Arch Virol (1997) 142: 939–952

Archives of Virology

Involvement of dsRNA virus in the protein composition and growth kinetics of host *Trichomonas vaginalis*

D. Provenzano,[1] **A. Khoshnan**[2], and **J. F. Alderete**[1]

[1]Department of Microbiology, The University of Texas Health Science Center, San Antonio, Texas, U.S.A., [2]Department of Microbiology, University of Southern California, Los Angeles, California, U.S.A.

Accepted November 20, 1996

Summary. *Trichomonas vaginalis* harbors a double-stranded (ds)-RNA virus, and the presence of virus is related to upregulated expression and phenotypic variation of a prominent immunogen (Khoshnan A, Alderete JF (1994) J Virol 68: 4035–4038). To further test the influence of virus on *T. vaginalis*, virus-infected (V^+) isolates were compared to virus-free (V^-), agar-cloned progeny trichomonads derived from the parental isolates for accumulation of total proteins and cysteine proteinases. Comparative high resolution two dimensional (2D)-SDS-PAGE was performed of trichomonads grown in a chemostat under identical conditions. At least 47 proteins were identified as specifically expressed by representative V^+ isolate 347, and ~41 spots were specific to the corresponding V^- progeny, showing an association between virus and the presence and absence of parasite proteins. Qualitatively and quantitatively dissimilar cysteine proteinase patterns were detected from numerous V^+ isolates and the V^- progeny. A 2D analysis for isolate 347 showed the appearance of unique proteinase activities for parental parasites and presence of at least one proteinase in the V^- progeny. Finally, the V^+ *T. vaginalis* isolate 347, but not the V^- isolate 347 progeny nor other V^+ isolates, underwent fluctuations in density during chemostat growth allowing for purification of virus particles from the V^+ isolate 347 supernatants during decreased parasite density.

Introduction

Trichomonas vaginalis is a protozoan parasite responsible for a sexually transmitted disease (STD) manifested as a non-self-limiting vaginitis in women [31]. Symptomatology varies among infected women. Symptomatic individuals have a foul-smelling discharge, severe inflammation and discomfort. Recent findings suggest that trichomonosis is associated with adverse pregnancy

outcomes [14, 30] and enhanced predisposition to HIV [24]. A review of several epidemiological studies revealed a possible association between *T. vaginalis* and a risk of cervical neoplasia [43].

Analysis of > 500 fresh *T. vaginalis* clinical isolates in our laboratory has demonstrated the presence of a segmented double-stranded (ds)-RNA virus (V^+) in one-half of the isolates, [10, 38, 42]. Complicating matters is the discovery of small-sized satellite dsRNAs in some, but not all, V^+ isolates [20, 23]. Recent reports have begun to dissect the interrelationship between the protozoan and the dsRNA virus. High levels of trichomonal transcription and synthesis of a prominent immunogen, called P270, were found associated with infection by dsRNA virus [22]. P270 undergoes phenotypic variation between surface and cytoplasmic expression, and loss of virus due to prolonged batch passaging of the parasite results in only cytoplasmic expression of P270 [1, 7, 10, 22, 42].

Infection by the dsRNA virus appears to be persistent, as no reports on lytic cycles for infected trichomonads have been described [21, 23, 41, 42]. Virus transmission appears to occur vertically, and no virus particles have been purified from batch-grown logarithmic phase cultures. Only transient infections of virus-free parasites have been achieved [20].

Another recent report evaluated the ability of cysteine proteinases of *T. vaginalis* to degrade human immunoglobulins (Ig) [37]. It was evident that V^+ isolates were more heterogeneous in the expression of Ig-degrading proteinases than were the V^- isolates. This heterogeneity in proteinase expression is not unlike early comparisons between V^+ and V^- isolates, which showed major differences in the surface expression and synthesis of immunogens among the two isolate types [4, 7–10].

In this report we show the influence of the dsRNA virus on the total protein composition of *T. vaginalis* organisms, including cysteine proteinases known to be produced in the vaginal environment of patients and which modulate trichomonal virulence and pathogenesis [3, 5, 6, 11, 12, 18, 32]. Interestingly, among several V^+ isolates examined, only *T. vaginalis* isolate 347 [22] displayed periodic fluctuations in cell density during batch and steady-state chemostat growth, which paralleled recovery of virus from cell-free culture supernatants. The implications of our results are discussed.

Materials and methods

Parasite cultures

V^+ *T. vaginalis* isolates 21201, T068-II, NYH 286, AL 8, AL 10, 347, and 8 111 and V^- progeny [1, 7, 22] were cultured axenically in complex Trypticase-yeast extract-maltose (TYM) medium supplemented with 10% heat-inactivated (HI)-horse serum (HS) [17, 37]. Only parasites from batch cultures at mid- to late-logarithmic phase of growth were utilized [36].

A chemostat (New Brunswick Scientific, New Brunswick, NJ) was inoculated with 1.25×10^8 parasites of either V^+ parental or V^- progeny first grown in batch cultures and resuspended in 5 ml medium for inoculation of a 1.25 L vessel [23, 27, 40]. Trichomonads

were then maintained aerobically at 37 °C at a doubling time of 24 h and pH 5.5, as recently described [20, 23, 27]. Parasite morphology and viability were monitored by microscopic wet mount examination. Cell number was obtained by averaging two determinations performed independently in an improved Neubauer hemocytometer. Cells and supernatants were each harvested at different times during growth and frozen at −70 °C until use.

Agar cloning of single trichomonads

Progeny virus-free agar clones of single trichomonads were derived as described before [1, 7, 22]. Briefly, sterile TYM medium containing 0.65% noble agar was warmed to 50 °C. Prewarmed HIHS was added (10% final concentration), and the temperature quickly brought to 37 °C, at which time trichomonads at a density of 20 to 40 cells ml^{-1} were added to the volume of 35.5 ml TYM-agar-HIHS. After gentle mixing, the contents were poured into autoclaved glass petri dishes. Growth of trichomonads occured under anaerobic conditions at 37 °C for 7 to 8 days before viewing. Visible colonies were harvested and transferred to TYM-10% HIHS for growth and freezing.

Detection of viral dsRNA, purification of virus, and examination of the genome of V^+ isolate and corresponding V^- progeny organisms

Presence or absence of the virus dsRNA was determined by agarose gel electrophoresis of purified RNA derived from each V^+ and corresponding V^- progeny, as detailed elsewhere [20–23, 41, 42]. Absence of viral dsRNA in V^- trichomonads was further confirmed by Northern analysis using riboprobes from cloned cDNAs derived to each of the three dsRNA segments [21], as has been specified recently [20, 22, 23]. Southern analysis was performed on the genome of both the V^+ isolate and corresponding V^- progeny examined using probes to several known genes [2, 16, 34], one of which is to the phenotypically varying P270 immunogen [16, 22]. This was important in order to show the isogenic nature of the V^- progeny based on these gene probes.

Virus was purified from supernatants by centrifugation of 25 ml of parasite culture at 500 × g for 10 min to remove intact cells. The supernatant was then further clarified of debris by centrifugation at 27 000 × g in a SS34 rotor for 30 min. Viral particles were precipitated from the supernatant by slow addition of polyethylene glycol (MW 8 000) and NaCl at a final concentration of 10% and 0.6 M, respectively. The precipitate was resuspended in 12 ml TNM buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl and 5 mM $MgCl_2$), equilibrated to a density of 1.35 g/ml with CsCl, and centrifuged at 100 000 × g in a SW41 rotor for 24 h. Fractions containing the virus were pooled and dialyzed in TNM buffer [21]. Viral particles were pelleted by centrifugation at 100 000 × g for 2 h, re-suspended in TNM buffer, and treated with 50 μg/ml proteinase K for 30 min at 65 °C. Viral dsRNA was extracted by phenolchloroform, precipitated by ethanol, and analyzed by ethidium-bromide staining after electrophoresis in 1% agarose as described before [20, 21, 42].

High resolution analysis of trichomonad proteins

Parasites for 2D analysis of total proteins [4, 35] were prepared by first treating trichomonads for 5 min at 4 °C with PBS containing 1mM N-α-p-tosyl-L-lysine chloromethyl ketone (TLCK) (Sigma Chemical Corp., St. Louis, MO) [11, 32, 33, 37]. This treatment neutralized the numerous trichomonad cysteine proteinases [32] and allowed for reproducibility of patterns. Organisms were then washed three times (3X) in

PBS by centrifugation at 500 × g for 5 min prior to freezing of pelleted trichomonads at −70 °C.

Protein samples and all reagents for electrophoresis were prepared as per the instructions provided by the manufacturer of a high resolution 2D apparatus (Oxford Glyco Systems, Bedford, MA). Briefly, frozen samples of 2×10^7 trichomonads were each resuspended in 240 µl sample buffer 1 (0.3% sodium dodecyl sulfate (SDS), 200 mM dithiothreitol (DTT), 28 mM Tris-HCl, and 22 mM Tris-base; final pH 8.0) and boiled at 100 °C for 5 min. Samples were placed on ice for 5 min before the addition of 24 µl sample buffer 2 (24 mM Tris-base, 476 mM Tris-HCl, 50 mM $MgCl_2$, 0.25 mg/ml RNase A (Sigma), and 1 mg/ml DNase 1 (Sigma); final pH 8.0). Samples were then precipitated in 210 µl ice-cold acetone and incubated on ice for 20 min followed by centrifugation at 4100 × g for 10 min. The supernatants were discarded, and the protein pellets were resuspended in 48 µl sample buffer 1 and 192 µl sample buffer 3 (9.9 M urea, 4% NP-40, 2.2% ampholytes pH 3–10 (Millipore Corporation, Bedford, MA) and 100 mM DTT). Samples of 5 µl equivalent to 4.2×10^5 cells were then loaded onto 1 mm diameter glass capillary tubes containing polymerized IEF gel solution (9.5 M urea, 2.0% nonidet P-40, 4.1% acrylamide, 5 mM CHAPS (US Biochemical Corp., Cleveland, OH), 5.8% ampholytes pH 3–10, and 0.006% ammonium persulfate). IEF was then performed as described previously [4] using a pH range in from 3 to 10. Electrophoresis in the second dimension was accomplished on 10% acrylamide using standard conditions. Gels were stained with silver as recommended by the manufacturer (Oxford). Electrophoresis of V^+ and V^- trichomonads was always performed in parallel using the same batch of ampholytes and other reagents. Protein patterns of gels obtained from the same run were compared, and reproducible patterns were obtained from electrophoresis of duplicate frozen samples evaluated at different times.

Substrate-gel electrophoresis for proteinase detection

For proteinase analysis 2×10^7 viable parasites were also washed three-times in cold PBS as described above prior to storage at −70 °C [13, 28, 32, 37]. No effect on proteinase activities for both parasite lysates and supernatants were seen by storage for extended periods, as before [37].

Substrate-gel electrophoresis was performed to compare proteinase degradation patterns between V^+ and V^- parasites [13, 28, 32, 37]. Separating gels of 7% acrylamide were copolymerized with 625 µg/ml each of fibronectin (Collaborative Research Inc., Becton Dickenson Labs, Bedford, MA), extracellular matrix proteins (ECM) (Matrigel; Collaborative), and human IgG (Sigma). Stacking gels of 4% acrylamide were utilized. Samples were prepared by triturating 2×10^7 trichomonads in electrophoresis dissolving buffer (125 mM Tris-HCl, pH 6.8, 4% SDS, 20% glycerol, 10% bromophenol blue) [37]. The homogenate was centrifuged for 1 min in a high speed microfuge to remove insoluble debris. Sample volumes of 3 µl equivalent to 3×10^5 trichomonads were electrophoresed at 120 V in electrophoresis buffer (25 mM Tris-HCl, pH 8.7, 192 mM glycine, and 10% SDS).

For 2D substrate-electrophoresis, IEF gels were cast in 1 by 75 mm capillary tubes by mixing 0.5 ml of 0.5 M CHAPS and 10% NP-40 with 5.75 ml of 15 M urea, 7.8% acrylamide, 0.2% bis-acrylamide-acrylamide, 7.0% ampholines pI 3–10, and 1.7% ampholines pI 4–8 (Millipore). Each solution was mixed and de-gassed at 37 °C before adding 10 µl TEMED and 20 µl (10%) ammonium persulfate. The mixture was loaded onto capillary tubes and polymerization occurred at RT in ≤1.5 h. After IEF, gels were loaded onto 10% acrylamide separating slab gels and electrophoresed [4, 32].

Samples of 2×10^7 cells were solubilized in 50 µl of sample buffer I (350 mM SDS and 150 mM DTT) followed by addition of 200 µl sample buffer II (160 mM DTT, 80 mM

CHAPS, 9 M urea, and 6.25% ampholines pI 3–10) [19]. IEF tube gels were loaded with 40 μl of sample followed by an equal volume of overlay buffer (9 M urea, 2% ampholines pI 3–10, 0.5% ampholines pI 4–8, and 0.0025% bromophenol blue) and electrofocusing performed at 500 V for 10 min followed by 750 V for 3.5 h. Electrophoresis at 120 V was followed by a 2 h incubation of substrate gels in reducing buffer (2.5% Triton-X 100, 100 mM sodium acetate and 1 mM DTT, pH 5.5) warmed to 37 °C. Reducing buffer was changed after 1 h. Finally, gels were stained for 2 h in Coomassie brilliant blue R prepared in 40% methanol and 10% acetic acid before destaining.

Results

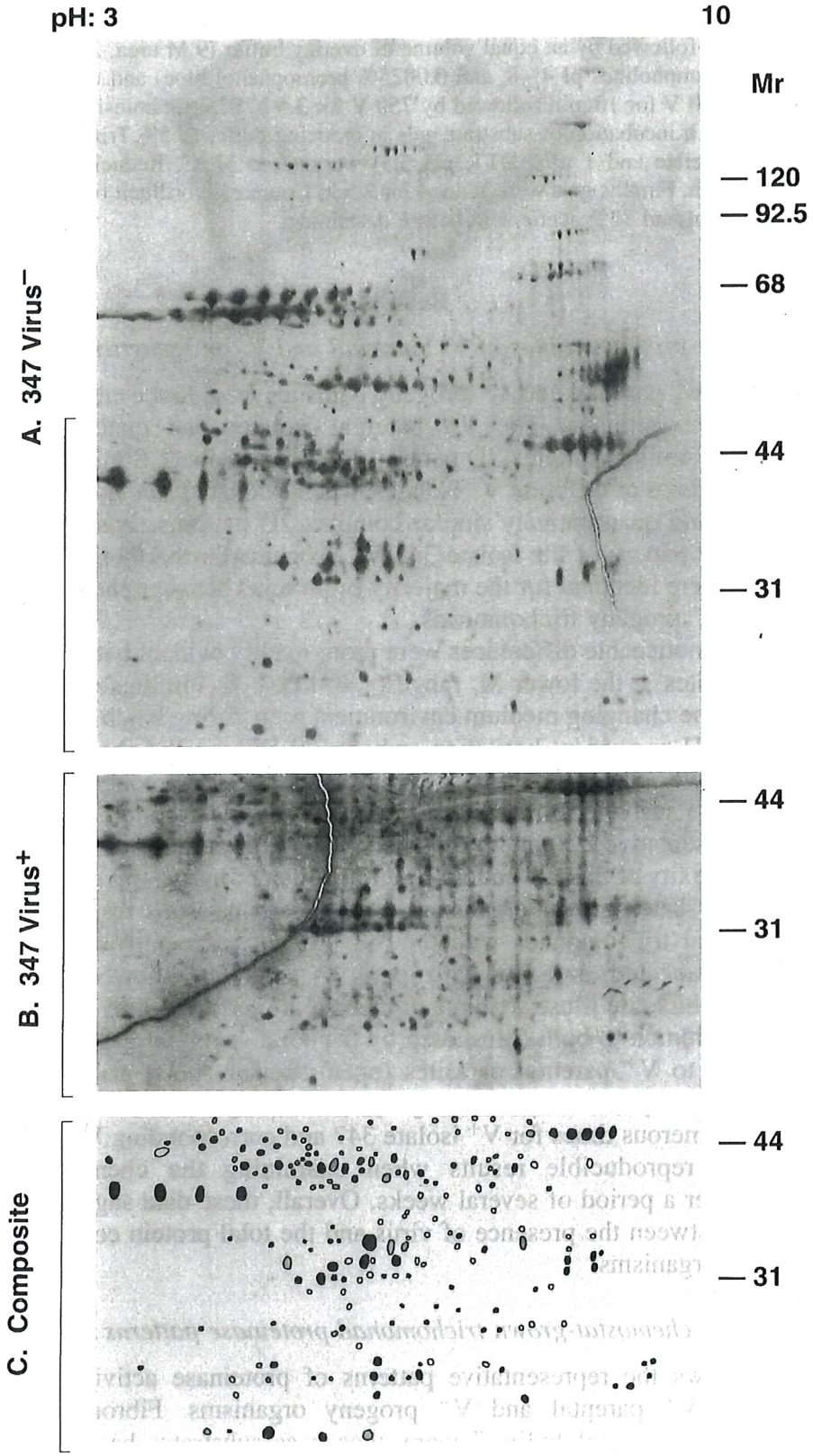
Comparative protein profiles of V^+ parental and V^- progeny trichomonads

Batch-grown V^+ parental and V^- progeny parasites were first evaluated by 2D-SDS-PAGE on minigels. The V^+ parental isolates had qualitatively and quantitatively similar complex 2D patterns (data not shown). Similarly, the V^- progeny organisms of different V^+ isolates, when evaluated side by side showed qualitatively and quantitatively similar complex 2D patterns, as shown in Fig. 1A for the 2D pattern of the isolate 347 V^- progeny. Furthermore, the overall 2D patterns were identical for the majority of proteins between the V^+ parental isolate and V^- progeny trichomonads.

However, noticeable differences were more readily evident between the V^+ and V^- parasites at the lower M_r range ($\leq$40-kDa). To eliminate the possible influence by the changing medium environment seen during batch growth, such as a drop in pH or nutrient limitation or both [20, 37], on the observed protein differences, we cultivated parasites in a chemostat under steady-state conditions. We also performed high-resolution 2D electrophoresis. Figure 1 shows a representative 2D pattern of isolate 347 V^- trichomonads (part A). The overall complexity of this 2D pattern resembled that of the V^+ parental as noted above for the batch-grown organisms. Careful comparisons of both the V^+ isolate parental trichomonads with the V^- progeny, especially in the region $\leq$50-kDa (bracketed area in both parts A and B), showed prominent differences, which are illustrated in a composite in part C. While many protein spots were common to both (illustrated by the solid spots), at least 47 proteins were specific to V^+ parental parasites (open circular spots) and 41 proteins were uniquely expressed in V^- progeny (shaded spots). This analysis was performed numerous times for V^+ isolate 347 and corresponding V^- organisms and yielded reproducible results when examining the chemostat-grown organisms over a period of several weeks. Overall, these data suggest a strong association between the presence of virus and the total protein composition of *T. vaginalis* organisms.

Batch- and chemostat-grown trichomonad proteinase patterns also differ

Figure 2 shows the representative patterns of proteinase activities of three batch-grown V^+ parental and V^- progeny organisms. Fibronectin, ECM proteins, and immunoglobulin G were chosen as substrates based on earlier


pH: 3
10
Mr
— 120
— 92.5
— 68
— 44
— 31
A. 347 Virus⁻
— 44
— 31
B. 347 Virus⁺
— 44
— 31
C. Composite

work and their possible relevance to pathogenesis and immune evasion [12, 37]. Although the three V^+ parental isolates (lanes 2, 4 and 6) and V^- progeny trichomonads (lanes 1, 3, and 5) displayed qualitatively similar substrate-degradation patterns, differences distinguished the two types of trichomonads. Analysis of V^+ parasites, in all cases, resulted in the detection of higher numbers and greater amounts of different proteinase activities. Not surprisingly, this evaluation detected unique bands (arrows), consistent with the known specificity of the trichomonad cysteine proteinases for certain substrates [3, 33, 37].

To rule out the possibility that the cultivation of parasites, especially during the isolation of virus-free agar-cloned progeny trichomonads, was responsible for the observed changes, we examined for proteinase profiles among agar clones of V^+ and V^- isolates. No changes in any proteinase patterns were evident (data not shown), suggesting strongly that the loss of virus from the original V^+ parental organisms influenced the observed expression patterns.

Next, we performed 2D-substrate electrophoresis on extracts of organisms from chemostat-grown cultures. Figure 3 shows the total 2D proteinase patterns on gelatin, a substrate commonly used for analysis of trichomonad cysteine proteinases [6, 13, 28, 32], of isolate 347 V^+ parental (part B) versus V^- progeny (part A) parasites. Under these conditions, 12 readily detectable and prominent proteinase activities were identified for V^+ 347 parasites. A significant quantitative reduction in activity was apparent upon loss of virus. Only four major and some minor activities were readily discernible for V^- progeny trichomonads. The disappearance of three major proteinases (arrows) in the M_r range of 40 kDa to 80 kDa from V^+ organisms upon loss of virus was especially noteworthy, as was the appearance of a new activity for the V^- progeny in the same region (asterisk). Also conspicuous was the decrease in four major low M_r activities in V^-progeny, as illustrated within the boxed region. As with the 2D total protein patterns, the patterns were reproducible among either the same preparation of duplicate samples assayed at different times or among different preparations of organisms harvested at various times during culture under the same conditions. This is important, as it shows that the steady-state growth conditions allowed for reproducible expression of defined

Fig. 1. High resolution two dimensional (2D) electrophoresis on 10% acrylamide of total proteins of the isolate 347 parasites grown in the chemostat. **A** The total 2D protein pattern of the V^- progeny trichomonads derived from agar clones (see Materials and methods). Absence of dsRNA virus among V^- progeny was determined as described in Materials and methods. **B** The low M_r proteins by 2D electrophoresis under identical conditions as shown for part A of the parental V^+ 347 organisms. In both of these 2D profiles of V^- and V^+ parasites, patterns were derived from sample volumes of 5 µl equal to 4.2×10^5 trichomonads electrophoresed in parallel using the same reagents. **C** The composite shows spots common to both V^- and V^+ parasites (solid circular) versus those unique to the V^- (shaded) and V^+ (open) parasites. Molecular weight protein markers (BioRad Laboratories, Richmond, CA) are included on the right as kilodaltons

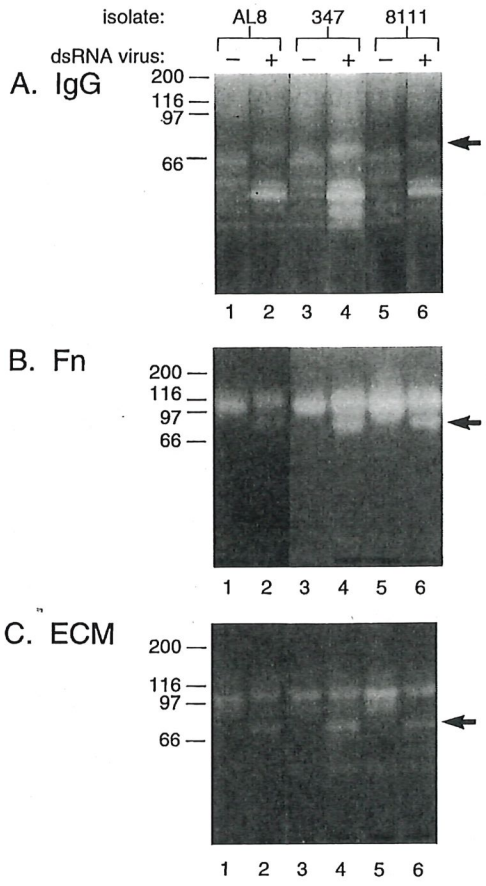


Fig. 2. Substrate-gel electrophoresis for comparative evaluation of proteinase activity among representative V^+ parental isolates (lanes labeled plus) and the corresponding agar-cloned V^- progeny (lanes labeled minus). Optimal resolution of proteinase activities was achieved from sample volumes of 3 µl equivalent to 3×10^5 trichomonads, which were electrophoresed on 7% acrylamide gels copolymerized with the indicated substrates. IgG (**A**), Fn (**B**), and ECM (**C**) refer to human immunoglobulin G, fibronectin, and extracellular matrix proteins, respectively (see Materials and methods). A prominent proteinase activity unique to the V^+ isolate trichomonads and visualized on all substrates is indicated by arrows. Relative electrophoretic mobilities (M_r) of standard protein markers (BioRad) are shown on the left in kDa

proteinases. Finally, all proteinase activities reported here were inhibited by TLCK, a known inhibitor of the trichomonad cysteine proteinases [3, 11, 32, 33, 37].

Growth fluctuations and purification of virus from supernatants of isolate 347

Figure 4 shows the growth curves of chemostat cultures of V^+ isolates 347 and T068-II over an extended time period. V^+ isolate 347 parasites (open squares), but not V^+ organisms of isolate T068-II (open triangles) and several other isolates (not shown), displayed reproducible periodic decreases in cell density throughout, albeit to different levels, as evidenced at 60 h and more dramatically at 400 h. During each decrease in density, trichomonal lysis and dense cellular debris were evident in the supernatants. The insert shows the higher cell density of 6.4×10^6 ml^{-1} recorded for the V^- cloned progeny, which never showed fluctuations under the same experimental steady-state conditions (solid circles). The peak cell density of 5.0×10^6 (at ~500 h) for the parental V^+ 347 trichomonads (open squares) never reached that of the virus-

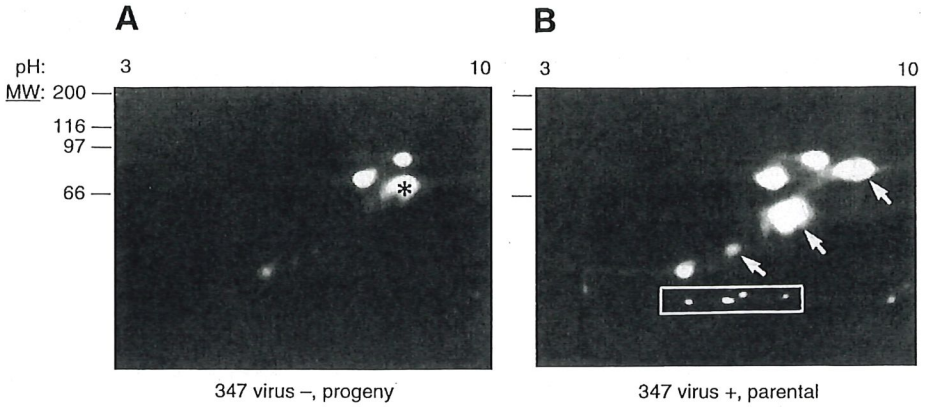


Fig. 3. Comparative 2D proteinase patterns of *T. vaginalis* isolate 347 between V^+ parental organisms (**B**) and V^- parasites of a representative agar clone (**A**). Patterns reflect sample volumes of 40 µl corresponding to 3.2×10^6 trichomonads. Isoelectric focusing was done for 3.5 h in a pH 3 to pH 10 ampholine gradient. Electrophoresis was performed on 10% acrylamide gels co-polymerized with 1.2 mg/ml substrate. Arrows and blocked regions for the V^+ parental profiles illustrate activities lost or greatly diminished following loss of the dsRNA virus. The asterisk for the V^- progeny organisms indicates the appearance of a new proteinase activity. These 2D patterns were reproducible for duplicate samples handled similarly and for new samples obtained at later times from trichomonads cultivated under identical conditions in the chemostat

free progeny. Interestingly, each decrease in cell numbers was succeeded by a correspondingly higher cell density but, again, the density never reached that of the V^- cloned progeny over the ~600 h test period. Importantly, these observations were recorded for isolate 347 on chemostat runs performed on several separate occasions. A decrease in parasite number corresponding to cell lysis was also observed during batch culture at the mid-logarithmic phase of growth, and the culture recovered to maximal cell numbers of $\sim 2 \times 10^6$. No similar fluctuations in density were observed during batch growth of numerous other V^+ as well as with previously studied V^- isolates [3, 20–23, 27].

Finally, culture supernatants of the V^- progeny trichomonads were examined for the presence of virus dsRNA. Figure 4B presents two separate experiments showing purification (Materials and methods) of virus from cell-free supernatants (lane 3) at times of cell lysis corresponding to 96 h (expt. 1) and 432 h (expt. 2). No similar recovery of dsRNA was possible at times when the fluctuations in cell density were not evident. Not unexpectedly, washed pelleted organisms (lane 1) and contaminating debris from the supernatants (lane 2) gave prominent dsRNA bands after agarose gel electrophoresis of extracted RNA. No virus was ever recovered from the supernatants of other V^+ isolates, such as NYH 286, T068-II, AL 10 and 21 201, grown under identical chemostat steady-state conditions nor, as expected, from other V^- isolates that have been studied recently [11, 16, 22] (data not shown). Fluctuations in cell numbers were not evident in these other isolates. Also, V^- progeny of isolate

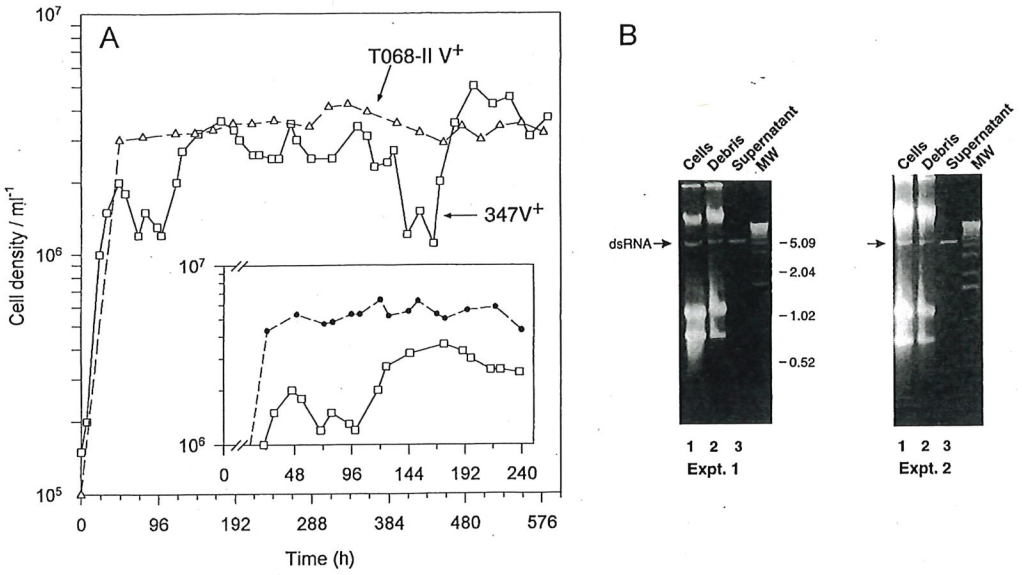


Fig. 4. Growth kinetics of parental *T. vaginalis* isolates 347 and T068-II (**A**) and the ability to isolate viral dsRNA from the supernatant of isolate 347 during growth fluctuations (**B**). The insert presents the growth kinetics up to 240 h under identical conditions comparing isolate 347 parental V^+ (□) and progeny V^- parasites (•). All isolates and progeny organisms were grown in a chemostat cultured under steady-state conditions of pH 5.5 and 24 h generation time. **B** shows two separate and representative experiments on the recovery of the dsRNA recovered from cells (*1*), debris (*2*), and cell-free supernatant (*3*). The dsRNA extracted from each sample was electrophoresed in 1% agarose and visualized by ethidium-bromide staining. Relative position of 1 kb molecular size markers (MW; GIBCO-BRL, Life Technologies Inc., Gaithersburg, MD) are shown on the right

347 (insert, solid circles) grown under identical conditions in batch and chemostat cultures never had detectable virus or dsRNA in cell-free culture supernatants.

Discussion

That the dsRNA virus influences total protein composition of the parasite is suggested by the dramatic differences in protein and proteinase profiles between V^+ parental and V^- progeny organisms. The reproducibility among chemostat-grown parasites of protein and proteinase patterns and the finding of identical profiles among agar clones derived from single trichomonads suggest that the variation in protein content detected is associated with the presence of virus and not environmental modulations, such as medium pH, which is known to change dramatically during batch growth [23, 27]. These findings are an extension of and consistent with recent experimental evidence showing that V^+ *T. vaginalis* isolates have higher levels of transcript and amounts of P270 immunogen than did V^- progeny [9, 16, 22].

Trichomonad cysteine proteinases are known virulence factors that contribute to the survival of the parasite in numerous ways. Iron-inducible proteinases degrade complement C3 [3] made available to the site of infection during menstruation and, in addition, the proteinases degrade all classes of immunoglobulins (Igs) [37] found in the vagina to specific trichomonad immunogens during infection [4, 8]. The evaluation over the years of V^+ and V^- isolates has shown different but reproducible Ig-degradation patterns [38], with V^+ isolates producing more heterogeneity in proteinase activities. In addition, proteinases promote cytoadherence [11, 12], mediate contact-dependent cytotoxicity [12], and hemolyze erythrocytes after hemagglutination as part of a nutrient acquisition system [15, 26]. Thus, any relationship between the presence/absence of virus with qualitative and quantitative levels of cysteine proteinases may be significant and result in direct influence of some or all of these properties, which in turn may affect virulence and pathogenesis. It will be of interest in the future to determine whether any of these proteinases are encoded by the virus, especially given the fact that, for most V^+ isolates, the virus is comprised of three dsRNA segments in addition to satellite dsRNAs [21, 23].

The use of the chemostat for cultivation of *T. vaginalis* was ideal for comparing V^+ isolates and corresponding agar-cloned V^- progeny trichomonads. We observed that V^+ *T. vaginalis* isolate 347 underwent periodic fluctuations in cell density, unlike the other V^+ isolates examined. As such, this may be the first time that a lytic event has been reproducibly recorded for a virus-harboring isolate of *T. vaginalis*. The culture density never equaled levels recorded for the V^- progeny even after 25 days (600 h) in the chemostat, perhaps indicating influence by the virus on parasite metabolism and/or utilization of nutrients.

Since only isolate 347 had growth fluctuations, the observations described here suggest diversity among the virus-harboring isolates, something consistent with the known divergence in the dsRNA genomes among trichomonad viruses [39]. It is equally plausible that other isolate-specific chemostat growth conditions, yet to be determined, such as a different pH, generation time or nutrient deprivation, could also produce fluctuations in growth kinetics for the other V^+ isolates. This possibility reinforces the need to continue examining the in vivo environmental cues or stresses that enhance dsRNA virus influence of expression of trichomonad proteins.

It is conceivable that the virus may encode for regulatory proteins that affect expression of trichomonad genes. This possibility would not be surprising since the V^+ isolates, compared to V^- parasites, have up-regulated levels of expression of the P270 immunogen [22] in addition to the property of phenotypic variation [7, 10]. This possibility also has precedence. The expression of regulators that modulate virulence-associated properties for the dsRNA virus infecting *Endothia parasitica* has been described [25]. Even more intriguing is the role of an endoribonuclease activity, should it exist in *T. vaginalis* as recently reported for the *Leishmaniavirus* [29], as a possible

regulator of the expression of trichomonad proteins. It has been suggested for *Leishmaniavirus* [29] that the viral capsid-endoribonuclease may cleave both virus and parasite mRNAs, thus influencing expression of cleavage-susceptible viral and trichomonad mRNAs [29]. In this scenario the virus may have an effect above that seen from encoding a regulator that acts directly on promoters for gene expression. Collectively, this analysis reinforces the idea that this virus is involved, either directly or indirectly, in the modulation of expression of trichomonad proteins.

Acknowledgements

We acknowledge Mike Lehker for helpful discussions, Jean Engbring for her critical review of the manuscript, Marie Crouch for kindly donating purified fibronectin, and Andrew P. Alderete for his dedicated technical assistance with the electrophoresis assays and affinity purification of fibronectin. We appreciate the excellent secretarial assistance of Tonya Williams. This study was supported by Public Health Service grants AI 39803 and AI 18768 from the National Institute of Allergy and Infectious Diseases, National Institutes of Health.

References

1. Alderete JF (1988) Alternating phenotypic expression of two classes of *Trichomonas vaginalis* surface markers. Rev Infect Dis 10 [Suppl 2]: S408–S412
2. Alderete JF, O'Brien JL, Arroyo R, Engbring JA, Musatovova O, Lopez O, Lauriano C, Nguyen J (1995) Cloning and molecular characterization of two genes encoding adhesion proteins involved in *Trichomonas vaginalis* cytoadherence. Mol Microbiol 17: 69–83
3. Alderete JF, Provenzano D, Lehker MW (1995) *Trichomonas vaginalis* resistance to complement is mediated by iron-inducible cysteine proteinase. Microbiol Pathol 19: 93–103
4. Alderete JF, Garza G, Smith J, Spence M (1986) *Trichomonas vaginalis*: electrophoretic analysis and heterogeneity among isolates due to high-molecular weight trichomonad proteins. Exp Parasitol 61: 244–251
5. Alderete JF, Newton E, Dennis C, Neale KA (1991) Antibody in sera of patients infected with *Trichomonas vaginalis* is to trichomonad proteinases. Genitourin Med 67: 331–334
6. Alderete JF, Newton E, Dennis C, Neale KA (1991) The vagina of women infected with *Trichomonas vaginalis* has numerous proteinases and antibody to trichomonad proteinases. Genitourin Med 67: 469–474
7. Alderete JF, Kasmala L, Metcalfe E, Garza G (1986) Phenotypic variation and diversity among *Trichomonas vaginalis* isolates and correlation of phenotype with trichomonal virulence determinants. Infect Immun 53: 285–293
8. Alderete JF, Suprun-Brown L, Kasmala L, Smith J, Spence M (1985) Heterogeneity of *Trichomonas vaginalis* and discrimination among trichomonal isolates and subpopulations by sera of patients and experimentally infected mice. Infect Immun 49: 463–468
9. Alderete JF, Suprun-Brown L, Kasmala L (1986) Monoclonal antibody to a major surface immunogen differentiates isolates and subpopulations of *Trichomonas vaginalis*. Infect Immun 52: 70–75

10. Alderete JF, Demes P, Gombosova A, Valent M, Janoska A, Fabusova H, Kasmala L, Metcalfe EC (1987) Phenotype and protein/epitope phenotypic variation among fresh isolates of *Trichomonas vaginalis*. Infect Immun 55: 1037–1041
11. Arroyo R, Alderete JF (1989) *Trichomonas vaginalis* proteinase activity is necessary for parasite adherence to epithelial cells. Infect Immun 57: 2991–2997
12. Arroyo R, Alderete JF (1995) Binding of specific *Trichomonas vaginalis* cysteine proteinases to host cells. Arch Med Res 26: 279–285
13. Coombs GH, North MJ (1983) An analysis of the proteinases of *Trichomonas vaginalis* by polyacrylamide gel electrophoresis. Parasitology 86: 1–6
14. Cotch MF, Pastorek JG, Nugent RP, Yerg DE, Martin DH, Eschenbach DA (1991) Demographic and behavioral predictors of *Trichomonas vaginalis* infection among pregnant women. The vaginal infection and prematurity study group. Obstet Gynecol 78: 1087–1092
15. Dailey DC, Chang T, Alderete JF (1990) Characterization of *Trichomonas vaginalis* haemolysis. Parasitology 101: 171–175
16. Dailey DC, Alderete JF (1991) The phenotypically variable surface protein of *Trichomonas vaginalis* has a single, tandemly repeated immunodominant epitope. Infect Immun 59: 2083–2088
17. Diamond LS (1957) The establishment of various trichomonads of animal and man in axenic cultures. J Parasitol 43: 488–490
18. Garber GE, Lemchuck-Favel LT (1989) Characterization of extracellular protease of *Trichomonas vaginalis*. Can J Microbiol 35: 903–909
19. Hochstrasser DF, Harrington M, Hochstrasser AC, Miller MJ, Merril CR (1988) Methods for increasing the resolution of two-dimensional protein electrophoresis. Anal Biochem 173: 424–435
20. Khoshnan A, Alderete JF (1995) Characterization of double-stranded RNA satellites associated with the *Trichomonas vaginalis* virus. J Virol 69: 6892–6897
21. Khoshnan A, Alderete JF (1993) Multiple double-stranded RNA segments are associated with virus particles infecting *Trichomonas vaginalis*. J Virol 67: 6950–6955
22. Khoshnan A, Alderete JF (1994) *Trichomonas vaginalis* with a double-stranded RNA virus has upregulated levels of phenotypically variable immunogen mRNA. J Virol 68: 4035–4038
23. Khoshnan A, Provenzano D, Alderete JF (1994) Unique double-stranded RNAs associated with the *Trichomonas vaginalis* virus are synthesized by viral RNA-dependent RNA polymerase. J Virol 68: 7108–7114
24. Laga M, Manoka A, Kivuvu M, Malele B, Tuliza M, Nzila N, Goeman J, Behets F, Batter Vm Alary M, Heyward WL, Ryder RW, Piot P (1993) Non-ulcerative sexually transmitted diseases as risk factors for HIV-1 transmission in women: results from a cohort study. AIDS 7: 95–102
25. Larson TG, Choi GH, Nuss DL (1992) Regulatory pathways governing modulation of fungal gene expression by a virulence-attenuating mycovirus. EMBO J 11: 4539–4548
26. Lehker ML, Chang TH, Dailey DC, Alderete JF (1990) Specific erythrocyte binding is an additional nutrient acquisition system for *Trichomonas vaginalis*. J Exp Med 171: 2165–2170
27. Lehker MW, Alderete JF (1990) Properties of *Trichomonas vaginalis* grown under controlled chemostat conditions. Genitourin Med 66: 193–199
28. Lockwood BC, North MJ, Scott KI, Bremner AF, Coombs GH (1987) The use of a highly sensitive electrophoretic method to compare the proteinases of trichomonads. Mol Biochem Parasitol 24: 89–95

29. MacBeth KJ, Patterson JL (1995) Single-site cleavage in the 5′-untranslated region of *Leishmaniavirus* RNA is mediated by the viral capsid protein. Proc Natl Acad Sci USA 92: 8994–8998
30. Minkoff H, Gruenebaum AN, Schwartz RH, Feldman J, Cummings M, Cromleholme W, Clark L, Pringle G, McCormick M (1984) Risk factors for prematurity and premature rupture of membranes: a prospective study of the vaginal flora in pregnancy. Am J Gynecol 150: 965–972
31. Müller M (1983) *Trichomonas vaginalis* and other sexually transmitted protozoan infections. In: Holmes KK, Mardh P (eds) International perspectives of neglected sexually transmitted disease. York Hemisphere Publ New York, pp 113–124
32. Neale KA, Alderete JF (1990) Analysis of the proteinases of representative *Trichomonas vaginalis* isolates. Infect Immun 58: 157–162
33. North MJ, Robertson CD, Coombs GH (1990) The specificity of trichomonad cysteine proteinases analyzed using fluorogenic substrates and specific inhibitors. Mol Biochem Parasitol 39: 183–194
34. O'Brien JL, Lauriano C, Alderete JF (1996) Molecular characterization of a third malic enzyme-like AP65 adhesin gene of *Trichomonas vaginalis*. Microb Pathogen 20: 335–349
35. O'Farrell PH (1975) High resolution two-dimensional electrophoresis of proteins. J Biol Chem 250: 4007–4021
36. Peterson KM, Alderete JF (1982). Host plasma proteins on the surface of pathogenic *Trichomonas vaginalis*. Infect Immun 37: 755–762
37. Provenzano D, Alderete JF (1995) Analysis of human immunoglobulin-degrading cysteine proteinases of *Trichomonas vaginalis*. Infect Immun 63: 3388–3395
38. Provenzano D, Alderete JF (1996) Unpublished observations
39. Tai J-H, Su HM, Tsai J, Shaio MF, Wang CC (1993) The divergence of *Trichomonas vaginalis* virus RNAs among various isolates of *Trichomonas vaginalis*. Exp Parasitol 76: 278–286
40. Tempest DW (1970) The continuous cultivation of microorganisms. 1. Theory of the chemostat. Methods Microbiol 2: 259–276
41. Wang A, Wang CC (1986). The double-stranded RNA in *Trichomonas vaginalis* may originate from virus like particles. Proc Natl Acad Sci USA 83: 7956–7960
42. Wang AL, Wang CC, Alderete JF (1987) *Trichomonas vaginalis* phenotypic variation occurs only among trichomonads infected with the double-stranded RNA virus. J Exp Med 166: 142–150
43. Zhang Z-F, Begg CB (1994). Is *Trichomonas vaginalis* a cause of cervical neoplasia? Results from a combined analysis of 24 studies. Int J Epidemiol 23: 682–690

Authors' address: Dr. J. F. Alderete, Department of Microbiology, UTHSCSA, 7703 Floyd Curl Drive San Antonio, TX 78284-7758, U.S.A.

Received August 7, 1996