

TRICHOMONAS VAGINALIS IS DEPENDENT ON UPTAKE AND DEGRADATION OF HUMAN LOW DENSITY LIPOPROTEINS

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Trichomonas vaginalis is the microbial agent responsible for trichomonal vaginitis, a major sexually transmitted disease throughout the world (1-4). The complex events involved in trichomonad parasitism of host cells are poorly understood. We have focused on the ability of *T. vaginalis* to selectively acquire host-derived macromolecules in order to better define host-trichomonad interactions (5-8). Trichomonad surface receptors are responsible for the selective and specific acquisition of host proteins, and the biologic properties conferred upon *T. vaginalis* by these host proteins appear to contribute to the parasitic attributes of these microorganisms (6-8). For example, trichomonads display proteolytic inhibitory properties after α_1 -antitrypsin binding (6). Also, iron uptake and subsequent enhancement of important metabolic pathways follow *T. vaginalis* acquisition of human lactoferrin (8).

Another class of host molecules essential for trichomonal growth and multiplication are lipids (7, 9). These host macromolecules are used by *T. vaginalis* presumably for membrane maintenance and synthesis. In a recent study, lipoproteins replaced plasma as a growth supplement (7). This report characterizes further the interaction of human low density lipoproteins with *T. vaginalis*. These data show the presence of specific receptors on *T. vaginalis* surfaces for lipoprotein binding and uptake. We believe these parasite-host protein interactions are important factors that may promote infection and disease pathogenesis, and we discuss the biological implications of our work.

Materials and Methods

Growth of Organisms. *T. vaginalis* strain NYH 286 was passaged daily in Diamond's trypticase yeast extract-maltose (TYM)¹ medium (10) supplemented with 10% normal human plasma. Other strains used in this study included NYH 272, ATCC 30001, 30236, RU 375, IR 78, JHHR, and JHHW (8), and these isolates exhibited similar growth kinetics. Only late log stage parasites were used in these studies (5). *Trichomonas tenax* (ATCC 30207) was passaged every 48 h and was grown at 36°C in trypticase, panmead soy broth (North American Biologicals, Miami, FL) supplemented with vitamins (North

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¹Abbreviations used in this paper: EDTA, ethylenediaminetetraacetic acid; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; PMSF, phenylmethylsulfonyl fluoride; SDS, sodium dodecyl sulfate; TCA, trichloroacetic acid; TYM, trypticase-yeast extract-maltose; Z(3-12), Zwittergent 3-12.

American Biologicals), and 10% normal human plasma. For certain experiments, low density lipoproteins at levels equivalent to those found in plasma were added to TYM medium (7). A semi-defined medium consisting of trypticase (20 g/l), maltose (5 g/l), nucleic acid precursors (9), vitamins (9), iron (8), KH_2PO_4 (0.48 g/l), K_2HPO_4 (0.48 g/l), cysteine (1.0 g/l), ascorbic acid (0.2 g/l), and low density lipoproteins (7), pH 6.2, was also used where indicated for cultivation of *T. vaginalis*.

Low Density Lipoprotein Preparation. Human low density lipoproteins ($d = 1.006$ – 1.063 g/ml) were prepared from pooled normal human plasma in 0.1% (wt/vol) EDTA. Very low density lipoproteins were first removed by ultracentrifugation (11) in a Beckman 60 Ti rotor (50,000 rpm) at 10–15°C for 24 h. Following removal of the very low density lipoproteins, the remaining plasma was adjusted with solid KBr to a density of 1.063 g/ml and subjected to centrifugation for an additional 24 h. The low density lipoprotein was removed and resuspended in a 1.063 g/ml KBr solution and centrifuged again. Finally, the low density lipoproteins were removed and dialyzed against four changes of 4 liters PBS (137 mM NaCl, 2.7 mM KCl, 4.6 mM Na_2PO_4 , and 1.5 mM KH_2PO_4), pH 7.4. For growth studies, low density lipoproteins were adjusted to the original starting plasma volume.

Radioiodination of Low Density Lipoproteins. Low density lipoproteins were radioiodinated using an oxidative iodine monochloride technique recently described (12). Briefly, an equal volume of 2 M glycine-NaOH buffer, pH 10, was added to the low density lipoprotein solution (10 mg/ml). 2 mCi of Na^{125}I (Amersham Corp., Arlington Heights, IL) was added followed by 300 μl of a solution of 100 mM NaI and 10 mM NaIO_3 in 7 N HCl diluted 1:100 in 2 M NaCl. The reaction was allowed to proceed for 1 min and then placed on a Sephadex G-25 column (1 \times 10 cm) equilibrated in PBS containing 0.01% (wt/vol) EDTA. Efficiency of radioiodination was determined by trichloroacetic acid (TCA) precipitation (8). The lipid-associated radioactivity was determined by chloroform/methanol (2:1, vol/vol) extraction (13) and represented ~9% of incorporated radioactivity.

Cell Binding Assays. Live, motile trichomonads washed twice in PBS were resuspended to a density of 1×10^8 cells/ml. 100- μl aliquots were then added to 1.5-ml conical polypropylene microfuge tubes pretreated with 1% horse serum (8). Various amounts of radioiodinated lipoproteins were added to the parasites for a 500 μl final volume. Incubation was carried out at both 4°C and 37°C for various times with occasional gentle shaking. Competition experiments were performed similarly with unlabeled proteins added to the parasites for 10 min before addition of ^{125}I -low density lipoproteins. Trichomonads were separated from unbound material by gentle centrifugation (8) followed by three washings in ice-cold PBS. Finally, pelleted organisms were resuspended and transferred to another tube for determination of avidly bound cpm.

***T. vaginalis* Degradation of Apoproteins and Accumulation of Lipids.** Trichomonads were incubated with ^{125}I -low density lipoproteins for 30 min at 4°C followed by three washes in PBS. The parasites (1×10^6) were resuspended in 500 μl PBS and incubated at 37°C for various lengths of time. The organisms were then pelleted, the supernatant removed, and the cells resuspended in 500 μl PBS. Apoprotein degradation was determined by TCA precipitation and chloroform extraction (14) of the parasites and the supernatant after the indicated time points. Briefly, 70 μl of 100% TCA was added to 500 μl of parasites or 500 μl supernatant to achieve a 12% final TCA concentration. After a 15-min incubation at 37°C, the mixtures were centrifuged at 10,000 rpm in a microcentrifuge for 2 min. 5 μl 40% KI and 25 μl 30% H_2O_2 was then added to supernatants. After a 5-min incubation at room temperature, 800 μl of chloroform was added and shaken for 5 min. This mixture was then centrifuged, and the radioactivity in the aqueous and chloroform phase was determined. Trichomonads handled similarly at 4°C were used as controls.

Affinity Purification of *T. vaginalis* Lipoprotein Receptor. A total of 5×10^9 organisms (~400 mg) radiolabeled with [^{35}S]methionine (Amersham), [^3H]amino acids (Amersham), or Na^{125}I as before (8) were resuspended in 10 ml PBS containing 1% (wt/vol) Zwittergent 3-12 (Calbiochem-Behring Corp., La Jolla, CA) detergent with 0.01% (wt/vol) PMSF

(Sigma Chemical Co., St. Louis, MO). Parasites were solubilized by homogenization (15) and the insoluble material removed by centrifugation at 40,000 g in a Sorvall RC-5B refrigerated superspeed centrifuge for 60 min. The soluble extract was then diluted in PBS-0.01% PMSF to give a final Z3-12 concentration of 0.001%. Chromatography was performed on a 1 × 2 cm column of low density lipoprotein-Sephrose prepared as before (8) using CNBr-activated Sepharose (Sigma). After extensive washing with PBS-0.01% PMSF, the adherent material was eluted with a 2% SDS-0.01% β ME solution.

SDS-PAGE. One-dimensional SDS-PAGE was performed according to Laemmli (16), using 1.5-mm slab gels consisting of a 3% stacking and 7.5% separating gel (5-8). Following electrophoresis, the gels were fixed and dried for exposure to x ray film or processed for fluorography (5). Molecular weight standards were from BioRad Labs (Richmond, CA).

HeLa Cell Membrane Preparations. HeLa cells were disrupted by placing 1×10^9 cells in 10 ml of deionized water followed by 20 strokes in a tissue homogenizer. Nuclei were separated by centrifugation at 1,500 rpm in a Sorvall RT6000 tabletop centrifuge. Crude membranes were then pelleted by centrifugation at 42,000 g. The membranes were resuspended in 10 ml of PBS and pelleted once more. Finally, the membrane pellet was resuspended in PBS at a concentration of 200 mg/ml. Between 0.1 and 3.0 ml of this preparation was then added to 6 ml TYM medium for growth studies.

Results

Time Course and Temperature-dependent Low Density Lipoprotein Binding. The time course binding of 125 I-labeled human low density lipoproteins to *T. vaginalis* at both 4°C and 37°C is shown in Fig. 1. At 4°C an apparent equilibrium between association and dissociation was reached after 30 min. At 37°C, the

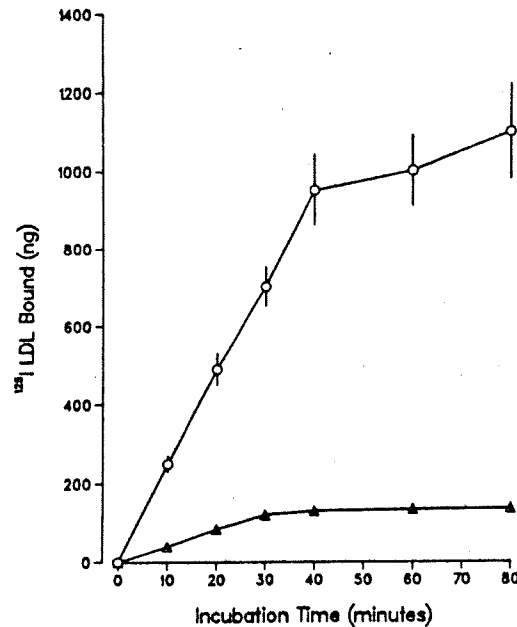


FIGURE 1. Representative time course binding of 125 I-labeled human low density lipoproteins by *T. vaginalis*. Organisms were suspended at a density of 1×10^7 parasites in a 0.5 ml volume and incubated with 150 μ g of 125 I-low density lipoproteins at 4°C (Δ) or 37°C (O). The amount of cell-associated radioactivity was determined as described in Materials and Methods. Individual points are the mean of triplicate determinations from a single experiment, and standard deviations for time points at 4°C never exceeded 1% of the mean.

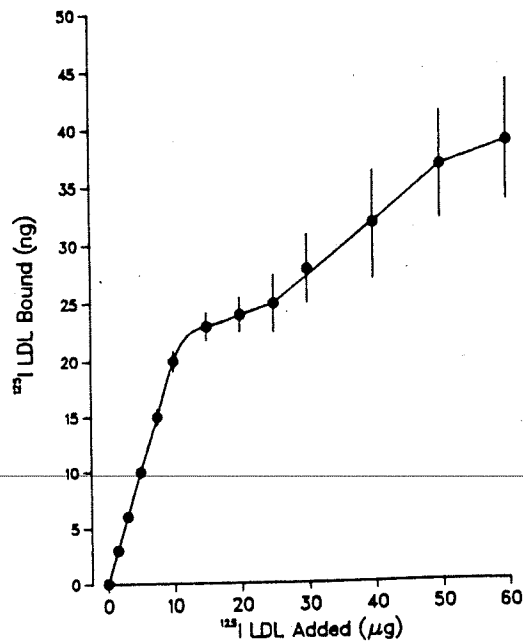


FIGURE 2. Concentration-dependent binding of ^{125}I -low density lipoproteins (LDL) by *T. vaginalis*. Cells were suspended at a density of 1×10^7 organisms in 0.5 ml PBS and incubated with varying concentrations of ligand for 40 min at 4°C . Parasite-bound radioactivity was determined as described in Materials and Methods. Each data point represents the mean value of duplicate samples in three separate experiments.

cell-associated radioactivity increased rapidly for 40 min and then increased gradually for the remaining 40 min. Consistent with a recent report by us (7), trypsin treatment after incubation with iodinated low density lipoproteins failed to remove cell-associated radioactivity from parasites handled at 37°C , suggesting that these parasites are internalizing surface-bound low density lipoproteins.

Concentration-dependent Acquisition of ^{125}I -Low Density Lipoproteins. As is illustrated in Fig. 2, addition of increasing concentrations of radioiodinated low density lipoproteins to 1×10^7 organisms at 4°C resulted in an initial stoichiometric increase in the amount of surface-bound low density lipoproteins, followed by a leveling off, indicating a possible saturation of high-affinity trichomonad binding sites. At high low-density lipoprotein concentrations, a nonsaturable low-affinity binding component was observed and may represent nonspecific lipid-lipid interactions at these elevated concentrations between ligands and trichomonads.

*Apoprotein Degradation and Lipid Accumulation by *T. vaginalis*.* The release of TCA-soluble radioactivity in supernatant by trichomonads during a representative pulse-chase experiment is shown in Fig. 3. The degradation of apoproteins and subsequent release of TCA-soluble radioactivity into the supernatant corresponded to a reduction in parasite-associated radioactivity. The amount of lipid-associated radioactivity in the chase supernatant was negligible throughout the experiment and accounted for no more than 1% of the total cpm bound by *T.*

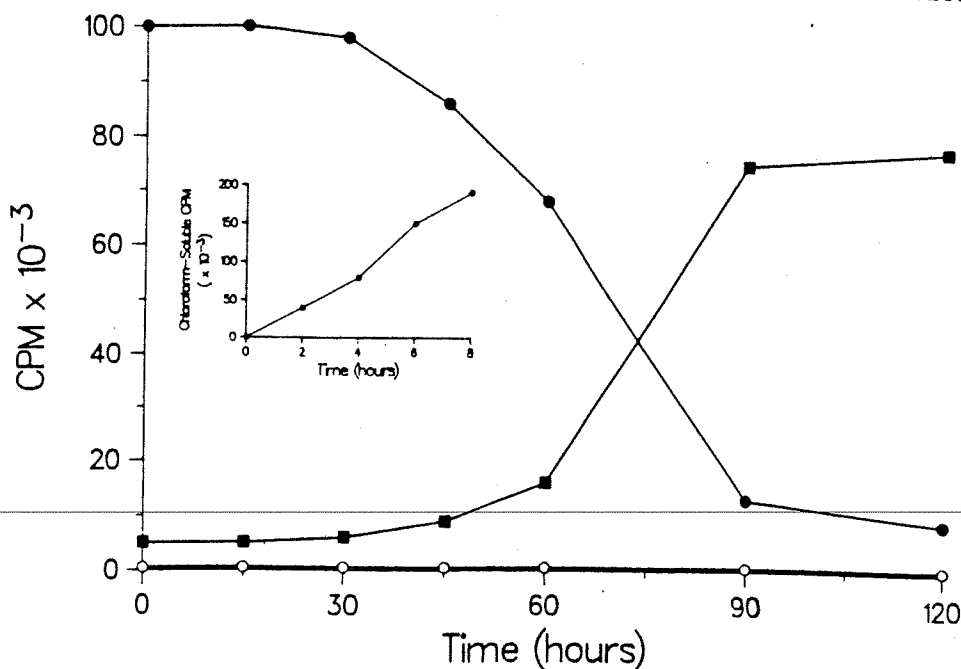


FIGURE 3. Representative time course degradation of apoproteins and accumulation of lipids by 1×10^8 *T. vaginalis* incubated with $500 \mu\text{g}$ radioiodinated low density lipoproteins as described in Materials and Methods. Protein-bound radioactivity was detected by TCA precipitation and chloroform methanol extraction of trichomonads (●) and supernatants (■) from duplicate samples at various time points. Lipid-associated radioactivity in the supernatant from the same experiment (○) was also compared with long-term accumulation of iodinated lipids by *T. vaginalis* organisms (●, inset) at 37°C .

vaginalis. The data indicate that trichomonads rapidly degrade the apoprotein components of low density lipoproteins.

Evidence for parasite accumulation of lipids was obtained by incubating trichomonads with ^{125}I -low density lipoproteins in culture medium for extended periods of time. The insert to Fig. 3 shows an increase in lipid-associated, chloroform-soluble radioactivity at the indicated time points. After extended incubation, 50% of the label in these cells was in the lipid fraction. These data showing lipid accumulation along with apoprotein degradation reinforce the importance of low density lipoprotein uptake for maintenance of parasite membrane integrity.

Trichomonad Low Density Lipoprotein Receptor Specificity. Earlier observations indicated that trichomonads are also able to interact with high density lipoproteins and very low density lipoproteins (7). Competitive binding experiments were performed using unlabeled lipoprotein subfractions (11) with iodinated low density lipoproteins in order to determine whether these three lipoprotein subfractions possess a common ligand recognized by a parasite receptor. As can be seen in Fig. 4, high density, low density, and very low density lipoproteins effectively compete with ^{125}I -low density lipoprotein binding to *T. vaginalis* surfaces. Albumin, another lipid-carrying plasma constituent, was una-

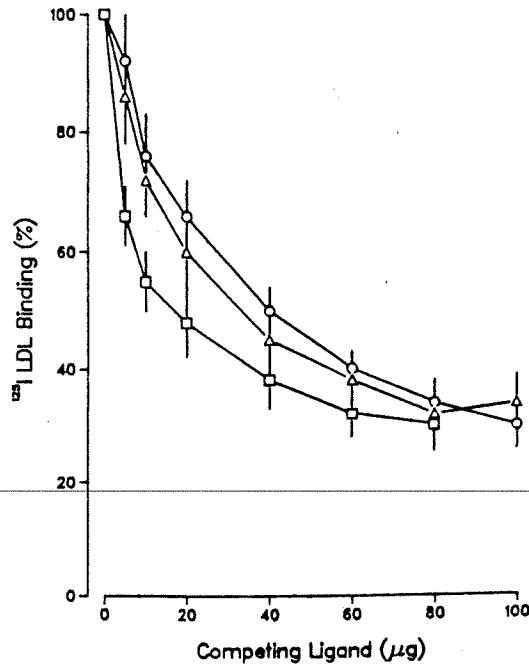


FIGURE 4. Competition of ^{125}I -low density lipoprotein acquisition by *T. vaginalis* with unlabeled lipoprotein subfractions. A 0.5 ml suspension of 1×10^7 trichomonads was incubated for 10 min at 4°C with increasing amounts of unlabeled high density lipoproteins (O), low density lipoproteins (Δ), and very low density lipoproteins (\square) before addition of $25 \mu\text{g}$ of ^{125}I -low density lipoproteins and incubation for another 30 min at 4°C . The amount of ^{125}I -low density lipoproteins bound to trichomonads in the absence or presence of competing ligands was determined as described in Materials and Methods. Each data point represents the mean value of duplicate samples from two separate experiments.

ble to prevent iodinated low density lipoprotein acquisition by trichomonads (data not shown). These results indicate that a component common to all three lipoprotein fractions is involved in lipoprotein binding and uptake.

Additional competition studies were performed using unlabeled apoproteins in an attempt to determine the apoprotein which mediates lipoprotein binding to intact organisms. Fig. 5 shows the effective competition for trichomonal binding of iodinated low density lipoproteins by an aqueous preparation of a chloroform-methanol extraction of lipoproteins. This aqueous extract consisted of a mixture of apolipoproteins (data not shown); as can be seen in Fig. 5, apolipoprotein CIII, a protein common to all lipoprotein subfractions, effectively competed for the acquisition of low density lipoproteins by *T. vaginalis*. In contrast, apolipoprotein A1, a protein found only in high density lipoproteins, was unable to compete for ^{125}I -low density lipoprotein binding.

Affinity Purification of Trichomonad Low Density Lipoprotein Receptors. Identification of the parasite membrane components responsible for low density lipoprotein binding was attempted using affinity chromatography. As can be seen in Fig. 6 (lanes 1B, 2B, and 3B), trichomonad proteins with molecular weights $>250,000$ were found specifically adsorbed to the lipoprotein affinity matrix. These same trichomonad proteins did not bind to albumin-Sepharose (data not

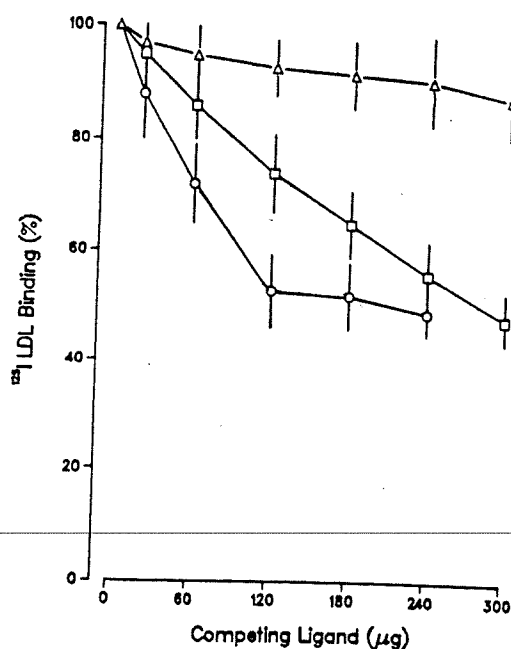


FIGURE 5. Apolipoprotein inhibition of *T. vaginalis* binding of ¹²⁵I-low density lipoproteins. Competitive inhibition of ¹²⁵I-low density lipoproteins binding to *T. vaginalis* as described in the legend to Fig. 4 was performed using a protein extract of total plasma lipoproteins (□) and apolipoprotein CIII (○). Lack of inhibition of ¹²⁵I-low density lipoprotein acquisition was demonstrated using apolipoprotein A₁ (Δ). Avidly bound radioactivity was determined as described in Materials and Methods. Each data point represents the mean value of duplicate samples from a representative experiment.

shown). Radiolabeled proteins in the detergent extract are illustrated in lanes 1A, 2A, and 3A. The ability to purify identical intrinsically or extrinsically labeled proteins (lanes 1B, 2B, and 3B) reinforces the specific interactions between receptor and lipoprotein ligands.

T. vaginalis Growth in a Semi-defined Medium. As can be seen in Fig. 7, a semi-defined medium providing a source of amino acids (trypticase), vitamins, nucleic acid precursors, and low density lipoproteins promoted 90% of the levels of trichomonal growth and multiplication compared with serum-supplemented medium. This medium has been used to maintain cultures for extended periods and to successfully cultivate fresh vaginal isolates of *T. vaginalis*.

Because of the specific mechanism for procurement of essential lipids via lipoprotein uptake and processing, we investigated the use of host cell membranes as a source of lipids for *T. vaginalis*. Trichomonads were inoculated into tubes containing TYM-medium supplemented with different amounts of HeLa cell membrane preparations. No growth of *T. vaginalis* was observed after numerous attempts. Furthermore, no viable parasites were detected by the third passage. These results indicate a strict requirement for host lipoproteins by pathogenic human trichomonads.

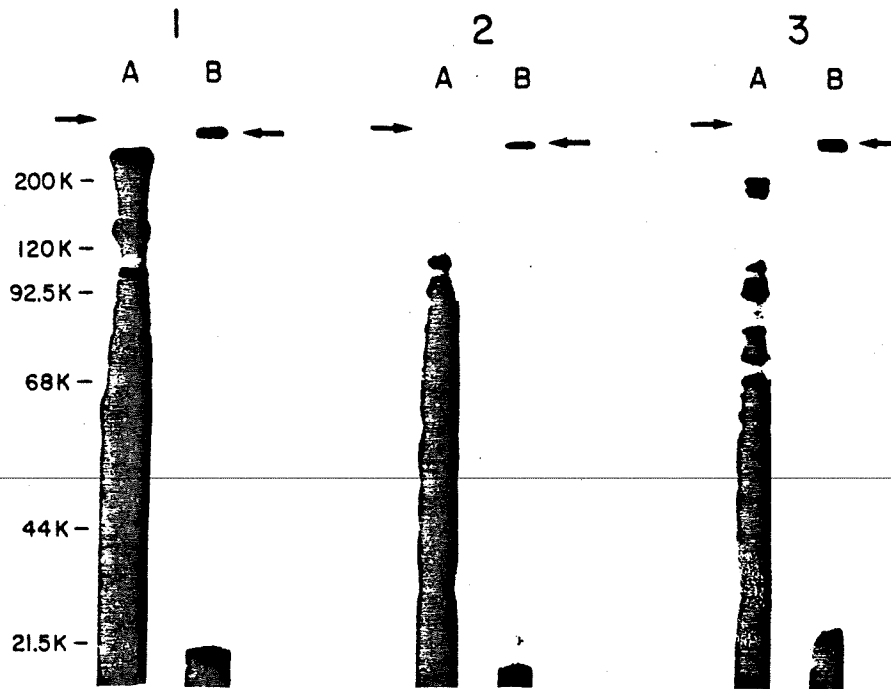


FIGURE 6. SDS-PAGE-radioautography of radiolabeled *T. vaginalis* proteins purified by low density lipoprotein-Sepharose chromatography. Radiiodinated (1), [35 S]methionine (2), and [3 H]amino acid-labeled (3) parasite proteins selectively bound to the lipoprotein column are shown in lane B (arrow). The patterns in lane A show the respective profiles of radiolabeled *T. vaginalis* proteins in Z3-12 detergent extracts, and arrows on the left of lane A denote the top of the acrylamide gel. Molecular weight markers: myosin, 200,000 (200 K); beta-galactosidase, 120 K; phosphorylase B, 92.5 K; bovine serum albumin, 68 K; ovalbumin, 44 K; soybean trypsin inhibitor, 21.5 K.

Discussion

The lipid metabolism of *Trichomonas vaginalis* is poorly understood. Trichomonads may be incapable of forming fatty acyl groups or sterols *de novo* and may not convert or retroconvert certain long chain fatty acids or cholesterol (17, 18). Other studies using a defined medium demonstrated the importance of protein carriers for effective uptake of cholesterol and fatty acids by trichomonads (9). The ability of plasma lipoproteins to supplement complex media and the inability of lipoprotein-deficient plasma to support active parasite growth suggests that plasma lipoproteins are an essential source of lipid material for these parasites (7). We, therefore, felt it was important to examine lipoprotein-*T. vaginalis* interactions to better understand the contribution of host lipid-carrying material to the metabolism and growth of these parasites.

T. vaginalis appears to possess receptors for human low density lipoproteins. These receptors also have specificities for human high density lipoproteins and very low density lipoproteins consistent with the presence of common apolipoprotein components in these lipid subfractions. The rate of association of low density lipoproteins with these parasites at both 4°C and 37°C also indicates that receptors are responsible for ligand (lipoprotein) binding. The nonsaturable

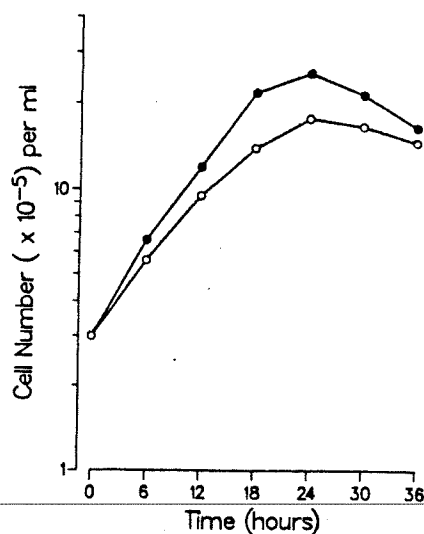


FIGURE 7. Representative growth characteristics of *T. vaginalis* NYH 286 in a semi-defined medium of amino acids, vitamins, and nucleic acids supplemented with normal human plasma (●) or low density lipoproteins (○). Details of medium composition are described in Materials and Methods.

binding component at high concentrations of lipoproteins suggests that lipid-lipid interactions between the lipoprotein particle and trichomonad membranes contribute to nonspecific lipoprotein binding. The ability of purified apolipoprotein CIII to compete for low density lipoprotein acquisition implicates this apoprotein component as a mediator of receptor recognition. The inability to achieve a >50% competition with apolipoprotein CIII ligand is consistent with the idea that lipid-lipid hydrophobic associations can contribute to low density lipoprotein binding. Chromatography of an extract of intrinsically or extrinsically labeled *T. vaginalis* on a low density lipoprotein-Sepharose affinity matrix resulted in the purification of a high molecular weight trichomonad protein, implicating this polypeptide as the putative lipoprotein receptor.

Processing of lipoproteins by *T. vaginalis* is very similar to mammalian cell binding, uptake, and degradation of host lipoproteins (19-22). Our data also show trichomonad-mediated acquisition of lipoprotein via apoprotein components. In contrast to human fibroblasts that possess a receptor specific for apolipoprotein B (21, 22), the trichomonad low density lipoprotein receptor appears to recognize apolipoprotein CIII, a protein common to the three subclasses of lipoproteins studied. Previous data (7) demonstrated that very low density lipoproteins are internalized by *T. vaginalis*, but do not allow for parasite growth and multiplication. This lack of growth may be due to a diminished amount or absence of certain lipids in this subfraction (23). Thus, studies using lipoprotein subfractions with defined components (23) may be useful for studying the lipid requirements of this parasite.

Our data suggest that the binding and uptake of low density lipoproteins by *T. vaginalis* allows for these parasites to assemble new membranes (Figs. 3 and 7). The apparent inability of extracts of HeLa cell membranes to support *T.*

vaginalis growth in a complex medium is noteworthy. These data suggest an *in vivo* requirement by *T. vaginalis* for key lipids supplied predominantly by host lipoproteins. It is possible that mammalian cell alteration of important lipids prevents trichomonad utilization for successful membrane biosynthesis. Alternatively, the lack of receptor-recognizable protein ligands on host cells may prevent efficient uptake and processing of lipid-containing components such as cell membranes.

Information on the effect of *T. vaginalis* alteration of the lipid environment of the host may also be important. Uptake by this parasite of large amounts of lipoproteins, molecules that affect host membrane composition and cellular responsiveness (24), may lead to host cytopathology. We have observed no differences in the ability of numerous isolates of trichomonads (8), including *Trichomonas tenax*—an avirulent organism of the normal flora of the oral cavity of man (2), to bind and internalize purified low density lipoproteins. While these data do not distinguish between virulent and avirulent trichomonads, they do indicate the important role of low density lipoprotein uptake mechanisms for essential maintenance of parasite membranes.

The ability of low density lipoproteins to supplement a semi-defined medium makes it possible now to grow *T. vaginalis* in a more simplified serum-free medium. Since trypticase represents a mixture of amino acids (25) from acid hydrolysis of casein, it also seems possible now to determine precise amino acid requirements, if any, which may enhance growth or promote other aspects of *T. vaginalis* virulence. Finally, *T. vaginalis* parasites grown in a serum-free medium may facilitate biochemical (26) analysis of the parasite, providing insight into unique metabolic pathways or enzymes for pharmacological (27) drug targeting.

Summary

Human plasma low density lipoprotein uptake by the urogenital pathogen, *Trichomonas vaginalis*, was examined. Rapid binding and internalization of ¹²⁵I-labeled low density lipoproteins by live *T. vaginalis* was observed at 37°C. Data showing parasite degradation of the internalized apoproteins and lipid accumulation following low density lipoprotein uptake was obtained. Acquisition of low density lipoproteins was by a trichomonad surface protein that possessed a molecular weight of >250,000. The receptor is specific for apolipoprotein CIII, a component of high, low, and very low density lipoprotein subfractions. Low density lipoproteins in a semi-defined medium of trypticase, nucleic acid precursors, vitamins, and maltose promoted *T. vaginalis* growth and multiplication at rates and levels equal to the yeast extract-trypticase-serum complex medium routinely used for culture of trichomonads. HeLa cell membranes as a source of lipids were unable to sustain *T. vaginalis* organisms. These data demonstrate host lipoprotein internalization by *T. vaginalis* via a specific uptake mechanism.

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