haemocytometer chamber using erythrosin B as a viability marker (Philips, 1973). Only parasite suspensions exhibiting viability indexes higher than 90% were used in the following experiments.

LMN-1 immobilization on glass surfaces. Pre-cleaned glass slides were uncoated or coated with 5, 10, 20, 30 or 40 µg LMN-1 ml⁻¹ (LMN from Engelbreth-Holm Swarm Tumour; Sigma) (Silva-Filho *et al.*, 1988) made in Ca²⁺-, Mg²⁺- and Mn²⁺-containing 50 mM HEPES free acid, pH 6.6, for 2 h at 37 °C. Subsequently, the unbound LMN-1 was aspirated and discarded.

[³H]Thymidine labelling of *T. foetus*. Parasites cultured at 37 °C for 24 h were collected by centrifugation, washed and resuspended in TYM medium plus serum containing 30 µCi [³H]thymidine (Alderete *et al.*, 1995; Rocha-Azevedo *et al.*, 2005). After 24 h incubation the resulting radiolabelled parasites were collected by centrifugation (450 g), and washed three times with 0.01 M phosphate-buffered 0.145 M NaCl (PBS), pH 7.2, in order to remove the unassociated radioactive tracer.

Binding to immobilized LMN-1. [³H]Thymidine-labelled parasites were suspended in 10 mM HEPES plus 125 mM sucrose containing 0.01 M CaCl₂, MgCl₂ and MnCl₂, or without divalent cations, followed by an additional incubation with or without 5 mM EDTA. The parasites were allowed to interact with uncoated and LMN-1-coated slides for 1 h at 37 °C. In some assays sucrose was replaced by 125 mM β-lactose or galactose. Following interaction with LMN-1, the unbound parasites were aspirated and discarded; those which remained associated with the slides were washed twice and solubilized by incubation in a lysis solution (0.2% SDS in 0.2% NaOH). Radioactivity (c.p.m.) was measured by liquid scintillation. The resulting c.p.m. values were converted to the percentage of parasites attached to LMN-1 according to Rocha-Azevedo *et al.* (2005): % attachment = (c.p.m._{experimental}/c.p.m._{positive control}) × 100.

Scanning electron microscopy. Parasites were allowed to interact with glass coverslips either coated with 20 µg LMN-1 ml⁻¹ or without LMN-1 for 60 min at 37 °C, and then carefully washed three times

with PBS following fixation with 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.2. The samples were then washed sequentially with PBS, post-fixed with 1% osmium tetroxide in 0.1 M sodium cacodylate buffer (pH 7.2), dehydrated in a graded series of ethanol, critical-point dried by using CO₂, and sputter-coated with gold. The samples were examined in a JEOL JSM-5310 scanning electron microscope. The morphological types found among the parasites were scored and counted as follows: tear-shaped or slightly round (not amoeboid) and spread (amoeboid), in 10 randomly chosen fields at 1000 × magnification.

Trypsin and periodate treatments. Untreated parasites and parasites treated with 3 mg trypsin ml⁻¹ for 30 min at 37 °C, and parasites treated with 1 mM sodium periodate for 30 min at 4 °C (Crouch & Alderete, 1999), were washed with PBS, and their viability indices estimated as previously detailed.

Dot blot analyses. Parasites were disrupted in lysis buffer (Galán *et al.*, 1992) and the resultant cell extracts were then incubated with or without 10, 50 or 100 mg trypsin ml⁻¹ for 15 min at 37 °C or with 5, 10, 20 or 50 mM sodium periodate for 30 min at 4 °C. The untreated and treated extracts were then blotted onto nitrocellulose membranes and incubated overnight with TBST blocking buffer (Tris-buffered saline with 0.1% Tween, 20.5% non-fat dry milk and 1% BSA). Just after this, the membranes were sequentially loaded with 10 µg LMN-1 ml⁻¹ for 2 h in Tris-buffered NaCl (TBS) containing 1% BSA plus 0.01 M of each one of CaCl₂, MgCl₂ and MnCl₂, and sequentially incubated for 1 h with a polyclonal anti-LMN-1 antibody (1:100) and a secondary antibody conjugated to peroxidase (1:8000), in TBST blocking buffer.

Competition with LMN-1-related peptides. Prior to the interaction with immobilized LMN-1, parasites were pre-incubated for 30 min at 4 °C without added peptides or with 30 µg ml⁻¹ of one of the following LMN-1-related peptides (Table 1): AG73, C16, A208 and A13 (a kind gift from Dr M. Nomizu; Nomizu *et al.*, 1995, 1997, 1998). Alternatively, each of the peptides was added to the interaction medium. After the incubation period the wells were washed twice to remove non-attached parasites, and those which remained associated with the LMN-1-coated slides were counted by light microscopy.

Indirect immunoblotting. Parasites were disrupted in lysis buffer (Galán *et al.*, 1992) and incubated overnight at 4 °C with 10% trichloroacetic acid (Grimbleby & Ntalianas, 1961). The resulting proteins were resuspended in dissolving buffer (16.5 mM Tris, 0.5% SDS, 0.5% β-mercaptoethanol, pH 6.8), quantified according to Bradford (1976), diluted in sample buffer (Laemmli, 1970), and assayed or stored at -20 °C. SDS-PAGE analysis was performed in 7.5% polyacrylamide mini-gels with 30 µg protein per slot. Protein bands which were separated by SDS-PAGE were electrotransferred to nitrocellulose membranes (Towbin *et al.*, 1979). After protein blotting, the membranes were stored at 4 °C or immediately incubated with TBST blocking buffer followed by sequential incubations with LMN-1 (20 µg ml⁻¹), a LMN-1 goat antibody

Table 1. LMN-1 related peptides (Nomizu *et al.*, 1995, 1997, 1998)

Synthetic peptide	Sequence	Active sequence	Location
AG73	RKRLQVQLSIRT	LQVQLSIR	α-Chain globular domain
A13	RQVFQVAYIIKA	Not determined	α-Chain
A208	AASIKVAVSADR	IKVAV	α-Chain
C16	KAFDITYVRLKF	YVRL	γ-Chain

(1:100, Santa Cruz Biotechnology), and an anti-goat antibody conjugated to peroxidase (1:8000, Sigma), as described above.

Epithelial cell culture. HeLa cells were cultured in RPMI 1640 medium (Gibco Laboratories) supplemented with 25 mM HEPES and 10 % fetal bovine serum. Cells were seeded in plastic flasks, and incubated under a 5 % CO₂ atmosphere at 37 °C until confluent. The resulting cell monolayers were disrupted with 0.2 % trypsin and 0.58 mM EDTA in PBS. The cells were then washed twice in culture medium plus serum and then distributed into 96-well plates (10⁵ cells per well).

Cytotoxicity assay. HeLa cell monolayers were labelled with [³H]thymidine [4 µCi (148 kBq) per 10⁵ cells per well] overnight, according to Singh *et al.* (1999). The culture medium was then aspirated, and the wells were washed twice with warm PBS prior to the interaction with *T. foetus*. Previously washed parasites, which were suspended in RPMI medium without serum, supplemented or not with 20 µg LMN-1 ml⁻¹, were added to the HeLa cell monolayers (10 parasites:1 cell). After 5 h of parasite–cell interaction at 37 °C, the interaction medium was collected, and the released [³H]thymidine was evaluated by liquid scintillation counting (Melo-Braga *et al.*, 2003). The cytotoxicity indices were expressed as a percentage of the positive control (monolayer lysed by 0.2 % SDS in 0.2 % NaOH). The serum-free medium in which HeLa cells were maintained was collected and also used as negative control (spontaneous release). The percentage cytotoxicity was determined by the following formula: % cytotoxicity = (c.p.m._{experimental}/c.p.m._{positive control}) × 100.

Statistics. All data were analysed by using Student's *t*-test to identify the significance of differences between experimental and control conditions (*P* ≤ 0.05).

RESULTS

Binding of *T. foetus* to immobilized LMN-1

LMN-1 was immobilized onto glass slides at different concentrations (5, 10, 20, 30 or 40 µg ml⁻¹), and *T. foetus* trophozoites were added to uncoated and LMN-1-coated slides. As can be seen in Fig. 1, parasite attachment further

Fig. 1. Binding of [³H]thymidine-labelled *T. foetus* to LMN-1 with different amounts of LMN-1 (0, 5, 10, 20, 30 and 40 µg ml⁻¹). The number of parasites bound to immobilized LMN-1 was determined as detailed in Methods. The data represent the means of at least three independent experiments. Bars represent standard deviations of the mean from experiments carried out in triplicate.

increased concomitantly with addition of LMN-1 at amounts up to 20 µg ml⁻¹. Thus, we used 20 µg LMN-1 ml⁻¹ to coat glass slides throughout.

Morphological aspects of LMN-1-bound *T. foetus*

T. foetus was allowed to interact with uncoated and LMN-1-coated slides. In less than 30 min most of the parasites interacting with immobilized LMN-1 exhibited morphological alterations. Many amoeboid forms with rough surfaces and unusual cytoplasmic projections were observed among parasites attached to LMN-1-coated slides (Fig. 2B, D, arrows), while the usual tear-like shape was predominant in controls (Fig. 2A, C). In order to estimate the number of amoeboid parasites adhered to LMN-1 and adhered on uncoated and LMN-1-coated glass, a quantitative analysis was performed by recording the numbers of amoeboid (spread) and non-amoeboid forms of *T. foetus*. Contact with LMN-1 rendered more amoeboid parasites (25.3 per field) than those attached to uncoated control slides (3 per field; *P* < 0.05, Student's *t*-test).

Chemical nature of the parasite molecules involved in the binding to immobilized LMN-1

Before interaction with LMN-1, parasites were treated or not with either trypsin (3 mg ml⁻¹; 30 min at 37 °C) or sodium periodate (1 mM; 15 min at 4 °C). After 60 min of interaction with immobilized LMN-1, the trypsin-treated parasites were less associated with the protein (48.0 ± 2.3 %; *P* < 0.05) than the controls (100 %). However, previous incubation of the parasites with sodium periodate did not significantly influence their association with the immobilized protein (98.0 ± 1.9 %; *P* < 0.05).

Indirect dot-blot analyses identified the presence of LMN-1-reactive molecules in *T. foetus* (Fig. 3). When whole extracts of the parasite were pre-treated with 10, 50 or 100 mg ml⁻¹ trypsin the spots corresponding to those LMN-1-binding molecules progressively disappeared (Fig. 3). By contrast, whole extracts of *T. foetus* pre-treated with sodium periodate did not exhibit significant alterations in the reaction (Fig. 3).

Role of cations and sugars in the binding of *T. foetus* to LMN-1

Interaction of the parasites with LMN-1-coated slides in the presence of Ca²⁺, Mg²⁺ and Mn²⁺ resulted in a significant increase (61.2 %) in the number of parasites adhered to immobilized LMN-1 (Fig. 4; *P* < 0.05, Student's *t*-test). The specificity of this cation-dependent cell binding was confirmed by addition of the cation chelator EDTA to the interaction medium (Fig. 4).

In order to investigate possible modulation by LMN-1 sugar moieties in the attachment performed by *T. foetus* to immobilized LMN-1, parasites were allowed to interact with LMN-1 in the presence or absence of 125 mM

Fig. 2. Scanning electron micrographs of parasites adhering to glass coverslips coated (B, D) or uncoated (A, C) with LMN-1. Arrows indicate cytoplasmic projections.

β -lactose or galactose. After 1 h of interaction, no significant alterations in the rates of parasites associated to LMN-1-coated slides were observed when the interaction was carried out in the presence ($3.87 \pm 0.68 \times 10^4$ adhered parasites) or absence ($3.12 \pm 0.63 \times 10^4$ adhered parasites) of β -lactose. Galactose also had no effect on binding to LMN-1 (data not shown).

Fig. 3. Dot-blot analysis of *T. foetus* whole-cell extracts that were treated or not (control, CTL) with trypsin (100, 50, 10 $\mu\text{g ml}^{-1}$; 15 min at 37 °C) or periodate (5, 10, 20, 50 mM; 30 min at 4 °C). The extracts were dotted onto a nitrocellulose membrane and sequentially incubated with LMN-1, anti-LMN-1 antibody (1 : 100) and a peroxidase-conjugated secondary antibody (1 : 8000).

Identification of LMN-1 peptides recognized by *T. foetus*

Parasites were incubated with or without the LMN-1-derived peptides AG73, C16, A208 or A13 (Table 1) before the interaction with immobilized LMN-1. Alternatively, the same amount of each of these four peptides was added to the interaction medium. When the peptides were included in the interaction medium the following parasite binding rates were obtained: 49.0 %, 11.0 %, 29 % and 20.3 %, for AG73, C16, A208 and A13, respectively (Fig. 5). When the parasites were pre-incubated at 4 °C for 30 min with the peptides, and then poured into contact with LMN-1-coated slides the parasite binding rates decreased to 3.7 %, 12.3 %, 7.3 % and 9.0 %, for AG73, C16, A208 and A13, respectively (Fig. 5).

Identification of LMN-1-binding proteins

Whole-protein extracts of *T. foetus* were submitted to immunoblotting analysis in order to identify the LMN-1-binding proteins present in the parasite. This resulted in the appearance of five bands, of around 220, 200, 130, 125 and 80 kDa (Fig. 6). As a control, when the incubation step with LMN-1 was omitted, the reaction with both primary

Fig. 4. Adhesion of *T. foetus* to immobilized LMN-1 in the presence or absence of EDTA or divalent cations. Parasites were incubated with LMN-1-coated surfaces in the presence or absence (control) of divalent cations (Ca²⁺, Mg²⁺ and Mn²⁺) with or without EDTA. The data represent the means of at least three independent experiments. Bars represent standard deviations of the mean from experiments carried out in triplicate (*, $P < 0.05$).

and secondary antibodies did not result in any non-specific labelling.

Role played by LMN-1 in the parasite–cell interaction

Since certain ECM glycoproteins seem to improve both adhesion and toxicity exerted by trichomonads to mammalian cultured cells (Silva-Filho *et al.*, 1988; Crouch & Alderete, 1999) we investigated the possible role played by

Fig. 5. Adhesion of *T. foetus* to LMN-1 in the presence or not of LMN-1-binding sequences AG73 (RKRLQVLSIRT), C16 (KAFDITYVRLK), A208 (AASIKVAVSADR) or A13 (RQVFQVAYIIIIKA). Parasites were previously incubated (30 μ g ml⁻¹) or not (control) with each one of the peptides for 30 min at 4 °C before association with LMN-1-coated glass slides. Alternatively, competition assay parasites were added on LMN-1-coated surfaces which had been overlaid with the peptides (30 μ g ml⁻¹). The data represent the means of at least three independent experiments. Bars represent standard error of the mean from experiments carried out in triplicate.

Fig. 6. Indirect immunoblotting of *T. foetus* LMN-1-binding protein. Whole-protein extracts were electrophoresed and transferred to nitrocellulose membranes. The sheets were incubated with LMN-1, anti-LMN-1 antibody (1:100), and a peroxidase-conjugated secondary antibody (1:8000). The positions of molecular mass markers are indicated.

LMN-1 in the cytotoxicity exerted by *T. foetus* against HeLa epithelial cells. Prior to the interaction with cell monolayers, suspended parasites were incubated with or without LMN-1, and allowed to interact with [³H]thymidine-labelled HeLa cells (parasite:cell ratio of 10:1). Following 5 h of cell–parasite interaction we found that the cytotoxicity exerted by *T. foetus* was significantly increased when LMN-1 was present in the interaction medium. Monolayers formed by HeLa cells showed a cytotoxicity rate of $22.9 \pm 0.7\%$ when LMN-1-treated *T. foetus* was assayed, compared to a cytotoxicity rate of $15.9 \pm 0.4\%$ with untreated parasites.

DISCUSSION

T. foetus is an extracellular parasite usually found associated with the epithelium lining the urogenital cavities. Under certain conditions the parasite may invade host tissue (Parsonson *et al.*, 1976; López *et al.*, 2000). *T. foetus*, as well as other cavity parasites, appears to attach tightly to host tissue to overcome removal by physical forces. Therefore, the adhesion of *T. foetus* to both epithelial cells and ECM is an essential step for parasite maintenance in the host. The major component of basal membrane, LMN-1, may play a role during *T. foetus* binding and invasion of host tissue. As previously described (Silva-Filho *et al.*, 1988), we observed that after a few minutes of interaction between *T. foetus* and immobilized LMN-1, most of the parasites were physically associated with the glycoprotein (data not shown), and the number of parasites attached to LMN-1 was directly related to the time spent by the parasite to spread onto the LMN-1-coated surfaces. Additionally, a correlation between the number of parasites attached to LMN-1 and the amount of LMN-1 immobilized onto slides was observed (Fig. 1). The highest *T. foetus* adhesion index was obtained when 20 μ g LMN-1 ml⁻¹ was used to coat the slides. Such results led us

to consider that *T. foetus* attachment on LMN-1 is a specific process involving ligand–receptor binding.

A morphological alteration in *T. foetus* was observed when it was attached to immobilized LMN-1 (Fig. 2). The occurrence of amoeboid forms among parasites associated with LMN-1-coated slides strongly indicates that the association of *T. foetus* with LMN-1 results in an adhesive process, in which parasites are led to spread onto the immobilized glycoprotein. This seems to lead to a remodelling of both the parasite surface and also the meshwork formed by LMN-1. *Trichomonas vaginalis* also alters its morphology as soon as it adheres to host cells or some ECM glycoproteins (Schwebke & Burgess, 2004; Crouch & Alderete, 1999; Petrin *et al.*, 1998). Despite the potential importance of membrane trafficking and intracellular signalling, little is known about the molecular mechanisms underlying such morphological alteration phenomena (Arroyo *et al.*, 1993; Lal *et al.*, 2006).

Different types of LMN-1-binding molecules have been found in micro-organisms and mammalian cells, including LMN-binding proteins (Mercurio, 1995), integrins (Hynes, 2002; Rao *et al.*, 1992; Hernandez-Ramirez *et al.*, 2000) and receptins (Kronvall & Jonsson, 1999). In the present study we were able to identify the chemical nature of *T. foetus* LMN-binding molecules, and their dependence on divalent cations. The cation dependence of *T. foetus*–LMN-1 binding suggests the presence of integrin-like molecules in *T. foetus*, as already described in *Entamoeba histolytica* (Hernandez-Ramirez *et al.*, 2000). The occurrence of integrin-like molecules in *T. foetus* is currently under investigation in our laboratory.

The presence of different sites in the LMN-1 polypeptide chain as possible binding sites for prokaryotic and eukaryotic cells has been reported (Nomizu *et al.*, 1995, 1997, 1998). Furthermore, oligosaccharides associated with LMN-1 (Fujiwara *et al.*, 1988) have been also considered as important LMN-1 recognition sites by lectin-like molecules (Elola *et al.*, 2005; Ferreira *et al.*, 2006). Some lectin-like molecules recognize β -galactose and lactose residues of LMN-1 (Elola *et al.*, 2005; Woo *et al.*, 1990; Gu *et al.*, 1994); therefore the possibility that *T. foetus* may recognize LMN-1 through oligosaccharide chains on ECM molecule was explored. The presence of β -lactose or galactose in the interaction medium did not alter the binding of the parasite to LMN-1, excluding the possibility that *T. foetus* recognizes LMN-1 through these oligosaccharide moieties. On the other hand, the presence in the interaction medium of oligopeptides corresponding to amino acid sequences in the α 1 and γ 1 chains of LMN-1 partially inhibited the binding of the parasite to the immobilized protein (Fig. 5, competition assay). A high binding inhibition was also observed when *T. foetus* was pre-incubated with each of these peptides (Fig. 5, pre-incubation assay). These data indicate the possibility that *T. foetus* may recognize LMN-1 through different well-known amino acid binding sequences of the molecule, as previously observed for

other eukaryotic cells (Mercurio, 1995; Nomizu *et al.*, 1995, 1997, 1998; Ferreira *et al.*, 2006).

Five LMN-1-binding proteins, of approximately 220, 200, 130, 125 and 80 kDa, were detected in parasite homogenates. The number of LMN-1-binding proteins in *T. foetus* might be specifically related to the number of different cell recognition sites on the LMN-1 molecule, but the occurrence of a promiscuous surface glycoprotein of *T. foetus* involved in the recognition of LMN-1 cannot be ruled out. It remains unknown whether the chemical entity present in *T. foetus*, which we have named LMN-binding protein(s), corresponds to a multiligand recognition complex.

We demonstrated that the cytotoxicity of *T. foetus* to HeLa cells increased when the parasite interacted with LMN-1. The increase in cytotoxic effect might be related to an enhancement in cytoadhesion, since LMN-1-treated *T. foetus* adhered more to mammalian cells than did untreated ones (Silva-Filho *et al.*, 1988). However, it appears that the cytotoxicity exerted by *T. foetus* is not only a cytoadhesion-dependent phenomenon (Silva-Filho *et al.*, 1989). The binding of *T. foetus* to LMN-1 might trigger a signalling cue that could culminate in the upregulation of cytotoxic factors, such as the proteases released during interaction of trichomonads with mammalian cells (Alvarez-Sanchez *et al.*, 2000).

In summary, our studies identified five LMN-1-binding proteins in *T. foetus* that recognize LMN-1 by at least four different binding sequences. Taken together, these findings suggest an organized interaction complex in our parasitic protozoa model that is similar to those found in mammalian cells. *T. foetus* adhesion on LMN-1 biofilm was characterized as a cation-dependent phenomenon, by changes in *T. foetus* morphology and cytotoxicity, suggesting that the parasite–ECM interaction leads to an exchange of signals from ECM to the parasite. Moreover, *in vivo* LMN-1 could serve as a molecular track to *T. foetus*, probably guiding it to anatomic sites outside the urogenital tract.

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REFERENCES

- Alderete, J. F. & Garza, G. E. (1988). Identification and properties of *Trichomonas vaginalis* proteins involved in cytoadherence. *Infect Immun* **56**, 28–33.
- Alderete, J. F., Leher, M. W. & Arroyo, R. (1995). The mechanisms and molecules involved in cytoadherence and pathogenesis of *Trichomonas vaginalis*. *Parasitol Today* **11**, 70–74.

- Alvarez-Sanchez, M. E., Avila-Gonzalez, L., Becerril-Garcia, C., Fattel-Facenda, L. V., Ortega-Lopez, J. & Arroyo, R. (2000). A novel cysteine proteinase (CP65) of *Trichomonas vaginalis* involved in cytotoxicity. *Microb Pathog* 28, 193–202.
- Arrighi, R. B. & Hurd, H. (2002). The role of *Plasmodium berghei* ookinete proteins in binding to basal lamina components and transformation into oocysts. *Int J Parasitol* 32, 91–98.
- Arroyo, R., Gonzalez-Robles, A., Martinez-Palomo, A. & Alderete, J. F. (1993). Signalling of *Trichomonas vaginalis* for amoeboid transformation and adhesion synthesis follows cytoadherence. *Mol Microbiol* 7, 299–309.
- Bandyopadhyay, K., Karmakar, S., Ghosh, A. & Das, P. K. (2001). Role of 67 kDa cell surface laminin binding protein of *Leishmania donovani* in pathogenesis. *J Biochem* 130, 141–148.
- Bissell, M. J. & Barcellos-Hoff, M. H. (1987). The influence of extracellular matrix on gene expression: is structure the message? *J Cell Sci Suppl* 8, 327–343.
- BonDurant, R. H. (1997). Pathogenesis, diagnosis, and management of trichomoniasis in cattle. *Vet Clin North Am Food Anim Pract* 13, 345–361.
- BonDurant, R. H. (2005). Venereal diseases of cattle: natural history, diagnosis, and the role of vaccines in their control. *Vet Clin North Am Food Anim Pract* 21, 383–408.
- Bradford, M. M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* 72, 248–254.
- Corbeil, L. B., Hodgson, J. L., Jones, D. W., Corbeil, R. R., Widders, P. R. & Stephens, L. R. (1989). Adherence of *Tritrichomonas foetus* to bovine vaginal epithelial cells. *Infect Immun* 57, 2158–2165.
- Crouch, M. L. & Alderete, J. F. (1999). *Trichomonas vaginalis* interaction with fibronectin and laminin. *Microbiology* 145, 2835–2843.
- Diamond, L. S. (1957). The establishment of various trichomonads of animals and man in axenic culture. *J Parasitol* 43, 488–490.
- Eklom, P., Lonai, P. & Talts, J. F. (2003). Expression and biological role of laminin-1. *Matrix Biol* 22, 35–47.
- Elola, M. T., Chiesa, M. E., Alberti, A. F., Mordoh, J. & Fink, N. E. (2005). Galectin-1 receptors in different cell types. *J Biomed Sci* 12, 13–29.
- Ferreira, E. O., Lobo, A. L., Petrópolis, D. B., Avelar, K. E. S., Ferreira, M. C., Silva-Filho, F. C. & Domingues, R. M. C. P. (2006). A *Bacteroides fragilis* surface glycoprotein mediates the interaction between the bacterium and the extracellular matrix component laminin-1. *Res Microbiol* 157, 960–966.
- Fujiwara, S., Shinkai, H., Deutzmann, R., Paulsson, M. & Timpl, R. (1988). Structure and distribution of N-linked oligosaccharide chains on various domains of mouse tumour laminin. *Biochem J* 252, 453–461.
- Galán, J. E., Pace, J. & Hayman, M. J. (1992). Involvement of the epidermal growth factor receptor in the invasion of cultured mammalian cells by *Salmonella typhimurium*. *Nature* 357, 588–589.
- Ghosh, A., Bandyopadhyay, K., Kole, L. & Das, P. K. (1999). Isolation of a laminin binding protein from the protozoan parasite *Leishmania donovani* that may mediate cell adhesion. *Biochem J* 337, 551–558.
- Gordon, V. R., Asen, E. K., Vodkin, M. H. & McLaughlin, G. L. (1993). *Acanthamoeba* binds to extracellular matrix proteins in vitro. *Invest Ophthalmol Vis Sci* 34, 658–662.
- Grimbleby, F. H. & Ntalianas, H. A. (1961). Binding of trichloroacetic acid by protein. *Nature* 192, 835–836.
- Gu, M., Wang, W., Song, W. K., Cooper, D. N. M. & Kaufman, S. J. (1994). Selective modulation of the interaction of $\alpha 7 \beta 1$ integrin with fibronectin and laminin by L-14 lectin during skeletal muscle differentiation. *J Cell Sci* 107, 175–181.
- Hernandez-Ramirez, V. I., Anaya-Ruiz, M., Rios, A. & Talamas-Rohana, P. (2000). Tyrosine kinase activity induces by fibronectin through the $\beta 1$ -integrin-like molecule. *Exp Parasitol* 95, 85–95.
- Hynes, R. O. (2002). Integrins: bidirectional, allosteric signaling machines. *Cell* 110, 673–687.
- Kronvall, G. & Jonsson, K. (1999). Receptins: a novel term for an expanding spectrum of natural and engineered microbial proteins with binding properties for mammalian proteins. *J Mol Recognit* 12, 38–44.
- Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 227, 680–685.
- Lal, K., Noel, C. J., Field, M. C., Goulding, D. & Hirt, R. P. (2006). Dramatic reorganisation of *Trichomonas* endomembranes during amoebal transformation: a possible role for G-protein. *Mol Biochem Parasitol* 148, 99–102.
- Li, E., Yang, W. G., Zhang, T. & Stanley, S. L., Jr (1995). Interaction of laminin with *Entamoeba histolytica* cysteine proteinases and its effects on amebic pathogenesis. *Infect Immun* 63, 4150–4153.
- Lockwood, B. C., North, M. J. & Coombs, G. H. (1984). *Trichomonas vaginalis*, *Tritrichomonas foetus*, and *Trichomitus batrachorum*: comparative proteolytic activity. *Exp Parasitol* 58, 245–253.
- López, L. B., Braga, M. B., Lopez, J. O., Arroyo, R. & Costa e Silva-Filho, F. (2000). Strategies by which some pathogenic trichomonads integrate diverse signals in the decision-making process. *An Acad Bras Cienc* 72, 173–186.
- Melo-Braga, M. B., Rocha-Azevedo, B. & Silva-Filho, F. C. (2003). *Tritrichomonas foetus*: the role played by iron during parasite interaction with epithelial cells. *Exp Parasitol* 105, 111–120.
- Mercurio, A. M. (1995). Laminin receptors: achieving specificity through cooperation. *Trends Cell Biol* 5, 419–423.
- Meza, I. (2000). Extracellular matrix-induced signaling in *Entamoeba histolytica*: its role in invasiveness. *Parasitol Today* 16, 23–28.
- Nelson, C. M. & Bissell, M. J. (2006). Of extracellular matrix, scaffolds, and signaling: tissue architecture regulates development, homeostasis, and cancer. *Annu Rev Cell Dev Biol* 22, 287–309.
- Nomizu, M., Kim, W. H., Yamamura, K., Utani, A., Otaka, S. Y. A., Roller, P. P., Kleinman, H. K. & Yamada, Y. (1995). Identification of cell binding sites in the laminin $\alpha 1$ chain carboxyl-terminal globular domain by systematic screening of synthetic peptides. *J Biol Chem* 270, 20583–20590.
- Nomizu, M., Kuratomi, Y., Ponce, M. L., Song, S. Y., Miyoshi, K., Otaka, A., Powell, S. K., Hoffman, M. P., Kleinman, H. K. & Yamada, Y. (1997). Identification of cell binding sequences in mouse laminin $\gamma 1$ chain by systematic peptide screening. *J Biol Chem* 272, 32198–32205.
- Nomizu, M., Kuratomi, Y., Malinda, K. M., Song, S. Y., Miyoshi, K., Otaka, A., Powell, S. K., Hoffman, M. P., Kleinman, H. K. & Yamada, Y. (1998). Cell binding sequences in mouse laminin $\alpha 1$ chain. *J Biol Chem* 273, 32491–32499.
- Parsonson, I. M., Clark, B. L. & Dufty, J. H. (1974). The pathogenesis of *Tritrichomonas foetus* infection in the bull. *Aust Vet J* 50, 421–423.
- Parsonson, I. M., Clark, B. L. & Dufty, J. H. (1976). Early pathogenesis and pathology of *Tritrichomonas foetus* infection in virgin heifers. *J Comp Pathol* 86, 59–66.
- Petrin, D., Delgaty, K., Bhatt, R. & Garber, G. (1998). Clinical and microbiological aspects of *Trichomonas vaginalis*. *Clin Microbiol Rev* 11, 300–317.
- Philips, H. I. (1973). Dye exclusion tests for cell viability. In *Tissue Culture, Methods and Applications*, pp. 407–408. Edited by P. F. Kruse & M. K. Patterson. New York: Academic Press.

- Plow, E. F., Haas, T. A., Zhang, L., Loftus, J. & Smith, J. W. (2000). Ligand binding to integrins. *J Biol Chem* **275**, 21785–21788.
- Rao, S. P., Gehlsen, K. R. & Catanzaro, A. (1992). Identification of a β 1 integrin on *Mycobacterium avium*–*Mycobacterium intracellulare*. *Infect Immun* **60**, 3652–3657.
- Rocha-Azevedo, B., Melo-Braga, M. B. & Silva-Filho, F. C. (2005). Intra-strain clonal phenotypic variation of *Tritrichomonas foetus* is related to the cytotoxicity exerted by the parasite to cultured cells. *Parasitol Res* **95**, 106–112.
- Saarela, S., Wikstro, B. W., Rhen, M. & Korhonen, T. K. (1996). The GafD protein of the G (F17) fimbrial complex confers adhesiveness of *Escherichia coli* to laminin. *Infect Immun* **64**, 2857–2860.
- Schwebke, J. R. & Burgess, D. (2004). Trichomoniasis. *Clin Microbiol Rev* **17**, 794–803.
- Silva-Filho, F. C., Elias, A. C. & De Souza, W. (1986). Further studies on the surface charge of various strains of *Trichomonas vaginalis* and *Tritrichomonas foetus*. *Cell Biophys* **8**, 161–176.
- Silva-Filho, F. C., De Souza, W. & Lopes, J. D. (1988). Presence of laminin-binding proteins in trichomonads and their role in adhesion. *Proc Natl Acad Sci U S A* **85**, 8042–8046.
- Silva Filho, F. C., Breier-Saraiva, E. M., Tosta, M. X. & De Souza, W. (1989). *Trichomonas vaginalis* and *Tritrichomonas foetus* secrete a neuraminidase into culture medium. *Mol Biochem Parasitol* **35**, 73–78.
- Silva-Filho, F. C., Kasai, S., Nomizu, M., Lopez, L. B., Melo-Braga, M. B., Rocha-Azevedo, B., Petrópolis, D. B. & Horbach, I. S. (2002). How laminin-1 can be recognized by the protozoan parasite *Tritrichomonas foetus*: possible role played by the extracellular matrix glycoprotein in both cytoadhesion and cytotoxicity exerted by the parasite. *Parasitol Int* **51**, 305–307.
- Singh, B. N., Lucas, J. J., Beach, D. H., Shin, S. T. & Gilbert, R. O. (1999). Adhesion of *Tritrichomonas foetus* to bovine vaginal epithelial cells. *Infect Immun* **67**, 3847–3854.
- Towbin, H., Staehelin, T. & Gordon, J. (1979). Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc Natl Acad Sci U S A* **76**, 4350–4354.
- Woo, H. J., Shaw, L. M., Messier, J. M. & Mercurio, A. M. (1990). The major non-integrin laminin binding protein of macrophages is identical to carbohydrate binding protein 35 (Mac-2). *J Biol Chem* **265**, 7097–7099.

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