Properties of *Trichomonas vaginalis* grown under chemostat controlled growth conditions

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Abstract

***Trichomonas vaginalis* isolates NYH 286 and IR 78 were grown in continuous flow culture conditions in a complex trypticase-yeast extract-maltose medium supplemented with heat-inactivated horse serum. Parasites could be stably maintained in the chemostat at high densities ranging from 1×10^6 to 1×10^7 organisms ml^{-1}. Growth densities, acid production, and profiles of total versus secreted trichomonad proteins were characterised at different rates of growth and pH. Growth rate influenced the extent of parasite production of acid and the shedding of proteins into the medium but had no effect on overall parasite density. Lowering the pH from 6·0 to 5·0 resulted both in a decrease of cell density and acid production. At pH 4·5 isolate IR 78 but not NYH 286 was capable of growth and multiplication, showing the ability of some isolates to survive at the vaginal pH of healthy individuals. At this lower pH, however, isolate NYH 286 but not IR 78 synthesised new proteins which were detectable in stained gels. Also, inoculation of the chemostat with isolate NYH 286 comprising a mixture of fluorescent (positive, pos) and non-fluorescent (negative, neg) trichomonads as defined by monoclonal antibody reactivity to a surface immunogen resulted in a change in the parasite population to an almost homogeneous neg phenotype. These neg phenotype organisms switched back to pos phenotype after transfer to test tubes.**

Trichomonas vaginalis is a sexually transmitted flagellated protozoan of the human urogenital tract. Trichomoniasis in women ranges from an asymptomatic, carrier state to intense inflammation of the vaginal epithelium, which can be accompanied by itching, a purulent, white discharge, and severe abdominal pain.[1 2] Usually the infection in women is non-self-limiting, causing extensive morbidity.

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Possible virulence factors of *T vaginalis* include antigenic heterogeneity,[2] phenotypic variation,[3] haemolysis,[4] cytadherence,[5 6] cytotoxicity,[7 8] proteinases,[9] and secretion of immunogens into the culture medium.[10] The host environment may also influence or determine the extent of parasite virulence and disease pathogenesis. For example, host factors in the vagina including menstrual blood,[11] hormonal levels,[12] extent of inflammation,[13] normal flora,[2] oxygen tension,[14] and nutrient availability[15–17] can all contribute to the nature and extent of infection. Indeed the ability of complement in menstrual blood to kill trichomonads has been demonstrated,[11 18] and subpopulations of *T vaginalis* organisms refractory to the lysis by complement[11] represent surviving parasites with possibly a unique antigenic profile now present in the host.

Nutritional limitations, along with other related parameters such as slow growth rates and pH, as might be encountered in the host, can be examined through the use of a chemostat.[19] For this reason continuous flow culture might be useful for studying physiologic parameters that lead to expression of factors involved in establishment and maintenance of *T vaginalis* infection. In this paper we report on the growth of *T vaginalis* in a chemostat fermentor system. Trichomonads could be stably maintained for prolonged periods of time. Growth rate and pH were found to affect cell density and secretion of proteins into the medium. When compared with test tube grown trichomonads, major differences in total protein profiles were detected. In addition, isolates were differentiated on the basis of growth at pH 4·5, the normal vaginal pH.

Materials and methods

MEDIA AND GROWTH

T vaginalis isolates NYH 286 and IR 78 have been described elsewhere,[3 20–22] and were grown in trypticase-yeast extract-maltose (TYM) medium supplemented with 10% heat-inactivated horse serum[23] throughout this study. Late logarithmic-phase organisms[24] were then inoculated directly into the growth vessel containing TYM-serum medium to achieve an initial density of 1×10^5 org ml^{-1}. Trichomonads were then grown aerobically at 37°C at dilution rates of 0·07 $hour^{-1}$ (h), 0·013 h^{-1} and 0·007 h^{-1} which gave doubling times (t_d) of 10 h, 50 h and

100 h, respectively. The t_ds were calculated based on flow rates of TYM-serum medium added to the culture vessel.[25] For each analysis the chemostat was allowed to equilibrate by passing at least four complete changes of TYM-serum medium through the vessel. Culture pH was maintained automatically at pH 6·0, 5·0 or 4·5 with sterile 5N NaOH or 5N HCl and continuously monitored with an autoclavable pH electrode. Cells were enumerated microscopically with an improved Neubauer haemocytometer. Data obtained are the average of triplicate determinations of no less than two independent experiments.

INDIRECT IMMUNOFLUORESCENCE

Preparation of trichomonads for indirect immunofluorescence for detection of surface expressed immunogens was identical to a previously described procedure.[3 21 22] A monoclonal antibody (MAb) designated C20A3 was used to differentiate fluorescent (pos phenotype) from nonfluorescent (neg phenotype) subpopulations of isolate NYH 286. Isolate IR 78 synthesises but does not express the immunogen on the surface[3] and consists of homogeneous, stable neg phenotype trichomonads as defined by this MAb.[3 22]

SODIUM DODECYL SULFATE (SDS)-POLYACRYLAMIDE GEL ELECTROPHORESIS (PAGE) AND IMMUNOBLOT

The preparation of total trichloroacetic acid-precipitated proteins was done as previously detailed.[20] SDS-PAGE was also performed as described before using 3% stacking and 7·5% separating acrylamide gels.[20] Low and high molecular weight protein markers were purchased from BioRad Labs, Richmond, CA. Electrophoretic transfer of trichomonad proteins from acrylamide gels was also performed as before.[3] Nitrocellulose blots were then blocked with 5% nonfat dry milk in TBS (Tris, 20 mM; NaCl, 500 mM; pH 7·5) for 2 h at room temperature (RT). Rabbit anti-NYH 286 or IR 78 serum[20] diluted 1:500 in 5% milk-TBS was then added and incubated overnight at 4°C. These antisera did not react with any culture medium components. The blots were then washed three times with TBS at RT. Goat anti-rabbit immunoglobulin conjugated to horseradish peroxidase (BioRad Labs) was added to a final dilution of 1:2000. The blots were incubated at RT for 4 h, washed three times for 15 minutes (min) with TBS, and developed with TBS containing 4-chloro-1-naphtol (2 mg ml^{-1}), 20% methanol and 0·015% H_2O_2.

PREPARATION OF PARASITE ANTIGENS PRESENT IN CULTURE SUPERNATANTS

Trichomonads harvested from the chemostat were removed from the medium by centrifugation at 500 × *g* at 4°C for 15 min. Supernatant was carefully decanted without disturbing the cell pellet and centrifuged at 20,000 × *g* at 4°C for 10 min. Calcified supernatant was then filtered through a 0·45 μm Gelman aerodisk (Gelman Science Inc., Ann Arbor, MI) to remove any additional insoluble material. Supernatants were stored at −70°C until used. For SDS-PAGE the samples were combined with an equal volume of twofold concentrated dissolving buffer.[20] Volumes loaded onto gels were approximately 30 μl and corresponded to 2 × 10^4 organisms. As a control to monitor whether lysis of parasites accounted for the protein bands observed,

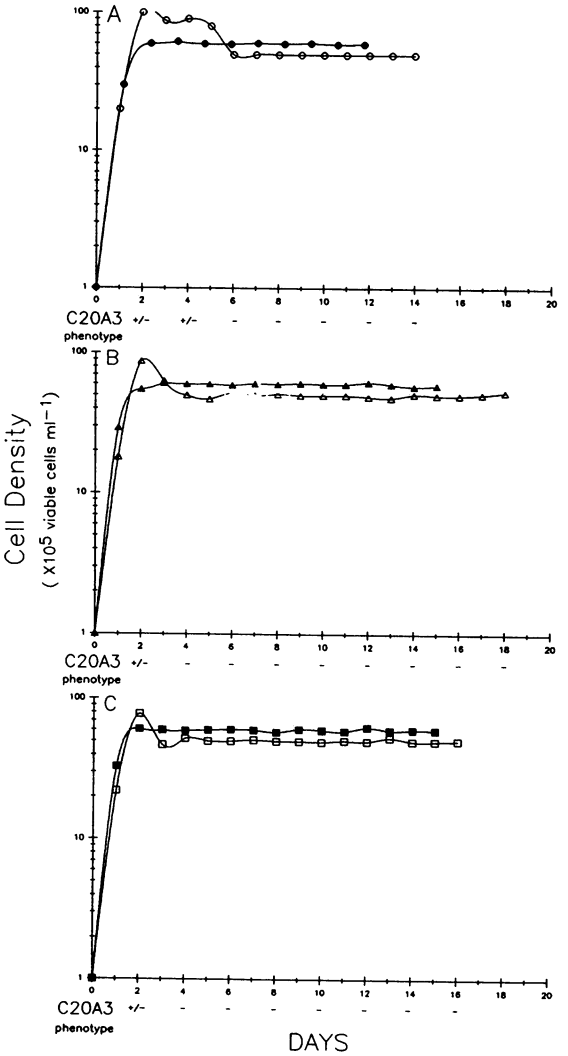

Figure 1 Growth of T vaginalis isolates NYH 286 (○, △, □) and IR 78 (●, ▲, ■) in a chemostat at generation times of 10 h (A), 50 h (B) and 100 h (C). The C20A3 phenotype refers to that of isolate NYH 286 and was determined by indirect immunofluorescent with monoclonal antibody C20A3 as described in Materials and methods. Each point represents the average of three independent determinations.

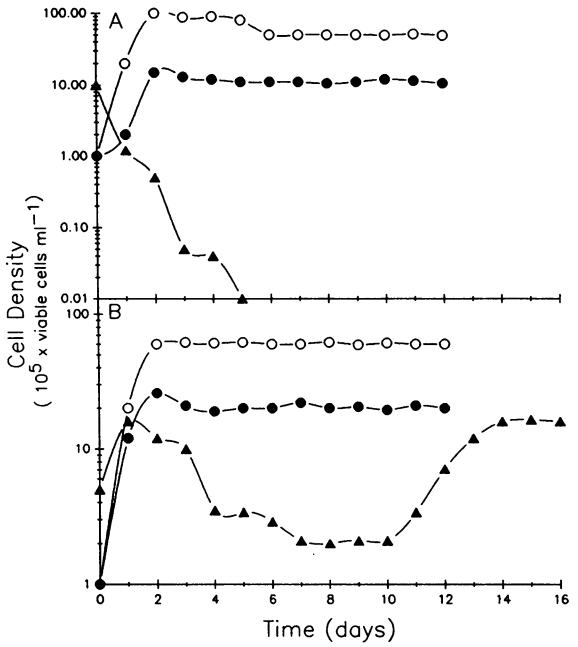

Figure 2 Growth characteristic of T vaginalis isolated NYH 286 (panel A) and IR 78 (panel B) in a chemostate at pH 6·0 (○), 5·0 (●) and 4·5 (▲). The pH was kept constant with an automatic pH control module by the addition of 5N NaOH.

an immunoblot was also done using total proteins from 2×10^4 trichomonads. This amount of trichomonad proteins failed to give any bands, consistent with earlier findings by us.[10]

Results

GROWTH OF *T VAGINALIS* ISOLATES NYH 286 AND IR 78 AT VARIOUS DILUTION RATES AND pH

Steady state growth for both isolates was achieved when parasite density as shown in fig 1 and 2 and acid production as presented in the table remained constant for each growth rate. Levels of cell densities of chemostat grown organisms ranged from 1×10^6 to 5×10^6 org ml^{-1} and compared favourably with previous reports by us on densities of trichomonads from batch cultures.[24]

We next wanted to determine whether this pathogenic protozoan could be grown at different generation times (t_ds). Compared with batch cultures where triochomonads have a t_d of 4–6 h,[24] *T vaginalis* in chemostat cultures had t_ds of up to 150 h. As shown in fig 1, different experiments were performed and parasite densities were unaffected by t_ds of 10 (part A), 50 (part B) and 100 h (part C) for either *T vaginalis* isolates NYH 286 or IR 78.

Lowering the pH from 6·0 to 5·0, however, resulted in a five-fold and three-fold decrease in cell density for isolates NYH 286 (fig 2A) and IR 78 (fig 2B), respectively. No multiplication of *T vaginalis* NYH 286 organisms was detectable at pH 4·5, and this resulted in parasite dilution due to removal of parasites from the growth vessel (fig 2A). Isolate NYH 286 also could not grow at pH 4·5 in batch cultures. In contrast, isolate IR 78 reached a cell density of $1·6 \times 10^6$ org ml^{-1} followed by a decrease to 2×10^5 org ml^{-1} at pH 4·5 (fig 2B). This cell number remained stable for four days followed by an increase to $1·6 \times 10^6$ org ml^{-1} by day 14, indicating growth and multiplication at pH 4·5. Isolate IR 78 could also grow in batch culture at pH 4·5 and reached a density of 5×10^5 org ml^{-1}. These results clearly differentiate *T vaginalis* isolates on the basis of their ability to survive at the normal vaginal pH.

ACID PRODUCTION

Batch culture pH decreases from 6·2 to 4·5 during growth of *T vaginalis*.[24] To establish if acid production is dependent on growth rate and medium pH, the consumption of NaOH to maintain a stable pH in the chemostat growth vessel was measured. Acid production declined when the generation time increased due to nutrient limitation or when the growth pH was decreased (table). The amount of acid produced was always higher for isolate IR 78 than NYH 286, and only isolate IR 78 produced acid at pH 4·5 (table).

PHENOTYPIC VARIATION

We also wanted to determine whether phenotypic variation for the expression of immunogens occurred in chemostat cultures as has been shown for batch-grown organisms.[3 22 26 27] A heterogeneous population was used to seed the culture vessel, and the parasites were monitored daily by indirect immunofluorescence using antibody. A shift of the parent heterogenous (30% pos phenotype) population to neg phenotype was always observed regardless of the culture t_d (fig 1). At no time were greater than 1% trichomonads fluorescent during the remainder of the experiment. The dramatic shift of the parent NYH 286 population to an almost homogeneous neg

Table The effect of various growth conditions on the amount of acid produced by T vaginalis NYH 286 and IR 78.

		*Acid produced fmol (h^{-1} org^{-1})**	
		isolate:	
Generation time	*Medium pH*	*NYH 286*	*IR 78*
10	6·0	170·0 SD 21·0	580 SD 52·0
50	6·0	84·0 SD 18·0	270 SD 33·0
⩾100	6·0	nm†	nm
50	5·0	5·4 SD 3·0	140 SD 17·0
50	4·5	nm	80 SD 24·0

*average of triplicate determinations done on three different experiments.
†nm, not measurable.

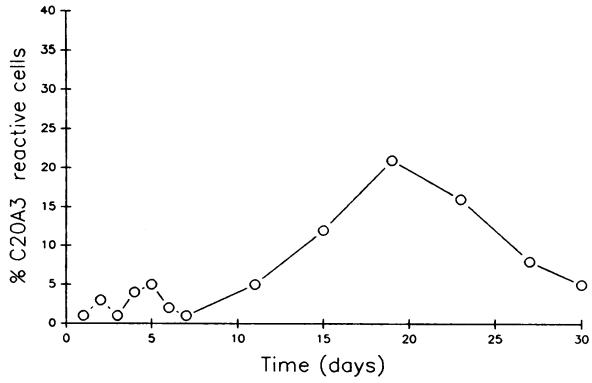

Figure 3 Reversion of T vaginalis NYH 286 to a heterogeneous phenotype after transfer of a homogenous neg phenotype grown in a chemostat to test tube culture. The phenotype was determined by indirect immmunofluorescence with monoclonal antibody C20A3. Each point represents three independent determinations of at least 100 organisms counted from at least ten different fields.

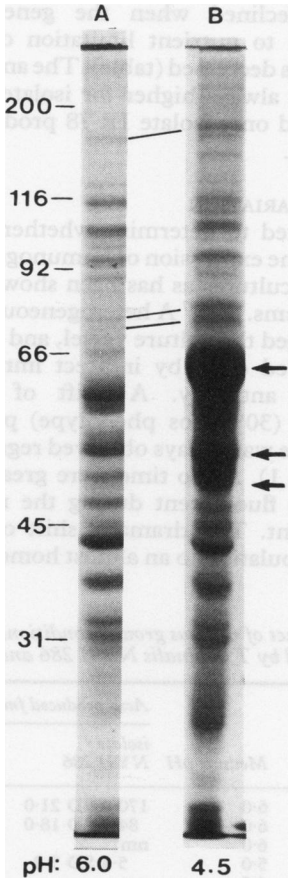

Figure 4 SDS-PAGE of total trichloroacetic acid-precipitated proteins of T vaginalis NYH 286 grown in a chemostat at pH 6 (lane A) or pH 4·5 (lane B).

phenotype was demonstrated during six independent experiments where the parent populations were comprised of different percentages (30% to 50%) of pos phenotype organisms.

Neg phenotype organisms were then removed from the chemostat at different times and propagated daily in test tubes. Figure 3 shows representative data of almost homogeneous neg phenotype parasites reverting to the pos phenotype. Clearly, chemostat conditions favoured the lack of surface expression of a prominent immunogen but did not select for a stable neg phenotype. As expected, isolate IR 78 remained neg phenotype throughout.[3]

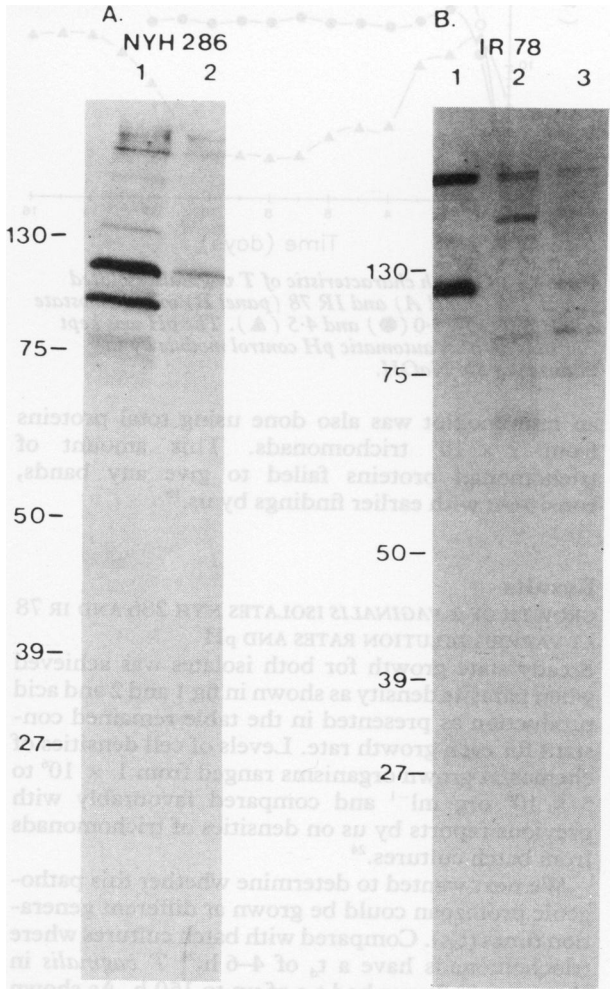

Figure 5 Immunoblot analysis of antigens released into the growth medium by T vaginalis isolates NYH 286 (A) and IR 78 (B). (A) Immunogens detected in supernatants of NYH 286 when grown in chemostat culture at a dilution rate of 0·07 h^{-1} (t_d=10 h) (lane 1) or dilutions rates greater than 0·07 h^{-1} (lane 2). (B) Trichomond proteins detected in supernatants of IR 78 when in chemostat culture at dilution rate of 0·07 h^{-1} (lane 1) or at 0·013 h^{-1} (t_d=50 h) (lane 2) or at pH 4·5 (lane 3).

SDS-PAGE AND IMMUNOBLOT OF TOTAL AND SECRETED PROTEIN

Qualitative and quantitative variation among certain proteins was apparent and reproducible when comparing gel profiles of isolate NYH 286 grown in the chemostat at different pH values. For example, SDS-PAGE of total trichloroacetic acid precipitated proteins from the same number of organisms[20] revealed dramatic differences in the protein profiles at pH 4·5 (fig 4, lane B). The protein pattern of trichomonads at pH 6·0 was identical to that previously determined for batch-grown parasites.[20] Similar dramatic differences in protein patterns were not seen for isolate IR 78 at these pHs.

The possibility that chemostat growth conditions influenced the release of proteins into the medium was also investigated. At all variables examined for both batch and chemostat cultures of NYH 286 (fig 5A), high Mr protein immunogens (>70-kDa) appeared to predominate in supernatants. Two intense bands of proteins of ~130-kDa were secreted into the growth medium only at a t_d of 10 h (fig 5A, lane 1). Much less secretion of immunogens appeared to occur when organisms were grown with t_ds > 10 h (lane 2). Finally, immunoblot using total proteins from 2×10^4 trichomonads, the equivalent cell number of supernatant used in these experiments, did not give any bands under the same conditions. These data show the selective enrichment for these immunogens in culture supernatants.

For isolate IR 78, two immunoblot patterns were observed of antigens released into the chemostat growth medium. The first pattern from chemostat cultures at a t_d of 10 h at pH 6·0 consisted of two prominent high molecular weight immunogens (>110-kDa) (fig 5B, lane 1). Another pattern was observed for parasites grown at a t_d of 50 h at pH 6·0 and indicated the release of several antigens ranging in molecular weight from 75-kDa to 200-kDa (lane 2). *T vaginalis* grown at a t_d of 50 h but at pH 4·5 gave a pattern similar to that seen with organisms grown at pH 6·0, although less amount of secreted trichomonad protein was seen (fig 5B, lane 3). Both isolates NYH 286 (fig 5A) and isolate IR 78 (fig 5B) demonstrated decreased secretion at longer t_ds.

Discussion

This study showed that *T vaginalis* can be grown successfully in a controlled chemostat system. High cell densities were achieved in the chemostat under various growth rates and pH as has been reported for test-tube grown cultures.[24] The generation times of chemostat trichomonads were much greater than those reported for batch cultures, which ranged from four to six hours.[2 24] For example, in the chemostat we were able to demonstrate a generation time for *T vaginalis* of up to 150 h. The in vivo generation time for this pathogen is unknown, but the doubling time might be expected to be considerably higher due to nutrient limitation and other factors in the vagina. This ability of the parasite to have long generation times as seen here may very well be relevant to the non-self-limiting nature of trichomoniasis.

The decrease in cell number for *T vaginalis* isolate NYH 286 grown at pH 4·5 in the chemostat (fig 2) indicates an adverse effect of pH on overall parasite physiology. This is consistent with known effects of pH on amount and extent of enzymatic activity and energy generation for other microorganisms.[28-30] Interestingly, isolate NYH 286 also produced dramatically less acid (table) than IR 78 at all generation times and medium pHs studied. Since lactate may be the major acid product of *T vaginalis* metabolism,[2 31] it is likely that a low pH provides a signal for decreasing acid production, which in turn results in decreased ATP generation. This then would be consistent with the observed diminished growth and elevated generation time for isolates like NYH 286. On the other hand isolate IR 78 appears more acid tolerant, as evidenced by the increase in cell density at pH 4·5 (fig 2B), and this suggests that low pH may not provide signalling to allow for the extent of decreased growth and higher generation times similar to NYH 286. These data may be meaningful to the in vivo situation, since isolates like NYH 286 are known to cytadhere optimally at elevated pH values (pH 5·5 to pH 6·0).[5] Some isolates like IR 78 may be at an advantage compared to other isolates in cases where the pH in patients remains low (pH ~4·5).[2 32]

Another finding of this study was that phenotypic variation of a major immunogen which occurs in batch cultures[23] was suppressed in organisms grown in the chemostat (fig 1). This observation was also documented for other isolates as well, including JH31A and AL20W, which are also heterogeneous for surface immunogens.[22] The fact that most fresh isolates from patients are negative for surface expression of this and other immunogens,[27] which correlates with increased virulence properties,[22] perhaps indicates that key selective pressures in vivo may in fact involve severe nutrient limitation leading to very extended generation times as shown here.

This study also shows that the overall protein composition of trichomonads may be influenced by environmental changes like pH and specific growth rate (fig 4). It is noteworthy that protein patterns of chemostat organisms at pH 6·0 were similar to previously published profiles of test tube parasites,[20 21] but dramatic qualitative and quantitative differences were observed in proteins of trichomonads of isolate NYH 286 grown in the chemostat at low pH (fig 5). The pH 4·5 protein pattern may actually be demonstrating a parasite response to stress resulting from the vaginal pH (fig 5, lane B), and this may be consistent with the

inability of this isolate to grow at this pH (fig 2). Isolates like IR 78 which do not give an altered protein pattern at pH 4·5 as do isolates like NYH 286 may represent parasites capable of growth under these conditions (fig 2). These results illustrate the importance of growth conditions on possible enhanced or new expression of immunogens, and it will be important to analyse these organisms grown under in vivo-like conditions in the chemostat for certain virulence properties. This is especially important, since researchers have utilised only test-tube grown organisms for studies involving antigen analysis,[2 3 9 10 27] antibody production,[3 21] and virulence.[4-8 22]

Shedding of antigens also appeared to be influenced by medium pH and *T vaginalis* growth rate (fig 5). Contrary to an earlier study of batch cultures which showed the release of many proteins ranging from 30-kDa to 300-kDa into the culture medium,[10] only few high molecular weight immunogens (>70 kDa) were shed by chemostat-grown organisms (fig 5). Although these experiments may be crucial for identifying selectively secreted or released proteins by *T vaginalis*, these results along with the ability of *T vaginalis* to have long generation times may indicate that soluble parasite immunogens may not be as common in the vagina as suggested earlier.[10]

Overall, continuous culture provides the most controlled method for growth of *T vaginalis*. This paper represents the foundation for our continued goal of understanding external signals which may be involved in expression of virulence factors for the pathogenic human trichomonads. It would appear that the effects of environmental changes on growth, product formation, and expression of virulence factors as reported for other microbial pathogens[33-35] can now be performed for this important sexually transmitted parasite.

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