Brief Definitive Report

SPECIFIC ERYTHROCYTE BINDING IS AN ADDITIONAL NUTRIENT ACQUISITION SYSTEM FOR *TRICHOMONAS VAGINALIS*

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Trichomonas vaginalis is a flagellated protozoan parasite responsible for a clinically recognized sexually transmitted disease. This microorganism resides within the human urogenital tract, where it is likely that key nutrients such as lipids (1) and iron (2) are limiting. To better understand alternative or additional mechanisms for sequestration of key host nutrients from those already described (1, 2), such as lipids and iron, utilized by these parasites, we examined the nature of the interaction between *T. vaginalis* and human erythrocytes and attempted to establish the biological significance of this interaction. Data are presented that show the specific nature of erythrocyte binding, and results suggest that erythrocytes provide both lipids and iron for parasite growth and multiplication.

Materials and Methods

Growth of T. vaginalis Organisms. Isolates NYH 286 and IR 78 (1, 2) were passaged daily in a trypticase yeast extract maltose (TYM) medium supplemented with 10% heat-inactivated horse serum (1) or with purified erythrocyte lipids, as described below.

Purification and Fixation of Erythrocytes. Human RBC were purified from defibrinated blood by standard protocols (3). After washing three times in isotonic phosphate buffer (IPB; 0.11 M, pH 7.4) (3), erythrocytes were fixed by incubation at 4°C for 1 h with 2.5% glutaraldehyde in IPB (4), followed by suspension of washed fixed erythrocytes in IPB-0.1 M glycine overnight.

Isolation of Erythrocyte Lipids. Lipids from RBC were obtained by chloroform/methanol (C/M) extraction (5). The lipid suspension was concentrated to 2 ml, transferred to a 10 × 185-mm test tube, and dried under nitrogen gas before solubilization by addition of 1 ml 100% ethanol. 10 ml TYM medium without serum was then added, and ethanol was removed by heating at 60°C for 10 min.

Cell-binding Assays. The trichomonad-erythrocyte interaction was visualized by brightfield and darkfield microscopy as previously described (4). Washed organisms (2×10^6) in 1 ml PBS-0.5% maltose (PBS-M) were aliquoted into siliconized microfuge tubes containing 50 μl of a 10% suspension of erythrocytes, and the mixture was incubated at different temperatures and times.

Alternatively, erythrocytes were attached to microtiter wells coated with polylysine (6). After removal of unbound erythrocytes, wells were treated with PBS-1% BSA, followed by

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addition of 50 μl PBS-M with different numbers of [^{3}H]thymidine-labeled trichomonads (4). After washing, wells were counted for radioactivity. Controls included incubating heat-killed parasites with fixed erythrocyte monolayers, and also organisms added to polylysine-coated wells treated with PBS-1% BSA. Microscopy did not reveal any nonspecific trichomonal associations.

Ligand Assay. The ligand assay for identifying the trichomonad erythrocyte-binding proteins (EBPs) was as recently described (4). Briefly, 4×10^7 radioiodinated or ^{35}S methionine-labeled trichomonads (4) were suspended in 300 μl NaCl-EDTA-Tris-HCl (NET) buffer (4), pH 7.4, with 3 mM N-α-*p*-tosyl-L-lysine chloromethyl ketone (TLCK). Then, 30 μl of 10% SDS, 300 μl of 10% Triton X-100, and 300 μl of a 10 mg/ml OVA solution in NET buffer were sequentially added to the extract with gentle homogenization. The extract was clarified and transferred into a microfuge tube containing 4×10^9 fixed erythrocytes. After overnight incubation at 4°C, the erythrocytes were pelleted, washed four times in NET-1 mM TLCK, and boiled in 50 μl electrophoresis-dissolving buffer (4). After SDS-PAGE using 3% stacking and 10% separating acrylamide gels (4), EBPs were visualized by autoradiography or fluorography (4).

Purification of and Antisera to EBPs. EBPs were purified by electroelution from acrylamide gels (7). Four mice were each immunized subcutaneously and intraperitoneally with 20 μg of the individual proteins in CFA. Boosters were given twice more using the same amount of protein in IFA. Prebleed sera and antisera were obtained by retroorbital bleeding.

Hemoglobin (Hgl) Cell-binding Assay. Hgl (Sigma Chemical Co., St. Louis, MO) (2.5 mg) in 0.5 ml water was iodinated by addition of Iodo-Beads (Pierce Chemical Co., Rockford, IL) and 2 mCi Na^{125}I (Amersham Corp., Arlington Heights, IL). Unbound radioactivity was removed by Sephadex G25 column chromatography and dialysis. ^{125}I-Hgl stored at −70°C was rechromatographed on Sephadex G25 before use. Cell-binding assays and competition experiments were as described previously (2).

Growth Stimulation Assay. *T. vaginalis* organisms (10^5/ml) were suspended in TYM serum medium containing 0.3 mM 2,2-dipyridal, which resulted in no detectable growth of trichomonads unless 250 μM exogenous ferrous ammonium sulfate prepared as previously described (8) was added to the medium. Hgl, lactoferrin (Ltf), and transferrin (Trf) (all from Sigma Chemical Co.) were then added at 100 μg/ml. Iron saturation of Ltf and Trf was achieved as before (2).

Results and Discussion

Specificity of T. vaginalis Binding of Erythrocytes. The specificity of erythrocyte binding to live trichomonads of isolates NYH 286 and IR 78 was evident by time, temperature, and saturation binding kinetics determined by either the microscopic evaluation or immobilized erythrocyte assays (see Materials and Methods). Using the microtiter well assay, saturation occurred after 15 min at 37°C with 2.5×10^5 trichomonads at a pH range of 5.0–6.0.

Interestingly, parasites monitored microscopically revealed internalization of fixed erythrocytes after a 30-min incubation of *T. vaginalis* with these host cells (Fig. 1). Internalized erythrocytes were differentiated from bound erythrocytes by the absence of agglutination due to antibody binding by erythrocyte blood group type-specific antiserum (data not shown).

Identification of EBPs of T. vaginalis. Autoradiograms of iodinated *T. vaginalis* surface proteins that bound to fixed erythrocytes showed two EBPs of relative molecular weights of 12,500 and 27,500 (Fig. 1, *IIA*). Erythrocyte recognition of only these trichomonad proteins was indicated by the binding of two proteins from a total detergent extract containing numerous iodinated proteins, as shown earlier (4). Fig. 1, *IIB* shows the reaction of mouse anti-27,500 protein with immunoblots performed

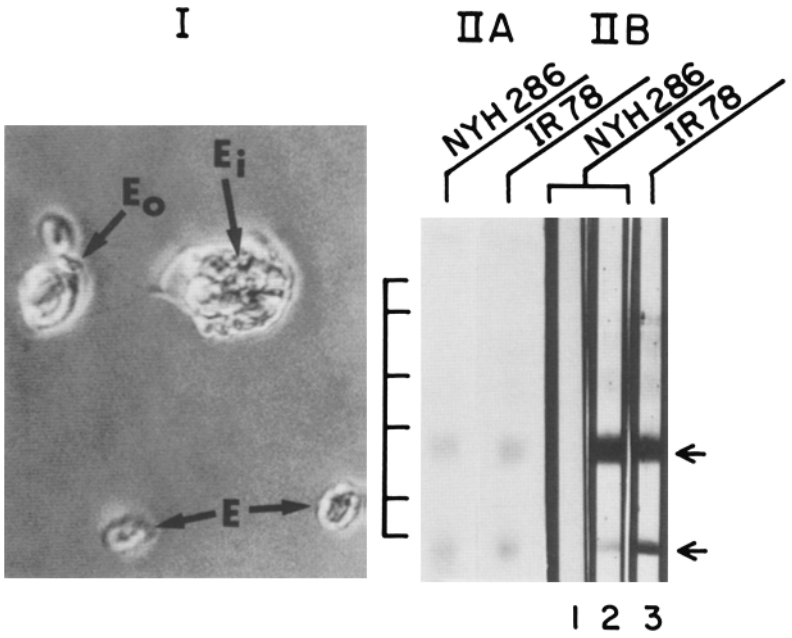

FIGURE 1. Internalization of glutaraldehyde-fixed erythrocytes by live parasites of *T. vaginalis* isolate NYH 286 (*I*) and identification of erythrocyte-binding trichomonad surface proteins (*II*). (*I*) E_o refers to adherent erythrocytes outside the organisms vs. internalized erythrocytes (E_i) and unbound erythrocytes in suspension (*E*). (*IIA*) Autoradiograms show binding to fixed erythrocytes of two trichomonad proteins (*arrows*) from a detergent extract of iodinated organisms. Molecular weight markers were from BioRad Laboratories (Richmond, CA). (*IIB*) The reaction of undiluted mouse antiserum to the 27,500 EBP of isolate NYH 286 with nitrocellulose blots of proteins from the ligand assay (see Materials and Methods) of isolates NYH 286 (lane *2*) and IR 78 (lane *3*). The reaction of prebleed mouse serum with a duplicate blot of NYH 286 EBPs is shown in lane *1*. Antiserum to the 12,500 EBP was identical by immunoblots with that shown for antiserum to the 27,500 EBP (lanes *2* and *3*).

as previously described (7), and mouse antiserum to each EBP crossreacted with the other molecular weight form.

Importantly, pretreatment of parasites with 15 μg/ml protein A-Sepharose-purified IgG of the antiserum to either of the two EBPs inhibited *T. vaginalis* attachment to erythrocytes by as much as 70% in the microtiter well adherence assay. A decreased erythrocyte binding was not observed with IgG from prebleed serum.

An explanation for the crossreactivity between the two EBPs is not known. No disulfide bond between the two molecular weight forms was detected, and no precursor-product relationships either between these adhesins or with a larger molecule were evident from pulse-chase experiments. It is conceivable that the EBPs have a common, functional, and antibody-crossreactive domain, as reported for other microbial models (7).

Also, undiluted sera from 40% (20/50) of patients gave strong immunoblot reactions of the EBPs when compared with sera from humans without a history of a

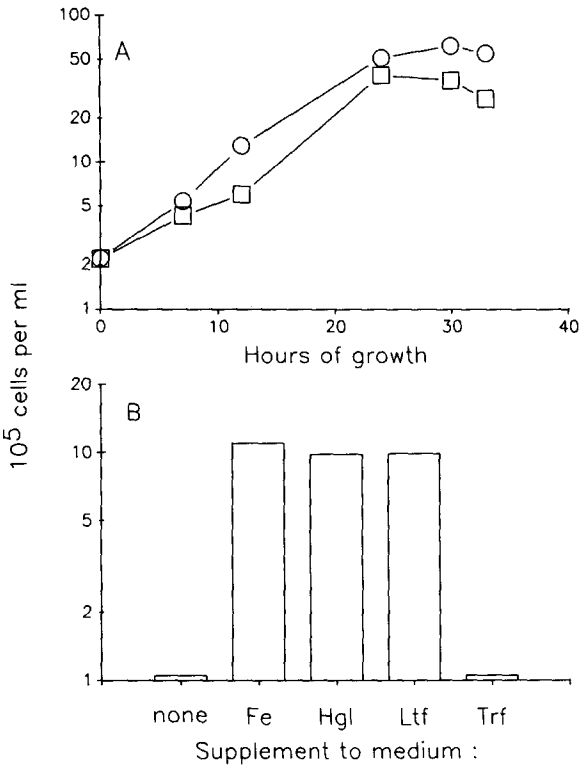

FIGURE 2. *T. vaginalis* NYH 286 growth in a serum-free medium supplemented with purified erythrocyte lipids (*A*) or in an iron-chelated medium supplemented with iron-binding proteins (*B*). (*A*) Growth kinetics for organisms grown in serum-supplemented medium (O) were compared with medium with purified erythrocyte lipids (□). Each point is the mean of three separate determinations. (*B*) Levels of growth of *T. vaginalis* NYH 286 incubated in medium in the presence of 0.3 mM 2,2-dipyridal and supplemented with exogenous ferrous ammonium sulfate (Fe), hemoglobin (Hgl), and lactoferrin (Ltf). Transferrin (Trf) is not bound by trichomonads (3) and was included as a negative control. Parasites were counted in a hemocytometer (Reichert Scientific Instruments, Buffalo, NY). Results represent the mean of triplicate samples handled identically.

sexually transmitted disease. These data show the in vivo expression of and host response to EBPs.

Erythrocytes Can Provide T. vaginalis with Essential Lipids. Purified erythrocyte lipids added to TYM medium resulted in growth kinetics similar to those seen in TYM serum medium (Fig. 2 *A*). Parasites incubated in medium with erythrocytes also achieved similar high densities, but at longer generation times, showing the ability to acquire lipids from intact erythrocytes. Confirmation of lipid acquisition under these growth conditions was obtained by showing the incorporation of radioactivity from iodinated erythrocyte lipids, as shown earlier (1). That mammalian epithelial cells are not a source of lipids for *T. vaginalis* (1) shows the highly selective mechanisms for lipid acquisition by this parasite.

Receptor Binding of Hgl is a Source of Iron for T. vaginalis. Addition of purified Hgl to the medium containing 0.3 mM 2,2-dipyridal (Fig. 2 *B*) resulted in parasite numbers like those seen by supplementation with exogenous ferrous ammonium sulfate. As expected, the addition of iron-saturated Ltf resulted in excellent growth levels, while iron-saturated Trf did not support growth (2).

The specific receptor-mediated binding of Hgl by *T. vaginalis* was then demonstrated. Fig. 3 shows saturation binding kinetics of ^{125}I-Hgl by trichomonads at 4°C, a temperature chosen to diminish uptake of the iron-binding protein (2). The bimodal pattern was reproducible in five different experiments and suggested that several receptors with different affinities or a receptor with multiple binding sites with

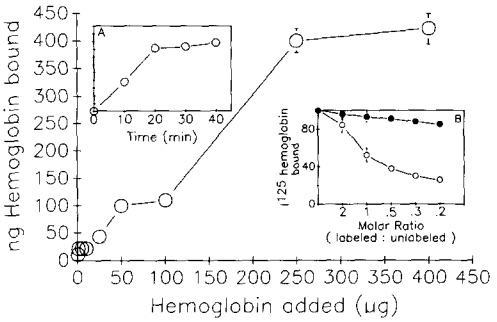

FIGURE 3. Concentration-dependent binding of ^{125}I-labeled hemoglobin to *T. vaginalis* NYH 286. The time course binding of ^{125}I-labeled hemoglobin to *T. vaginalis* NYH 286 is shown in inset *A* under conditions described in Materials and Methods. Inset *B* shows a representative competition experiment with increasing amounts of unlabeled hemoglobin (O) or lactoferrin (●). Results are of quadruplicate samples of three experiments.

different affinities for the ligand might be present. Importantly, Fig. 3 *B* illustrates that unlabeled Hgl competed with the iodinated ligand, whereas Ltf was ineffective in competing. The time-course and steady-state binding of iodinated Hgl to trichomonads at 4°C is also shown (Fig. 3 *A*).

T. vaginalis appears to be capable of utilizing hemoglobin as a source of iron (Fig. 2 *B*). Since free Hgl is readily bound by the serum protein haptoglobin and is not available in vivo (9), it is possible that the hemolysis of erythrocytes (10) results in immediate binding of Hgl by the trichomonad receptors (Fig. 3), thus preventing Hgl from being sequestered by host macromolecules. It has been shown that different microorganisms obtain heme iron from some but not all species of haptoglobin with bound Hgl (11). It is interesting to speculate that exascerbation of trichomoniasis among some patients during menstruation may be related to an ability of *T. vaginalis* to obtain iron from the haptoglobin species unique to those patients. Although unknown, the extraction of iron from the tetrapyrrole ring of heme by enzymatic reactions or the substitution of heme as electron carriers in trichomonal metabolism underscores the unique adaptation of this parasite to the vaginal-host environment.

Summary

Specific receptor-mediated binding by *Trichomonas vaginalis* of human erythrocytes was demonstrated. The ability of live parasites to internalize erythrocytes was also documented. In vitro growth assays during lipid-free and iron-limiting conditions that do not support the survival of *T. vaginalis* organisms showed that purified erythrocyte lipids and hemoglobin were each able to provide lipids and/or hemoglobin iron for trichomonal growth and multiplication. Parasites bound hemoglobin in a highly specific receptor-mediated fashion, and only the homologous unlabeled hemoglobin, but not lactoferrin and transferrin, competed with iodinated hemoglobin binding. Two antibody-crossreactive surface proteins of the parasites were identified as adhesins, and antibody to the individual adhesins inhibited *T. vaginalis* recognition and binding of erythrocytes. Finally, patient sera possessed antibody to the adhesins, showing the immunogenic nature and in vivo relevance of the trichomonad proteins during infection.

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References

1. Peterson, K. M., and J. F. Alderete. 1984. *Trichomonas vaginalis* is dependent on uptake and degradation of human low density lipoproteins. *J. Exp. Med.* 160:1261.
2. Peterson, K. M., and J. F. Alderete. 1984. Iron uptake and increased intracellular enzyme activity follow lactoferrin binding by *Trichomonas vaginalis* receptors. *J. Exp. Med.* 160:398.
3. Dodge, J. T., L. Mitchell, and D. J. Hanahan. 1963. The preparation and chemical characteristics of hemoglobin-free ghosts of human erythrocytes. *Arch. Biochem. Biophys.* 100:119.
4. Alderete, J. F., and G. E. Garza. 1988. Identification and properties of *Trichomonas vaginalis* proteins involved in cytadherence. *Infect. Immun.* 56:28.
5. Nelson, G. J. 1967. Lipid composition of erythrocytes in various mammalian species. *Biochim. Biophys. Acta.* 144:221.
6. Fisher, K. A. 1975. "Half" membrane enrichment: verification by electron microscopy. *Science (Wash. DC).* 190:983.
7. Thomas, D. D., J. B. Baseman, and J. F. Alderete. 1985. Putative *Treponema pallidum* cytadhesins share a common functional domain. *Infect. Immun.* 49:833.
8. Gorrell, T. E. 1985. Effect of culture medium iron content on the biochemical composition and metabolism of *Trichomonas vaginalis*. *J. Bacteriol.* 161:1228.
9. Javid, J. 1978. Human haptoglobin. *Curr. Top. Hematol.* 1:151.
10. Krieger, J. N., M. A. Poisson, and M. F. Rein. 1983. Beta-hemolytic activity of *Trichomonas vaginalis* correlates with virulence. *Infect. Immun.* 41:1291.
11. Helms, S. D., J. D. Oliver, and J. C. Travis. 1984. Role of heme compounds and haptoglobin in *Vibrio vulnificus* pathogenicity. *Infect. Immun.* 45:345.