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Contact-Dependent Disruption of the Host Cell Membrane Skeleton Induced by *Trichomonas vaginalis*

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This report presents evidence showing that the pathogenetic process of the protozoan parasite *Trichomonas vaginalis* involves degradation of the target cell membrane skeleton; spectrin, the most representative protein within this structure, has been identified as the main molecular target. Degradation of the target cell spectrin is accomplished only upon contact with the parasite, and immunochemical and immunofluorescence studies performed with the erythrocyte as a model demonstrate that degradation of the protein takes place before target cell lysis. A preliminary characterization of the effectors involved has led to the identification of a nonsecreted 30-kDa proteinase which is characterized by a high specificity for spectrin. This molecule is suggested as the main effector responsible for cytoskeletal disruption.

Microorganisms employ a combination of strategies in order to accomplish target cell disruption. Some commonly used mechanisms are phagocytosis, release of toxic molecules, invasion and killing of the target cell through disintegration of internal components, and secretion of proteinases for degradation and digestion of the host cell. In a recent study on the pathogenetic mechanisms of the protist *Trichomonas vaginalis*, we demonstrated that the microorganism lyses erythrocytes through insertion of ionic channels on the target cell membrane (17) under defined conditions (2, 14).

T. vaginalis is a flagellated protozoan parasite responsible for one of the most common sexually transmitted diseases in humans (27). Trichomoniasis affects mostly women, and its clinical manifestations range from an asymptomatic carrier state to a severe vaginitis. During protozoan infection, extensive damage to vaginal epithelium is observed (24). An in vitro cytopathic effect of *T. vaginalis* on epithelial cells is well documented (3, 4, 26); the protozoon is also able to lyse erythrocytes, which represent an important source of iron and fatty acids (29, 35). For our in vitro studies we utilized erythrocytes as a target for the evaluation of the *T. vaginalis* cytopathic effect, as these cells are easy to monitor for lysis; moreover, they represent the simplest model for studying membrane damage processes.

The goals of this work were to achieve a better understanding of the *T. vaginalis* pathogenetic mechanism and to shed light on the relationship between *T. vaginalis* and the target cell cytoskeleton. This cellular component is a specific target for numerous pathogens, for which it plays an important role as a mechanical means for entering the host cell as well as representing a direct target mediating disruption of the parasitized cell. In order to lyse the target cell, a close contact between *T. vaginalis* and target cell membranes is required. Here we describe by scanning electron microscopy (SEM) the occurrence of important morphological alterations, such as loss of shape and collapse, in erythrocytes in contact with the parasite surface. Similar alterations have been reported to occur in parasitized epithelial cell monolayers as well; cell rounding, detachment, and cell death have been observed (3, 4, 21, 26). This finding suggests that a pathogenetic mechanism based on cytoskeletal disruption of the target cell could be involved, which, together with the formation of functional pores, could be responsible for cytotoxicity.

The cytoskeleton of nucleate cells is a complicated latticework of microfilaments, intermediate filaments, and microtubules that provides motility, shape, and stability to the cell, with complicated interconnections between plasma membrane components and the cytoplasmic network. By contrast, erythrocytes lack the intracellular network that is characteristic of nucleate cells, and their shape, deformability, and structural stability are maintained by the membrane skeleton located just beneath the plasma membrane. Its relative simplicity has permitted its detailed characterization, and during the 1980s homologs of erythrocyte membrane skeleton proteins were found in a variety of tissues from several species. The well-defined characterization of the erythrocyte membrane skeleton and its homology with nonerythroid structures were the reasons for the utilization of erythrocytes as a cellular and molecular model throughout this work.

Our studies allowed the identification of a *T. vaginalis*-induced disruption of the host cell membrane skeleton, with the most representative protein, spectrin, being the main target. The characterization of the mode and kinetics of the phenomenon has led to the finding that the parasite accomplishes degradation of spectrin before lysis of the target cell takes place.

MATERIALS AND METHODS

Microorganisms and culture conditions. *T. vaginalis* isolates were obtained as vaginal specimens from women affected by trichomoniasis. After axenization, protozoa were cultured in Diamond's TYM (13) supplemented with 10% bovine serum, 100 mg of streptomycin sulfate ml^{-1}, and 100 IU of penicillin G per ml in a 5% carbon dioxide atmosphere at 37°C. Fresh isolates were stored in liquid nitrogen with 90% serum and 10% dimethyl sulfoxide. Cultured parasites were always monitored for motility, and only viable organisms (>99%) were used to perform the experiments. Further characterization experiments were performed with our isolate SS-22 as a representative strain. *Escherichia coli* and *Staphylococcus aureus* strains, isolated from urinary specimens of patients presenting with urologic infections, were grown in Luria-Bertani medium at 37°C.

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Erythrocytes. Group O Rh^+ erythrocytes were obtained from healthy human donors. Cells were either used fresh or stored until needed in glucose and citrate buffer at 4°C; only fresh erythrocytes were used for cytoskeletal integrity assays. Prior to use, erythrocytes were washed three times in isotonic buffer at 400 × *g* for 5 min. For preparation of ghosts, pelleted erythrocytes were washed and incubated in 5 mM phosphate lysis buffer (pH 7.4) supplemented with 1 μg of leupeptin ml^{-1}, 50 μg of *N*α-*p*-tosyl-L-lysine chloromethyl ketone (TLCK) ml^{-1}, and 10 mM EDTA in order to prevent the degradation of cytoskeletal components by erythrocyte-endogenous proteinases. Samples were incubated for 5 min on ice and centrifuged at 18,000 × *g* in a Sorvall refrigerated centrifuge at 4°C. Supernatants containing the released hemoglobin were discarded, and incubation and centrifugation cycles were repeated until an acceptable elimination of hemoglobin was achieved. After the last wash, ghosts were collected and used immediately.

SEM. For SEM experiments, *T. vaginalis* organisms were incubated with fresh erythrocytes in a ratio of 1:5. After 30 min, the cells were washed with Hanks solution and fixed for 20 to 30 min in 2% glutaraldehyde (electron microscopy grade) in 0.1 M sodium cacodylate buffer, pH 7.2. Cells were washed and postfixed in 1% osmium tetroxide in sodium cacodylate buffer for 30 min, dehydrated in a graded series of acetone (20 to 100%), and critical-point dried in a Polaron apparatus with amyl acetate as the intermediate fluid and CO_2 as the transition fluid. Samples were covered with gold in an Edwards S-150 sputter coater and observed with ISI-130 and JEOL JMS-35 electron microscopes at 15 and 30 kV (16).

Cell lysates. Parasite lysates were obtained by sonication in a Branson Sonifier B 12. A total of 3×10^6 cells ml^{-1} harvested in the late exponential phase of growth were washed three times in phosphate-buffered saline (PBS), pH 7.4, by centrifugation at 350 × *g*. The pH value was established at over 6.50 to avoid lysis of erythrocytes by protozoan perforins. Washed cells were resuspended to the desired density in PBS and subjected to five cycles of sonication for 10 s each at 50 W in an ice bath. Samples were then centrifuged at 18,000 × *g*, and supernatants were filtered through a 0.45-μm-pore-size filter membrane in order to perform the degradation assays.

Secreted proteins. Parasites harvested in the late exponential phase of growth were washed three times in PBS by centrifugation at 350 × *g*. Washed organisms were resuspended to a density of 3×10^6 ml^{-1} in PBS plus 15 mM maltose (PBS-M) (pH 7.4) and incubated at 37°C to allow secretion (14). After 90 min, the parasites were harvested by centrifugation; the recovered supernatant was centrifuged again at 15,000 × *g* and filtered to eliminate whole organisms. The pH was raised to 7.00 in order to prevent hemolysis. The proteins present in the cell-free supernatant were then concentrated 10 times in a Centricon (Amicon Division, Beverly, Mass.) unit with a 3-kDa cutoff. Parasites were always monitored for motility (>99%) during the incubation period. As determined by enumeration and trypan blue exclusion, these conditions did not result in cell lysis or parasite death.

Spectrin extraction. Freshly obtained ghosts were stripped by incubation for 5 min on ice with 1 N NaOH. After incubation, the suspension was promptly brought to neutrality with 1 M HCl. The sample was then centrifuged at 18,000 × *g* for 20 min, and the supernatant was collected. The released protein was concentrated in a Centricon concentrating unit with a 30-kDa cutoff, and the protein concentration was determined by the Bradford method (9) with the Bio-Rad (Hercules, Calif.) protein assay dye (Coomassie brilliant blue), with bovine serum albumin as a standard. Since spectrin integrity is not affected by short-term storage, the concentrated protein was stored at −20°C until needed. Prior to utilization, it was always tested for purity and integrity by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

Cytoskeletal degradation assays. *T. vaginalis* organisms from exponentially growing cultures were washed three times in PBS-M and resuspended at a concentration of 2×10^6 per ml. The suspension was then incubated with fresh washed erythrocytes in a ratio of 1:5, and vials were kept at 37°C to allow erythrocyte lysis. After 60 min, the supernatant fraction, which contained membranes from erythrocytes lysed by the microorganisms, was centrifuged several times at 350 × *g* to eliminate any remaining cells and checked by light microscopy to ensure clearance of microorganisms. Ghosts were then collected by centrifugation at 18,000 × *g* for 10 min in a refrigerated centrifuge. After protein quantification by the Bradford method, membranes were electrophoresed and blotted. The same amount of protein (15 μg per lane), derived from microorganism-lysed erythrocytes and control membranes, was loaded in a 10% polyacrylamide gel. SDS-PAGE and immunoblotting for this and the following experiments were performed as described previously (15). Anti-spectrin and anti-actin antibodies were purchased from Sigma (St. Louis, Mo.); anti-2.1 and anti-4.1 protein antibodies were kindly provided by P. Arese (University of Turin, Italy). All antibodies used for immunoblots were tested for absence of cross-reactivity with trichomonal and bacterial proteins.

The same procedure was carried out with *S. aureus* and a hemolytic *E. coli* strain, which were incubated with washed erythrocytes in a ratio of 20:1. Membranes from erythrocytes lysed in hypotonic buffer (see "Erythrocytes" above), quantified for protein concentration, were used as a control for background degradation by erythrocyte-endogenous proteinases.

Immunofluorescence assays. Exponentially growing *T. vaginalis* organisms were washed three times in PBS, resuspended in PBS-M at a concentration of 2×10^6 per ml, and incubated at 37°C with washed erythrocytes in a ratio of 1:5. At different time intervals, sets of 10-μl aliquots were spotted on 10-well multitest slides, air dried at 37°C, and fixed with ice-cold methanol. One well of each set was then incubated with an anti-cytoskeletal protein antibody against spectrin, actin, and proteins 2.1 and 4.1, respectively. Slides were then processed as described elsewhere (15).

Analysis of membrane skeleton-degrading activity in unlysed erythrocytes. *T. vaginalis* organisms from exponentially growing cultures were washed three times in PBS-M, pH 7.4, and resuspended at a concentration of 2×10^6 per ml. The suspension was then incubated with fresh washed erythrocytes in a ratio of 1:5 at 37°C. Every 20 min an aliquot of the sample was centrifuged at 350 × *g* for 5 min in order to collect parasites and unlysed erythrocytes. Intact cells were separated from *T. vaginalis* by a Ficoll-Hypaque gradient centrifugation at 350 × *g* for 20 min, washed several times, and then monitored by light microscopy for the absence of parasites as described previously (17). This procedure allowed the separation of intact erythrocytes from the already-lysed ones. Once free from parasites, unlysed erythrocytes were lysed in hypotonic buffer in the presence of proteinase inhibitors and electrophoresed (25 μg per lane). Immunoblotting was performed as described above with anti-spectrin, anti-protein 4.1, anti-protein 2.1, and anti-actin antibodies.

Degradation assays on purified spectrin. One hundred microliters of filtered parasite lysates (see "Cell lysates" above) or parasite secretions (see "Secreted proteins" above) obtained from different concentrations of exponentially growing *T. vaginalis* was incubated at 37°C with 20 μg of purified spectrin (see "Spectrin extraction" above) in the presence of 1 mM dithiothreitol. Controls consisted of 20 μg of purified spectrin resuspended in an equal volume of PBS-M. At different time points, 20-μl aliquots were collected, resuspended in Laemmli buffer (28), and boiled. Samples were then electrophoresed, Western blotted, and probed with anti-spectrin antibody (Sigma).

Substrate gel electrophoresis. Total *T. vaginalis* proteins for substrate SDS-PAGE (10, 25, 31) were obtained by pelleting 10^6 organisms and resuspending them in 50 μl of Laemmli buffer. Samples were centrifuged to remove insoluble material, and the supernatants were resuspended in Laemmli buffer without β-mercaptoethanol (40). Secreted protein samples were prepared by resuspending the previously concentrated cell-free supernatant (see "Secreted proteins" above) in Laemmli buffer.

Substrate gels were obtained by including the substrate protein in SDS–10% polyacrylamide gels. The gelatin-degrading activity was evaluated in a substrate concentration of 1.8 mg per ml, whereas spectrin was copolymerized in a concentration of 300 μg of protein per ml. After the electrophoretic run, gels were soaked in a 2.5% Triton X-100 solution for 1 h to allow protein renaturation and incubated for 2 h at 37°C in 100 mM sodium acetate buffer (pH 6.00) containing 1 mM dithiothreitol as a reducing agent. After incubation, the gels were stained with Coomassie blue. Experiments were performed in triplicate at different periods, and identical patterns were obtained.

RESULTS

Electron microscopy reveals morphological alterations in erythrocytes. SEM (Fig. 1) performed at early stages of interaction between *T. vaginalis* and erythrocytes revealed distinct morphological alterations occurring in parasitized erythrocytes, such as loss of shape and collapse. Such alterations could be observed only in erythrocytes in contact with the parasite surface, suggesting contact dependence. The morphological alterations were induced at early times of exposure; after 30 min of coincubation, erythrocytes would already display consistent abnormalities in shape.

Erythrocytes lysed upon contact with *T. vaginalis* undergo loss of spectrin. The morphological alterations revealed by SEM in erythrocytes were strongly suggestive of cytoskeletal damage. Therefore, membranes obtained upon parasite-induced lysis were electrophoresed, blotted, and probed with antibodies directed against the main membrane cytoskeletal proteins. As shown in Fig. 2 (lane 2), exposure of erythrocytes to live *T. vaginalis* induced the complete disappearance of spectrin from the target cell membrane. Spectrin disappeared in an extremely rapid fashion: after 60 min of erythrocyte exposure to the trichomonad cell, the protein would become undetectable. On the other hand, protein 4.1, protein 2.1, and actin (Fig. 2, lanes 3 to 5) were readily detectable after the same incubation time. In order to assess whether the disappearance of spectrin was specifically due to *T. vaginalis* and not merely to exposure to a hemolytic pathogen, the same procedure was carried out with *S. aureus* and an *E. coli* hemolytic strain. Ghosts obtained by exposure to these pathogens under-

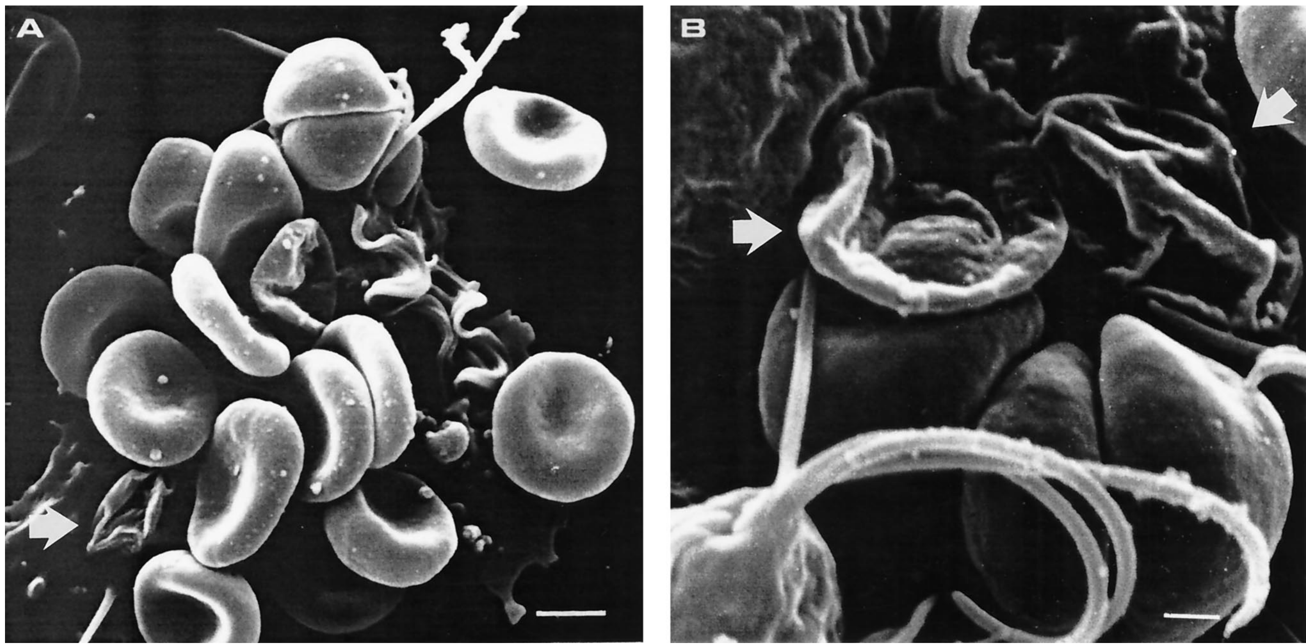

FIG. 1. Erythrocyte alterations in morphology following exposure to *T. vaginalis*. Scanning electron micrographs at early stages of erythrocyte exposure to *T. vaginalis* (parasite/target ratio, 1:5) show that erythrocytes adhering to the parasite surface undergo alteration in shape. The arrows indicate collapsed erythrocytes. Bars: A, 4 μm; B, 1 μm.

went only a light degradation of spectrin, whose high-molecular-weight fragments were still readily detectable even after extensive incubation times (Fig. 3, lanes 1 and 2). Erythrocyte membranes obtained by bacterium-mediated lysis displayed a spectrin degradation pattern similar to the one obtained following simple erythrocyte lysis in hypotonic buffer (Fig. 3, lane 3), suggesting an involvement of erythrocyte-endogenous proteinases rather than bacterial proteolytic activity. The finding that osmotic lysis and exposure to other hemolytic pathogens do not induce the dramatic disappearance of spectrin observed upon exposure of erythrocytes to *T. vaginalis* shows that endogenous erythrocyte proteinases themselves are not able to induce the spectrin loss observed upon contact with the parasite.

***T. vaginalis*-secreted proteins and cell lysates do not affect the internal membrane cytoskeleton of intact erythrocytes.** In order to test if the membrane skeleton damage was attributable to secreted effectors, washed erythrocytes were exposed to a protozoan-free supernatant obtained upon incubation of parasites for 90 min in PBS-M. After 3 h, erythrocyte membranes were electrophoresed and blotted. This experiment revealed that secreted proteins are not able to perform the cytoskeletal disruption observed upon direct contact with the parasite: the immunoblot patterns of spectrin, as well as the protein 4.1, protein 2.1, and actin ones, do not reveal any detectable difference between treated and untreated erythrocytes (data not shown). Incubation of erythrocytes with parasite sonicates led to exactly the same results obtained upon incubation with secreted proteins.

Disruption of spectrin occurs before lysis of the target cell. The finding that protozoan-free supernatants and protozoan lysates were ineffective in accomplishing cytoskeletal disruption suggested that the presence of live parasites was a prerequisite. An immunofluorescence study was performed in order to evaluate the kinetics of spectrin degradation inside erythrocytes exposed to live *T. vaginalis*. Highly motile parasites were incubated with erythrocytes in a ratio of 1:5, and the main skeletal proteins were observed at different times. The parasite/target ratio was optimized in order to ensure that upon brief incubation most of the parasites would come in contact with the erythrocytes.

Spectrin fluorescence, still readily detectable after 20 and 40 min (Fig. 4A and B), was observed to disappear within an hour (Fig. 4C), while protein 4.1, protein 2.1, and actin were detectable after the same incubation time. Figure 5 shows that fluorescence is still detectable after 60 min. Surprisingly, phase-contrast microscopy of the samples (Fig. 4C, right side)

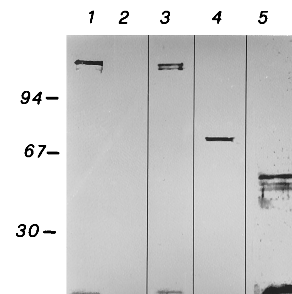

FIG. 2. Representative experiment showing the extensive degradation of spectrin upon exposure to *T. vaginalis*. Exponentially growing parasites were incubated with erythrocytes in a 1:5 ratio. Membranes from lysed erythrocytes were collected after 1 h, washed, quantified for protein content, resuspended in Laemmli buffer, and boiled. Samples were then electrophoresed in a 10% polyacrylamide gel and subjected to Western blotting; nitrocellulose membranes were probed with antibodies directed against the main erythrocyte cytoskeletal proteins. Lanes 2, 3, 4, and 5 represent the immunoblot patterns of spectrin, protein 2.1, protein 4.1, and actin, respectively, in erythrocyte membranes collected after 1 h of exposure to *T. vaginalis*. The positive control for spectrin, loaded in lane 1, was obtained by electrophoresing an equivalent amount of proteins from erythrocytes that were not exposed to the parasite. Molecular weights are shown on the left.

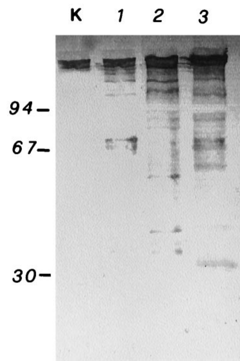

FIG. 3. Degradation pattern of spectrin obtained upon lysis of erythrocytes by hemolytic pathogens and in hypotonic medium. Erythrocyte membranes were electrophoresed in a 10% polyacrylamide gel, Western blotted, and then probed with an anti-spectrin antibody. Lane K, spectrin pattern obtained upon hypotonic lysis in the presence of proteinase inhibitors; lane 1, *E. coli*-mediated lysis; lane 2, *S. aureus*-mediated lysis; lane 3, erythrocytes lysed in hypotonic buffer without proteinase inhibitors. Molecular weights are shown on the left.

revealed that the loss of spectrin within erythrocytes in contact with the parasite occurred, although the hemoglobin content was still readily detectable, suggesting that target cell lysis was not a prerequisite for the disruption of spectrin.

In order to shed light on this phenomenon, sets of erythrocytes were allowed to interact with *T. vaginalis* for different lengths of time and unlysed erythrocytes were collected and immunoblotted. Blots were then probed with anti-spectrin antibodies (Fig. 6a). The results revealed that spectrin is degraded inside unlysed erythrocytes and that the phenomenon occurs almost immediately after contact with the parasite. In fact, unlysed erythrocytes collected after 20 min of exposure to *T. vaginalis* already display a regular cleavage of spectrin into high-molecular-weight fragments, and within 40 min the high-molecular-weight fragments are cleaved into lower-weight bands; after 60 min of erythrocyte exposure to the parasite the protein becomes undetectable, although cell lysis has not occurred yet. The same samples were then probed with anti-protein 4.1 (Fig. 6b), protein 2.1, and actin antibodies (data not shown). The results showed that degradation of these proteins does not occur until lysis has taken place.

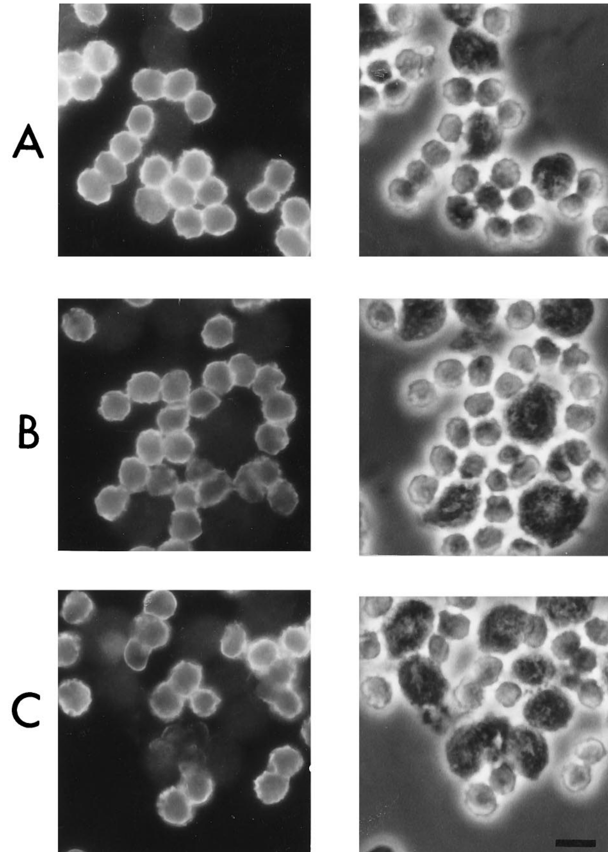

FIG. 5. Visualization by indirect immunofluorescence of the main membrane skeleton proteins inside erythrocytes exposed to *T. vaginalis* for 60 min. Indirect immunofluorescence patterns of parasitized erythrocytes treated with rabbit polyclonal antibodies directed against protein 4.1 (A), protein 2.1 (B), and actin (C) are shown on the left. Micrographs of the corresponding samples by phase-contrast microscopy are on the right. Bar, 8 μm.

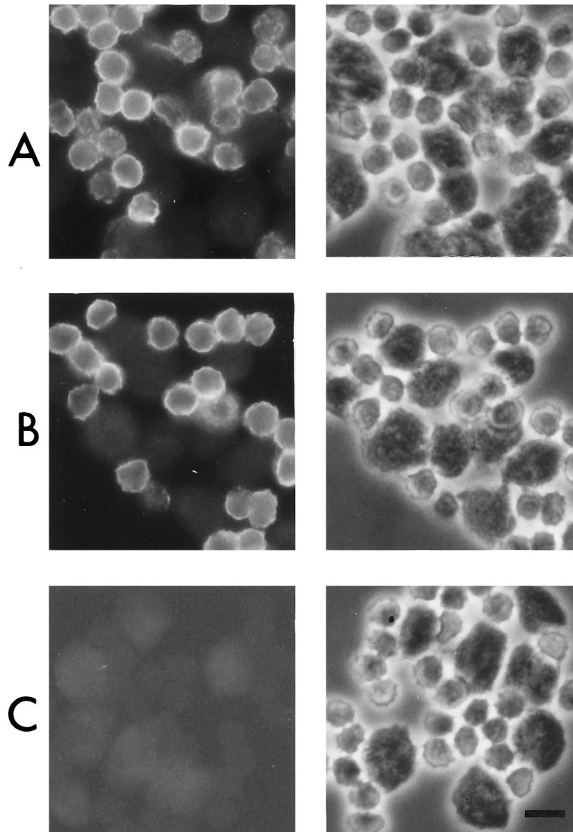

FIG. 4. Immunofluorescence patterns (left) of spectrin in erythrocytes exposed to *T. vaginalis* for 20 min (A), 40 min (B), and 60 min (C). Micrographs of the corresponding samples by phase-contrast microscopy are shown on the right. Bar, 8 μm.

Degradation of purified spectrin is accomplished in a time- and concentration-dependent fashion. Trichomonal lysates displayed a greater ability to degrade purified spectrin than did parasite supernatants. In fact, lysates derived from the lysis of 2×10^6 organisms were able to completely degrade 20 μg of purified spectrin within 1 h, while supernatants derived from the same number of organisms achieved only a slight degradation of the purified protein in the same time. Using lysates from 2×10^6, 2×10^5, and 2×10^4 *T. vaginalis* organisms, we observed a time and concentration dependency of spectrin degradation. In fact, after 1 h the lysate corresponding to 2×10^4 organisms was able to degrade only about 50% of the

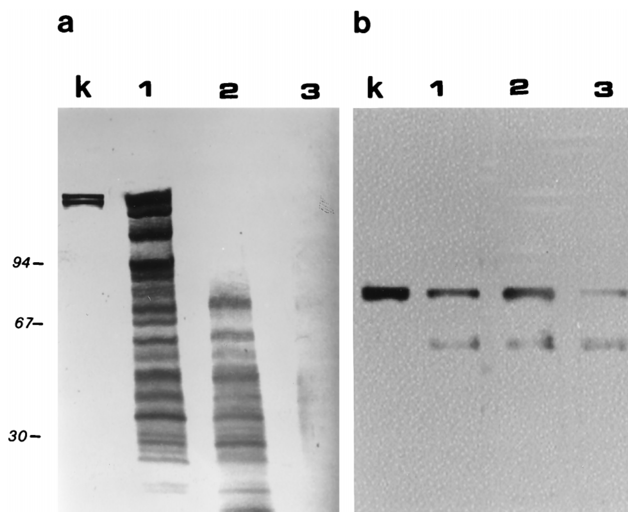

FIG. 6. Comparison of degradation modes and kinetics of spectrin (a) and protein 4.1 (b) in unlysed erythrocytes exposed to *T. vaginalis* and collected at the following times of incubation: lanes 1, 20 min; lanes 2, 40 min; and lanes 3, 1 h. Lanes K, control obtained by hypotonic lysis of erythrocytes in the presence of proteinase inhibitors. In panel a, lanes 1, 2, and 3, the sample was loaded in a 10-times-higher concentration in order to achieve a more detailed pattern of spectrin degradation fragments.

purified spectrin degraded by lysates obtained from 2×10^6 cells.

Substrate SDS-PAGE reveals the presence of a proteinase specific for spectrin. Substrate-gel electrophoresis (Fig. 7) was performed with spectrin (Fig. 7a) and the generic substrate gelatin (Fig. 7b) in order to identify and evaluate the specificity of the protozoan proteinases able to degrade spectrin. This analysis revealed that several proteinases were present which were able to degrade spectrin, both in lysates and secretions. The two patterns displayed a difference between cytoplasmic and secreted proteinases: a 30-kDa band was present in lysates which was never detected in the secreted protein samples (Fig. 7). Furthermore, the 30-kDa proteinase seems to be specific for spectrin, since it was not detectable when gelatin was used as a substrate. A strong inhibition was observed upon pretreatment with TLCK, suggesting a thiol proteinase nature (10, 39).

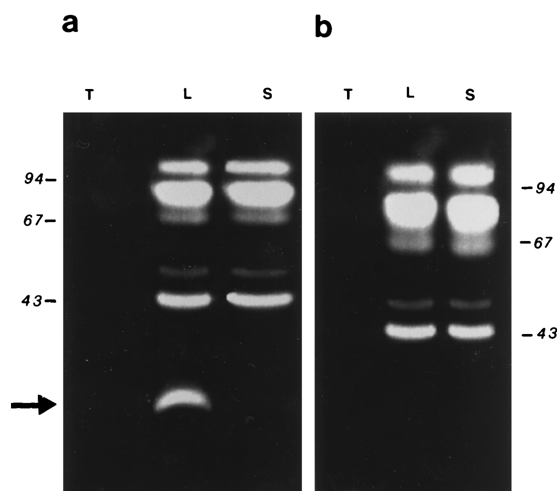

FIG. 7. Evaluation of proteinase activity by substrate SDS-PAGE with spectrin (a) and gelatin (b). Lanes: T, parasite lysate obtained in the presence of 1 mM TLCK; L, parasite lysate; S, secreted proteins. The arrow in panel a points to a 30-kDa proteinase present only in parasite lysates which was able to degrade spectrin but not gelatin. The proteinase pattern represented in the figure corresponds to that of isolate SS-22. Molecular weights are shown on the right and left.

DISCUSSION

The cytoskeleton is a target for many intracellular microorganisms. Some bacterial pathogens, such as *Yersinia*, are able to target the cytoskeleton without lysing or entering the cell, by means of secreted or directly injected proteins able to interact with the cellular cytoskeleton (42). This property is not exclusive to bacteria: the parasite *Trypanosoma cruzi*, for example, accomplishes cell invasion by triggering a rearrangement in the membrane skeleton, thus subverting cellular mechanisms and eventually leading to a combination of events which enable the parasite to enter the host cell (41). Moreover, the cytoskeleton plays a significant role in the replication of animal viruses, which interact with cytoskeletal elements inside infected cells at different stages of replication, often by means of virus-encoded proteinases, as in the case of Moloney murine leukemia virus or human immunodeficiency virus (32).

This report presents evidence showing that the host cell cytoskeleton is a target for the protozoan parasite *T. vaginalis* as well. It is noteworthy, however, that the molecular target within this structure has been identified as a component, spectrin, of the submembranal cytoskeleton. The most common targets for microbial pathogens which interact with the host cell cytoskeleton are in fact components of the cytoplasmic network, mostly actin, although the ability to target the host cell spectrin has been reported for the intracellular protozoa *Plasmodium falciparum* and *Plasmodium bergei* (12).

The degradation of spectrin within the target cell is achieved only upon exposure to live *T. vaginalis* parasites. Under these conditions, spectrin loss from the target cell is extremely fast and effective: in fact, the protein becomes undetectable within 60 min. Upon contact, spectrin degradation is achieved without lysis of the target cell as a prerequisite, as seen from immunofluorescence assay and immunoblotting performed on membranes of erythrocytes allowed to interact with the parasite and separated before lysis. Exposure of the target cell to protozoan secretions and lysates does not induce loss of spectrin within intact erythrocytes. Moreover, the ability of protozoan lysates to degrade purified spectrin has been observed to be far more effective than the degradation accomplished by secreted proteins.

The experiments performed in order to investigate the effectors involved in spectrin disruption led to the identification of an intracellular (or membrane-associated) 30-kDa proteinase able to degrade spectrin but not gelatin, suggesting a substrate specificity. The involvement of this protein in membrane skeleton disruption can be inferred. That the effectors responsible for the disappearance of spectrin could be only one or a few proteinases is in fact suggested by the degradation mode observed within unlysed erythrocytes, as shown in Fig. 5a. The molecular structure of spectrin in fact consists of repeated domains, and the ladder degradation into discrete subunits obtained within intact erythrocytes is likely the result of only one or a few cleavage sites. These findings, together with the observed high velocity of spectrin degradation and the ability of the enzyme to degrade spectrin but not a generic substrate such as gelatin, suggest a narrow specificity for this proteinase.

Several cysteine proteinases have been described in *T. vaginalis* that degrade different substrates (36, 38–40); using gelatin as a substrate, Garber and Lemchuk-Favel described 30-kDa

extracellular enzymes (19) that are produced by only a limited number of trichomonad isolates (20). In order to rule out any misinterpretation of the results, for characterization experiments we used the SS-22 *T. vaginalis* isolate, since it lacks these gelatin-degrading extracellular proteinases. We can therefore exclude an identity between the spectrin-degrading protease and the secreted 30-kDa proteases described by Garber and Lemchuk-Favel; nevertheless, we cannot rule out the possibility that the spectrin proteinase is one of the protozoan enzymes already described by others, using different substrates (39).

Since the 30-kDa spectrin proteinase appears to be nonsecreted, the proteolytic activity might be performed internally to the target cell following a release from the parasite, which may take place during the intimate contact that occurs between trichomonads and target cells. An intimate contact has been demonstrated to occur between membranes, with multiple contact focal points between parasite and host cell and numerous cytoplasmic projections interdigitating with the microvilli of the host cell plasma membrane (5, 22, 37). We observed by transmission electron microscopy that the same intimate contact occurs between *T. vaginalis* and erythrocyte membranes as well (unpublished data). Such a situation suggests that the effector delivery might take place through a membrane fusion event or by release through exocytotic microvesicles. It is also possible that a focal secretion of the spectrin protease could take place in the microenvironment between target and effector membranes, followed by the translocation of the molecule into target cells, as recently described for example for YopE of *Yersinia* (44) or for diphtheria (34) and ricin toxin (43).

Disruption of spectrin has fundamental implications for the host cell, since it arranges into a submembrane network largely responsible for maintenance of erythrocyte shape, membrane structural integrity, and reversible deformability (23). Spectrin accounts for 75% of the membrane skeleton protein mass in erythrocytes, and spectrin analogs are widely distributed among the majority of cell types (6).

We reported recently a *T. vaginalis* lytic mechanism mediated by pore-forming proteins, highly effective on erythrocytes (14, 17). Nucleate cells, though, are more resistant to osmotic lysis by perforins, and therefore an auxiliary mechanism, such as membrane skeleton disruption, might be needed to accomplish lysis. Contact-dependent cytotoxicity of *T. vaginalis* is well documented in the literature; on epithelial cell monolayers the parasite exhibits a direct cytotoxicity (3, 4, 21, 26) by inducing alterations in morphology as well as cell rounding, detachment, and cell death. The precise interrelationships between the perforin-mediated cytolytic mechanism and the proteolytic demolition of the submembrane network remain to be elucidated. The two mechanisms, though, need not be mutually exclusive; they may apply to different types of target cells as well as to the same target cells in a cooperative mechanism directed towards the accomplishment of lysis of nucleate cells, which are 6 to 100 times more resistant to osmotic lysis by perforins than are erythrocytes.

Spectrin seems to be involved in the association of intermediate filaments with the plasma membrane of epithelial cells and cultured fibroblasts (33). The proteolytic demolition of spectrin within these cells might induce a disaggregation of the membrane skeleton and of its connections with the cytoplasmic actin network, thus leading to an enhanced sensitivity of the nucleate cell to osmotic lysis mediated by the trichomonad perforins. Examples of perforin- or proteinase-mediated cytotoxicity are numerous, and such association has been observed to occur in other protozoan parasites, such as *Entamoeba histolytica* (30). In cytotoxic T lymphocytes both effectors are compartmentalized in microvesicles and released into the extracellular space formed between lymphocyte and target cell (7).

The study of microbial pathogenetic effectors is acquiring increasing importance as a tool for dissecting the molecular mechanisms of the host. An increasing number of recent findings on the molecular interactions between pathogens and their targets have provided useful insights into issues in cell biology (11). The understanding of actin behavior within the membrane skeleton, for example, has been greatly increased by the study of its interactions with several intracellular pathogens—*Salmonella* and *Shigella*, for example (1, 8, 11, 18). The *T. vaginalis* proteinase, being able to target spectrin in a specific fashion, could thus be suggested as an interesting tool for understanding the role spectrin plays in other cell types, such as within epithelial cells, in polarized membrane domains, and in lymphocyte capping, as well as its involvement in synaptic transmission among neuronal cells.

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