Molecular and Biochemical Parasitology, 12 (1984) 37–48
Elsevier

MBP 00438

SELECTIVE ACQUISITION OF PLASMA PROTEINS BY *TRICHOMONAS VAGINALIS* AND HUMAN LIPOPROTEINS AS A GROWTH REQUIREMENT FOR THIS SPECIES

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(Received 11 October 1983; accepted 28 December 1983)

Trichomonas vaginalis avidly bound numerous host macromolecules which were not removed by repeated washing in phosphate buffered saline. The use of radioiodinated Cohn plasma fractions in binding studies allowed the identification of plasminogen, fibrinogen, immunoglobulin G, lipoproteins A and B, transferrin, α_1-antitrypsin, and albumin on intact organisms. The binding of immunoglobulin G, albumin, transferrin, and lipoproteins to intact, motile trichomonads was further demonstrated using ^{125}I-labeled plasma that was chromatographically depleted of these proteins. Kinetic studies indicated that ^{125}I-labeled lipoproteins bind to *T. vaginalis* in a receptor-ligand-like manner. The surface localization and uptake of bound lipoproteins was shown by treatment of intact organisms with pronase at various times after incubation with lipoproteins. Purified lipoproteins could be substituted for plasma or serum as a growth supplement in a complex medium of trypticase/yeast extract/maltose and supported growth and multiplication rates equal to those in the same medium with plasma.

Key words: *Trichomonas vaginalis*; Host proteins; Cohn plasma fractions; Lipoproteins; Receptors

INTRODUCTION

While an extensive literature exists on various aspects of the pathogenic properties of *Trichomonas vaginalis*, no precise virulence factors have been identified [1–3]. One area of research in our laboratory has focused on the chemical and molecular interaction between *T. vaginalis* and avidly bound host proteins [4,5]. The ability of pathogenic trichomonads to selectively coat themselves with host materials may have relevance to this disease. For example, the acquisition of specific host molecules such

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Abbreviations: TYM, trypticase/yeast extract/maltose; SDS-PAGE, sodium dodecylsulfate-polyacrylamide gel electrophoresis; IgG, immunoglobulin G; PBS, phosphate-buffered saline; TCA, trichloroacetic acid; NHP, normal human plasma.

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as α_1-antitrypsin [4,5] or α_2-macroglobulin [5] may be properties which allow pathogenic trichomonads to circumvent nonspecific defense mechanisms through neutralization of host proteases.

In preliminary studies, several plasma proteins were found to readily and preferentially associate with intact pathogenic human trichomonads. In this report we present a strategy for identification of plasma proteins avidly associated with *T. vaginalis* following incubation of the trichomonads with plasma. We further demonstrate the contribution of acquired plasma lipoproteins to the growth properties of these parasites, and show that specific ligand-receptor-like interactions mediate plasma lipoprotein binding by trichomonads.

MATERIALS AND METHODS

Growth of T. vaginalis. T. vaginalis NYH 286 [6] was cultivated axenically using Diamond's trypticase/yeast extract/maltose (TYM) medium [7] supplemented with 10% (v/v) heat-inactivated human plasma as described previously [4].

Fractionation of human plasma. The initial separation of plasma into eight protein preparations was accomplished using the procedure of Cohn et al. [8]. Briefly, 25 ml of citrated human plasma containing approximately 2.2 g of protein was fractionated by precipitation of plasma components using cold-ethanol salt solutions. The ethanol precipitates were separated by centrifugation at 17 000 $\times$ *g*. All steps were carried out at recommended temperatures except that precipitates I and III were separated into fraction III-0 at 0°C. All fractions were dialyzed overnight against phosphate-buffered saline (PBS) (pH 7.4, 137 mM NaCl, 2.7 mM KCl, 4.6 mM Na_2HPO_4 and 1.5 mM KH_2PO_4), and used immediately. Proteins in individual Cohn fractions were characterized by sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and typical protein patterns of individual fractions are as presented in the Results section. The protein composition of the fractions obtained has been published [9] and is listed below along with protein concentration determined according to Bradford (BioRad Laboratories, Richmond, CA) and specific activity following radioiodination. I + III-3; plasminogen, fibronectin, fibrinogen (protein, 500 μg ml^{-1}; specific activity, 2 000 cpm ng^{-1}): II; γ-globulins (400 μg ml^{-1}; 2 500 cpm ng^{-1}): III-0; β-lipoproteins, euglobulins, ceruplasmin (400 μg ml^{-1}; 1 000 cpm ng^{-1}): III-1,2; prothrombin, isoagglutinins (200 μg ml^{-1}; 850 cpm ng^{-1}): IV-1; α-lipoproteins (300 μg ml^{-1}; 750 cpm ng^{-1}): IV-6,7; β-metal combining protein, α-2 mucoproteins, choline esterase, α-2 glycoprotein (300 μg ml^{-1}; 750 cpm ng^{-1}): V; albumin (500 μg ml^{-1}; 500 cpm ng^{-1}): and VI; α-1 glycoprotein, small proteins and peptides (50 μg ml^{-1}; 5 000 cpm ng^{-1}). Contaminating immunoglobulin G (IgG) was removed from fractions 1 + III-3, III-0, and III-1,2 by Protein A-Sepharose (Sigma Chemical Co., St. Louis, MO) column chromatography. Albumin was removed from fractions IV and VI by reactive blue 2-agarose (Sigma) chromatography. For certain experiments, whole plasma was depleted of both IgG

and albumin with Protein A-Sepharose and reactive blue 2-agarose. Chromatographic removal of plasma transferrin was achieved using the IgG fraction of monospecific high-titer antiserum to transferrin coupled to cyanogen bromide-activated Sepharose.

Purification of plasma lipoproteins. Human very low density lipoprotein ($d < 1.006$ g ml^{-1}), low density lipoproteins ($d = 1.006$–1.063 g ml^{-1}), high density lipoproteins ($d =$ 1.063–1.215 g ml^{-1}) were prepared from pooled normal human plasma in 0.1% (w/v) (EDTA) from fasted donors. Lipoproteins were fractionated by standard sequential ultracentrifugation [10] in a Beckmann 60 Ti rotor (50 000 rpm) at 5–10°C for 24 h using solid KBr for adjustment of densities. Briefly, 50 ml of freshly isolated plasma from fasted donors was dialyzed overnight against 4 l of PBS containing 0.1% (w/v) EDTA. The plasma was then diluted with distilled H_2O containing 0.1% (w/v) EDTA until a density of 1.006 g ml^{-1} was obtained. Following ultracentrifugation, the very low density lipoproteins were removed, and the remaining plasma adjusted with solid KBr to a density of 1.063 g ml^{-1} and subjected to recentrifugation. After removal of the low density lipoproteins, the remaining plasma was adjusted as before to a density of 1.215 gml^{-1} and centrifuged once again, followed by removal of the high density lipoprotein fraction. Isolated fractions were dialyzed against 4 changes of 200 vol of PBS containing 0.1% (w/v) EDTA, pH 7.4. Plasma separated from lipoproteins as above was also used in certain experiments. For growth studies, lipoproteins and lipoprotein subfractions were adjusted to the original starting plasma volume and dialyzed twice with 4 l of PBS.

Radioiodination of plasma proteins. Normal human plasma (NHP), Cohn plasma fractions, and plasma depleted of certain proteins were radiolabeled by lactoperoxidase-catalyzed radioiodination [11] using 1.0 mCi of $Na^{125}I$ (Amersham Corp., Arlington Heights, IL) as previously described [9]. The resulting labeled protein preparations were then placed in Spectrapor 10 000 MW cut-off dialysis tubing (Fisher Scientific Co., Houston, TX) and dialyzed overnight at 4°C against several changes of PBS.

Lipoproteins were iodinated with $Na^{125}I$ using a slight modification of the iodine monochloride method [12]. Briefly, 200 μl of 1 M glycine/NaOH buffer, pH 10, was added to 1 ml of lipoprotein (1 mg ml^{-1}) followed by the addition of 1.0 mCi of $Na^{125}I$. To this mixture was added 20 μl of iodine monochloride (400 mM in 2 M NaCl) followed by a 5 min incubation. Unbound radioactivity was removed by extensive dialysis against PBS containing 0.01% (w/v) EDTA, pH 7.4. Efficiency of radioiodination was determined by trichloroacetic acid (TCA) precipitation [11] and autoradiography after SDS-PAGE of radioiodinated materials.

Acquisition of avidly associated proteins. Live, motile trichomonads were washed at least 3 times in PBS to remove contaminating, loosely bound materials [4] and

resuspended to a density of 2×10^7 ml^{-1} in PBS. To siliconized microfuge tubes pretreated with 1% (v/v) horse serum [9], 100 μl samples were added. Various amounts of ^{125}I-labeled proteins in PBS were added to a final volume of 500 μl, followed by incubation at 37°C with occasional gentle shaking. After a 30 min incubation, the motile parasites were sedimented at 1 000 × g for 2 min in a Beckman microfuge B with a Dayton variable transformer at setting 40. The supernatant containing unbound proteins was discarded, and the organisms were washed 3 times in PBS by centrifugation. Parasites were continuously monitored by darkfield microscopy and remained viable throughout the acquisition experiments unless otherwise stated. Finally, the trichomonads were transferred to another microfuge tube and pelleted. Total proteins were precipitated with TCA [11],washed at 4°C with cold PBS, resuspended at room temperature in 200 μl of solubilizing buffer consisting of 60 mM Tris-base (pH 6.8), 2% (v/v) β-mercaptoethanol, 10% (v/v) glycerol, and 2% (w/v) sodium dodecylsulfate [13] and boiled for 3 min. Undissolved residue was removed by centrifugation prior to SDS-PAGE. Radioactivity was assessed using a Beckmann gamma 310 system counter. Counting efficiency among samples remained constant throughout these studies.

For measuring acquisition of ^{125}I-labeled plasma proteins at different pH values, 100 mM sodium acetate in PBS adjusted to designated pH values with 1 N NaOH was employed as buffer. No loss of viability of the organisms was observed during the incubation periods used. Since the autoradiographic profiles of ^{125}I-labeled NHP proteins bound to gluteraldehyde-fixed parasites were identical to those obtained using live trichomonads, fixed organisms were employed for some experiments in the presence of 1 M NaCl in the incubation mixture. Parasites were fixed with 0.1% (v/v) glutaraldehyde for 30 min at 4°C, and washed at least 4 times with PBS prior to use for binding studies.

Internalization of lipoproteins by live trichomonads was monitored by indirect immunofluorescence microscopy [14]. Briefly, 4×10^6 organisms, washed twice with PBS, were suspended in 0.5 ml ice cold PBS containing 10% (v/v) total plasma lipoproteins and incubated for 20 min at 4°C. The parasites were washed twice with cold PBS and suspended in 0.5 ml PBS containing 20 μl of rabbit antiserum to human lipoproteins or normal rabbit serum. After incubation for 20 min at 4°C the parasites were washed twice with cold PBS and resuspended in 0.3 ml of fluorescein isothiocyanate goat anti-rabbit immunoglobulin G (Cappel Labs, Cochranville, PA) diluted 1:5 (v/v) in PBS buffer. The suspension was placed at 4°C in the dark for 20 min before washing as above. The trichomonads were finally suspended in 0.2 ml PBS and monitored for fluorescence with a Zeiss IM 35 microscope equipped with fluorescence and darkfield optics. *T. vaginalis* organism remained viable throughout this procedure.

SDS-PAGE. Proteins dissolved in solubilizing buffer were loaded onto individual wells of 3% (v/v) and 7.5% (v/v) acrylamide stacking and separating gels, respectively

[4,5,9]. Electrophoresis was carried out on a 16 cm slab gel apparatus (BioRad Labs) using an initial constant current of 15 mA per gel which was increased to 30 mA per gel after penetration of the bromphenol blue tracking dye into the separating gel. Gels were then stained and destained prior to drying and exposure of gels to film for various lengths of time [4].

RESULTS

Selective acquisition of plasma proteins. Our earlier studies [4] demonstrated that *T. vaginalis* was able to acquire certain plasma proteins. First we examined the kinetics of binding of ^{125}I-labeled plasma to intact parasites. Acquisition was greater at 37°C than at 4°C (Fig. 1A). Time dependent acquisition was obtained at both temperatures and the continued slow increase of binding at 37°C at 60 and 90 min is indicative of internalization of some proteins in radioiodinated NHP as shown later (Table I). Interestingly, utilization of a sodium acetate buffer at various pH values showed increased radioactivity associated with the live trichomonads at pH 4.0 compared to pH 7.0. Subsequent experiments using glutaraldehyde-fixed organisms at pH 4.0 but in the presence of 1.0 M NaCl did not result in a reduction of bound labeled-NHP proteins, demonstrating that ionic interactions alone were not responsible for host protein uptake (data not shown). SDS-PAGE of ^{125}I-labeled proteins adsorbed from plasma at different pH values showed that pH 4 resulted in increased acquisition of the same proteins that were associated with trichomonads at pH 7.

Electrophoretic analysis revealed that approximately 10 ^{125}I-labeled proteins which had molecular mass ranges of 200 kDa to 40 kDa, and less than 30 kDa (Fig. 2, lane 9a) were acquired by live cells. Samples enriched for specific groups of proteins by Cohn fractionation were used to identify some of the acquired proteins. Protein patterns of

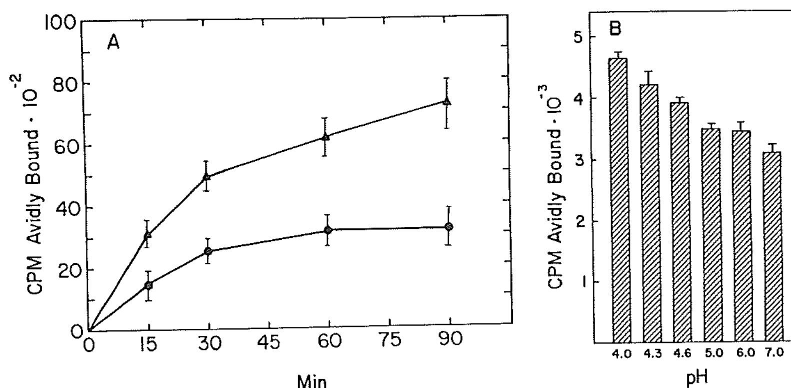

Fig. 1. Data showing acquisition by *T. vaginalis* of radioiodinated NHP proteins (protein concentration, 60 gl^{-1}; specific activity 1600 cpm μg^{-1}) incubated for increasing lengths of time (A) at 37°C (▲) and 4°C (●). Trichomonal binding of ^{125}I-labeled proteins was examined at various pH values at 37°C (B). Each point represents the average of triplicate samples from a representative experiment.

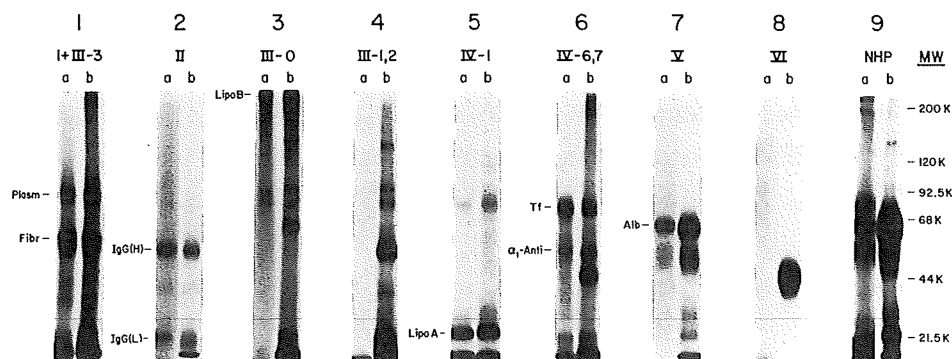

Fig. 2. SDS-PAGE autoradiography of representative profiles of proteins in Cohn fractions (lanes 1b through 9b). Radioiodinated proteins from individual Cohn fractions avidly associated with washed, motile *T. vaginalis* are illustrated in lanes 1a through 9a. Abbreviations: Plasm, plasminogen; Fibr, fibrinogen; IgG(H) and IgG(L), immunoglobulin G heavy and light chains respectively; Lipo B and Lipo A, lipoproteins B and A respectively; Tf, transferrin; α_1-Anti, α_1-antitrypsin; and Alb, albumin.

radioiodinated Cohn fractions after electrophoresis are as shown in Fig. 2 (lanes 1b through 8b), and, as can be seen from the gel profiles in lanes 1a through 8a, eight distinct proteins from respective Cohn fractions were acquired by live *T. vaginalis*. Importantly, these bound proteins possessed electrophoretic mobilities like those proteins associated with trichomonads when NHP was employed (Fig. 2, lane 9a). The inability of ^{125}I-labeled proteins in fractions III-1,2 and VI to be adsorbed onto intact *T. vaginalis* reinforced the specificity of trichomonal acquisition of certain plasma proteins. These and earlier data [4] from studies using commercially available preparations of pure plasma proteins implicated plasminogen, fibrinogen, immunoglobulin G, lipoproteins A and B, transferrin, α_1-antitrypsin [5] and albumin as plasma proteins acquired by *T. vaginalis*.

Additional binding experiments using plasma depleted of specific components confirmed the acquisition of transferrin, albumin, immunoglobulin G, and lipoproteins. As shown in Fig. 3A, corresponding protein bands in the autoradiographic profile of acquired plasma proteins were missing when radioiodinated plasma depleted of a given protein (Fig. 3B) was employed. For example, removal of albumin (Fig. 3B, lane c) from total plasma (lane a) resulted in a concomitant loss of a band co-migrating with ^{125}I-labeled albumin. Decreased intensities in the corresponding bands were obtained when plasma depleted of transferrin (lane b), IgG (lane d), and lipoproteins (lane e) were evaluated electrophoretically (Fig. 3B) or used for binding experiments (Fig. 3A). Incomplete removal of certain bands may indicate the presence of different proteins on trichomonal surfaces with similar electrophoretic mobilities.

Interaction of human plasma lipoproteins with live trichomonads. Because of the importance of plasma lipoproteins in the metabolic functions of eukaryotic cells [15,16] and

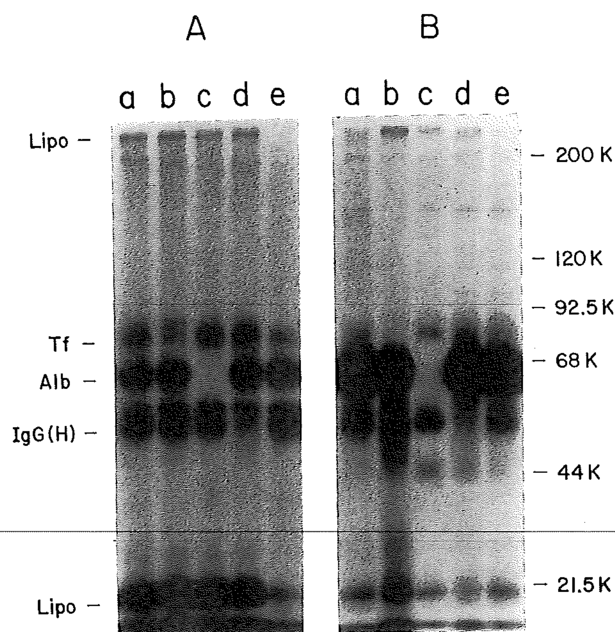

Fig. 3. Autoradiograms after SDS-PAGE of ^{125}I-labeled plasma proteins avidly associated with the surface of *T. vaginalis* (A) using NHP or plasma depleted of certain proteins (B). The gel profiles of radioiodinated NHP (lanes a), and plasma chromatographically-depleted of transferrin (lanes b), albumin (lanes c), IgG (lanes d), and lipoproteins (lanes e) used in a typical binding experiment are shown.

a recent report showing a requirement by trichomonads for lipids complexed with protein moieties [17], we attempted to define further the biochemical properties and biological role of the lipoproteins binding to *T. vaginalis.*

Washed, motile trichomonads were examined for their ability to acquire lipoproteins isolated from fresh human plasma. Incubation of increasing amounts of labeled human lipoproteins with a constant number of parasites at 4°C resulted in the saturation binding by trichomonads of human lipoproteins. Maximal acquisition was obtained for 1×10^7 parasites using 30 μg of labeled proteins (Fig. 4). These studies were carried out at 4°C to prevent internalization of acquired lipoproteins. Importantly, effective competition (greater than 70%) was only achieved when unlabeled lipoproteins were incubated with *T. vaginalis* prior to the addition of radioiodinated lipoproteins (Fig. 5). Unlabeled albumin, another lipid-carrying plasma protein, for example, failed to inhibit any binding of ^{125}I-labeled lipoproteins under identical conditions. Maximal inhibition of binding achieved under these experimental conditions was reasonable considering the complexity and heterogeneity of total lipoproteins used in binding studies and is consistent with other published reports on eukaryotic lipoprotein acquisition [18].

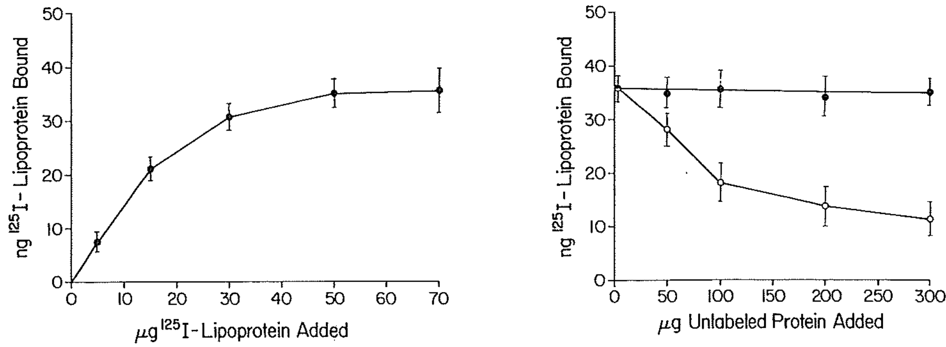

Fig. 4. Acquisition of lipoproteins by live *T. vaginalis* in a representative experiment. Approximately 1×10^7 parasites were incubated for 30 min at 4°C with the indicated amounts of ^{125}I-labeled lipoproteins followed by extensive washing of trichomonads and counting of bound radioactivity. Each point represents the average of triplicate samples.

Fig. 5. Inhibition of acquisition of saturating levels of ^{125}I-lipoproteins (60 μg 500 μl^{-1}) by *T. vaginalis* in the presence of increasing amounts of unlabeled albumin (●) or unlabeled total plasma lipoproteins (o). Unlabeled protein preparations (abscissa) were incubated with trichomonads for 15 min prior to the addition of radioiodinated lipoproteins.

Internalization of lipoproteins by T. vaginalis. In order to demonstrate uptake of lipoproteins by *T. vaginalis* under conditions optimal for internalization, time and temperature dependent binding of ^{125}I-labeled total lipoproteins were examined (Table I). Uptake of radioiodinated lipoproteins was much greater at 37°C than at 4°C. Lipoprotein binding, however, leveled off only at the lower temperature. Up to 60% of radioactivity was removed by pronase treatment for 30 min at room temperature (22°C), but only from trichomonads incubated with radiolabeled lipoproteins at 4°C, suggesting a surface localization for acquired lipoproteins at this temperature. No lysis of parasites was evident under these conditions. In contrast, protease treatment removed little or no radioactivity from organisms incubated with lipoproteins at 37°C indicating internalization of lipoproteins under these conditions. Greater than 95% radioactivity was recovered in the pellet after centrifugation of the *T. vaginalis* organisms incubated with lipoproteins at 37°C, showing that the results in Table I were due to internalization and not trichomonal release of bound radioactivity.

Additionally, indirect immunofluorescence studies using antibodies directed against apolipoproteins showed greatly diminished fluorescence following a 30 min incubation of parasites at 37°C after treatment of organisms with lipoproteins. Maximal fluorescence was obtained using parasites incubated with lipoproteins at 4°C throughout the experiment. These results indicate that *T. vaginalis* specifically ac-

TABLE I

Binding and protease removal of ^{125}I-labeled lipoproteins acquired by *T. vaginalis*

Incubation time prior to enzyme treatment[a] (min)	Temperature (°C)	^{125}I associated with *T. vaginalis*[b]		
		Control (cpm)	After pronase[c] treatment	
			cpm	% remaining
5	4	7090	2810	40
15	4	7050	3380	48
30	4	12200	5270	43
60	4	13500	4740	35
5	37	12100	11900	98
15	37	70200	70200	100
30	37	163000	174000	100
60	37	235000	231000	98

a 1×10^7 organisms in 0.5 ml PBS were incubated in 1.5 ml microfuge tubes with saturating amounts (60 µg) of ^{125}I-labeled lipoproteins (specific activity, 3.4×10^5 cpm ng^{-1} protein). Each value represents the mean of three samples in a typical experiment, and standard deviation did not exceed 8% of the mean for each sample.

b After incubation at the specified temperatures for the designated times, trichomonads were washed at least twice further with PBS and resuspended to 0.5 ml. Microfuge tubes were then measured for radioactivity in a Beckmann gamma counter.

c Lipoprotein-treated trichomonads were washed and incubated with 500 µg pronase prepared fresh in 1 ml PBS for 30 min at 22°C. Concomitant increases in radioactivity were found in cell-free supernatants after pronase treatment.

quires lipoproteins from a complex mixture of proteins such as plasma, and subsequently internalizes these host macromolecules.

Effect of lipoproteins and lipoprotein fractions on the growth of T. vaginalis. We were interested in whether lipoproteins or individual lipoprotein subfractions are able to promote in vitro growth and multiplication of *T. vaginalis*. As can be seen in Fig. 6, TYM-medium supplemented with an amount of total lipoproteins equivalent to that found in 10% (v/v) plasma yielded almost identical rates and levels of growth. In addition, high density lipoproteins and low density lipoproteins were also capable of supporting parasite multiplication in this complex medium, but very low density lipoproteins supported only low level rates of parasite growth. As expected, plasma deficient in lipoproteins failed to support any growth of motile trichomonads. These data demonstrate that lipoproteins represent plasma constituents essential for *T. vaginalis* growth and multiplication.

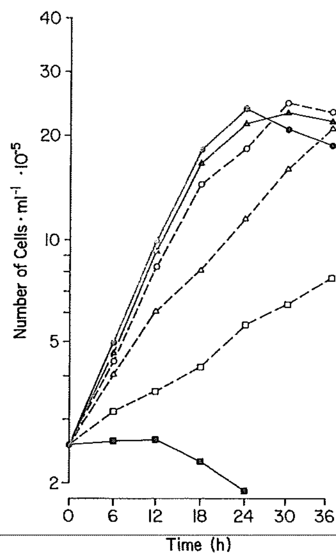

Fig. 6. Representative growth curves of *T. vaginalis* grown in TYM-medium supplemented with 10% (v/v) each of the following components: normal human plasma (●), lipoprotein-free plasma (■), total plasma lipoproteins (▲), high density lipoproteins (△), low density lipoproteins (○), and very low density lipoproteins (□).

DISCUSSION

We have been interested in examining the overall biological and immunological properties of membranes of pathogenic *T. vaginalis*, especially with regard to specific host-parasite interactions which may enhance parasite survival in host tissues and/or produce host cytopathologic reactions. We feel that the interaction of *T. vaginalis* and other parasites with host macromolecules is an important area of research inasmuch as beneficial properties conferred upon pathogenic microorganisms following acquisition of host substances have been documented in other microbial systems [9,19–22].

In this study, we present a strategy for the identification and characterization of human plasma proteins preferentially bound to *T. vaginalis*. The ability of *T. vaginalis* to acquire a small number of proteins from NHP at pH values found in the vagina was demonstrated. While the significance of parasite binding of human IgG, albumin, transferrin, and other host molecules remains undefined, we employed a combination of biochemical, fluorescence and microbiological techniques to examine further the interaction of plasma lipoproteins with live organisms.

The specific nature of lipoprotein acquisition was demonstrated, and high density lipoprotein and low density lipoprotein subfractions were found to support growth of these human trichomonads. These data are consistent with the reported lipid-carrier requirements of *T. vaginalis* in a totally defined medium [17]. It is noteworthy, however, that other investigators have used this defined medium for cultivation of the

pathogenic bovine trichomonad but only in the presence of dialyzed serum [23]. We have also been unable to grow *T. vaginalis* in this defined medium [17] using lipoproteins or lipoprotein subfractions (data not shown), suggesting a possible contribution by other associated plasma proteins to the growth capabilities of trichomonads. It is conceivable that other proteins abundant in the vagina and on mucosal surfaces, but not in plasma, may play significant roles in trichomonal survival in the urogenital environment. Experiments are in progress to define the contribution of other acquired proteins to the growth of *T. vaginalis* as well as to identify the components of high density and low density lipoproteins recognized by trichomonad membrane receptors.

The presence of receptors on *T. vaginalis* for binding lipoproteins was implicated through saturation, kinetic, and competition experiments using homologous and heterologous proteins. Receptors specific for plasma proteins required by *T. vaginalis* suggest that these molecules contribute to growth and may enhance the overall virulence of these parasites. In addition, parasite receptors with immunochemical properties similar to those found on host cells might produce autoimmune-like host cytopathologic reactions. On the other hand, trichomonal membrane components involved in host protein acquisition may be presumptive vaccinogen candidates. Clearly, the dissection of microbial components involved in host protein acquisition is essential prior to utilization of either whole cells or subfractions as vaccines [24,25].

The growth characteristics of fresh isolates versus isolates after several serial passages of *T. vaginalis* may be due to trichomonad adaptation to lipoproteins or other proteins in TYM-serum medium compared to the composition and quantity of proteins found at mucosal surfaces. Furthermore, the variation in concentration of substances such as lipoproteins at the site of infection in the vaginal mucosa are variables which may have direct impact on host susceptibility or resistance to infection. Thus, differences in essential nutrients or immunologic factors in the immediate urogenital environment in contact with pathogenic trichomonads represent considerations requiring further discussion and evaluation.

ACKNOWLEDGEMENTS

This work were supported by Public Service grants AI 18768 and AI 19142 from the National Institutes of Health to J.F.A. We thank Jill Sapp and Mary Devadoss for their excellent secretarial assistance.

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