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Phenotypes and Protein-Epitope Phenotypic Variation among Fresh Isolates of *Trichomonas vaginalis*

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Fresh isolates of *Trichomonas vaginalis* were examined for reactions to a panel of five monoclonal antibodies (MAbs). Four MAbs (C20A3, DM126, DM116, and C55) were to distinct surface immunogens and one MAb (L64) was to a cytoplasmic component. The fresh isolates were evaluated by indirect immunofluorescence (IF), immunoblotting, and radioimmunoprecipitation. IF assay with C20A3 MAb gave isolates which were homogeneous nonstaining (negative [Neg] phenotype) and isolates which were heterogeneous staining and nonstaining (positive [Pos] and Neg phenotype, respectively) organisms. Immunoblotting and radioimmunoprecipitation assays revealed that surface phenotypic heterogeneity among isolates with C20A3 MAb was due to the presence or absence of the immunogen from the parasite surface. IF assay with DM126 MAb also gave Pos and Neg phenotypes among parasites of some isolates. All of the isolates were always Neg phenotype with DM116 and C55 MAbs. The occurrence of Neg phenotype organisms with DM126, DM116, and C55 was due to epitope inaccessibility to their respective MAbs and not to the absence of the immunogen from trichomonal membranes. All isolates possessed the cytoplasmic protein recognized by L64 MAb. Paired isolates (taken 5 to 6 days apart) from 24 women were also studied. Four of the 24 paired isolates (16%) had different phenotype distributions at the two timepoints for C20A3. Fresh isolates also underwent phenotypic variation during in vitro growth and multiplication, as determined with C20A3. Also, 7 of the 24 paired isolates demonstrated dramatic changes in the accessibility of DM126 MAb to epitope binding. Lastly, 55 (90%) of 60 serum samples from patients with trichomoniasis evaluated in this study possessed antibody to the C20A3 reactive molecule. The data show that the fresh *T. vaginalis* isolates were predominantly Neg phenotype and confirm the occurrence of protein and epitope phenotypic variation for major immunogens among fresh isolates of the pathogenic human trichomonads.

The phenotypes of *Trichomonas vaginalis* isolates, as defined by indirect immunofluorescence (IF) assay with monoclonal antibodies (MAbs) on the basis of the presence on (positive [Pos] phenotype) or absence (negative [Neg] phenotype) of key immunogens from trichomonal membranes (5–8), may represent an important virulence determinant. It is interesting that trichomonads which are Neg phenotype still synthesize the molecule and can become Pos phenotype (7, 8). Recent data (6, 8), which show the ability of *T. vaginalis* isolates to undergo phenotypic variation, may help clarify a great deal of the reported antigenic heterogeneity of trichomonal isolates (11, 12, 17–21). Also noteworthy were correlations between the Neg phenotype of trichomonads and the enhanced contact-dependent cytotoxicity against HeLa cells (6).

Because only a few fresh isolates have been used in previous studies (3, 4, 6–8), it was important and necessary to evaluate a greater number of fresh isolates in order to better understand the role of phenotype in parasite virulence and disease pathogenesis. In this study, we used a number of immunologic assays, including IF, immunoblotting (IB), radioimmunoprecipitation (RIP), and flow cytofluorometry, along with five distinct MAbs to study further the phenotypic and other biological properties of 100 fresh isolates of *T. vaginalis*. We confirmed the findings of an earlier report (6) which showed the presence of only two types of trichomonal populations, i.e., those with homogeneous Neg phenotype and those with both heterogeneous Pos and Neg phenotypes, with respect to IF reactions with a specific MAb. We also demonstrated the phenotypic variation of isolates taken from the same patient at two different times and of certain isolates grown in vitro. Changes in the accessibility of an epitope of another major immunogen were also observed for trichomonads from patients. Finally, our data are very much in agreement with the previously reported extensive commonality of proteins in *T. vaginalis* isolates (2, 4, 6–8). The results of the present study reinforce the view that trichomonal antigenic distinctions are due to the expression of immunogens and, possibly, of key epitopes on trichomonal surfaces.

MATERIALS AND METHODS

Patients and parasites. The materials used in this study were obtained from patients attending the outpatient clinic at the Institute of Parasitology, Comenius University, Bratislava, Czechoslovakia, and from women being temporarily housed in a women's shelter in Bratislava. The women in the latter group were the source of paired isolates (taken 5 or 6 days apart) because these patients had no contact with males during the study. The clinical picture of the patients with trichomoniasis ranged from asymptomatic carriers to symptomatic carriers with inflammation and discharge.

Vaginal wash samples (2) were obtained from all patients. The trichomonads evaluated by IF and IB assays as described below were taken either directly from washes or after growth in Diamond medium (9) without agar. In the latter case, parasites were first cryogenically preserved after being extracted from washes and were grown in Diamond

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TABLE 1. Characterization of MAb reactions with *T. vaginalis* NYH286[a]

MAb	Reactions[b] with NYH286 and specific proteins (M_r)[c] by:		
	IF	IB	RIP
C20A3	+	+	+ (267,000)
DM126	+	+	+ (230,000)
DM116	−	+	+ (65,000)
C55	−	−	+ (65,000)
L64	−	+	− (31,300)

[a] NYH286 is a long-term-grown isolate used in our laboratory and has been characterized extensively (1–8).
[b] +, Positive reaction; −, negative reaction.
[c] M_r values were determined by electrophoretic characterization of the trichomonad protein antigens (7).

medium with agar and 1,000 U of penicillin and 1,000 μg of streptomycin per ml. Isolates were then incubated in Diamond medium (9) without agar but in the presence of antibiotics for no more than 3 days or until desired densities were achieved. Because past work (6) demonstrated the need for extended in vitro cultivation in order to detect phenotypic alterations, a period of no more than 3 days was considered reasonable to achieve the cell densities needed for analysis of fresh isolates. The representative isolates discussed in this report were also grown for several weeks in Diamond medium without agar and without antibiotics for flow cytofluorometric analysis (6).

IF, IB, and RIP assays. The IF assay was performed as recently described (1, 7, 8) with concentrated vaginal wash material or with trichomonads that had been axenically grown for ≤ 3 days, depending on when appropriate densities of parasites were obtained. Under no circumstances did the MAbs react with debris such as vaginal epithelial cells or with other contaminating microorganisms, including yeast, that could have been present in patient vaginal washes. At least 10 fields were examined for quantitation of fluorescent and nonfluorescent parasites. Test results were confirmed by the evaluations of at least two investigators.

The IB assay (22) was done for total protein precipitates of *T. vaginalis* (1, 13) grown as described above. Procedures for electrophoretic analysis of trichomonad proteins were as previously described (1, 13).

The RIP assay was performed with certain extrinsically labeled parasites of certain isolates as previously described (1). For the RIP assay, isolates with representative phenotypes, as defined by the IF assay, were grown in Diamond medium without agar for no longer than 5 days before cryogenic preservation. Trichomonads were then retrieved and grown overnight to desired densities for iodination, and RIP assay.

MAbs. Hybridomas synthesizing MAbs to *T. vaginalis* were produced as recently described (7), except when different antigen preparations as discussed below were used for immunization of BALB/c mice. Table 1 shows the MAbs and their reactions when evaluated by the various assays with the standard *T. vaginalis* NYH286 isolate used in our laboratory. The immunoglobulin G2a (IgG2a) MAb C20A3 directed to a trichomonad immunogen has been recently characterized (6, 7). MAb DM126 is an IgG2b which reacts with a surface proteinaceous component having an M_r of 230,000. DM116 and C55 MAbs are IgG1 and recognize comigrating surface polypeptides of 65 kilodaltons. L64 MAb is an IgG3 antibody to a 31.3-kilodalton cytoplasmic protein.

L64 was obtained from BALB/c mice immunized with trichomonad protein antigens of <65-kilodaltons. These antigens were generated by electroelution of proteins from preparative gels of total protein preparations of 4×10^7 trichomonads (1). Dialysis membranes in the sample cups of an electrophoretic concentrator (model 1750; ISCO, Lincoln, Nebr.) possessed M_r cutoffs of 3,000. Mice were immunized at monthly intervals for a total of 15 times, once with antigens in Freund complete adjuvant and 14 times with antigens in Freund incomplete adjuvant. Sera from the immunized mice were tested for antibody by enzyme-linked immunosorbent assay (2) before hybridization of spleen lymphocytes as recently described (7).

MAbs DM126 and DM116 were from mice that had been immunized with trichomonal membranes, partially purified as described for Leischmania sp. by Dwyer (10). Briefly, 5×10^8 parasites were washed three times in phosphate-buffered saline and were suspended to a 1-ml volume in 10 mM Tris hydrochloride (pH 8.0) containing phenylmethylsulfonyl fluoride, and the suspension was placed on ice for 20 min. The organisms were lysed with 15 strokes on a Dounce homogenizer, and the homogenate was centrifuged at 8,000 × *g* for 30 min at 4°C. The crude membrane pellet was then suspended to 4.0 ml in a solution of 146 mM sucrose prepared in 20 mM Tris hydrochloride–3 mM $MgCl_2$ (TM) buffer, pH 8.0. Membrane aggregates were visualized microscopically and were further disrupted by passage through a syringe or by vortexing. This material was centrifuged on a cushion of 73 mM sucrose for 30 min at 8,000 × *g*. The pellet containing enriched membranes was suspended to 0.24 to 0.48 ml in 146 mM sucrose-TM buffer, and portions of the mixture were centrifuged on a three-step sucrose gradient consisting of 0.75 ml (85%), 1.8 ml (75%), and 1.8 ml (60%) of sucrose. After 8 h at 42,000 × *g*, each fraction was aspirated and dialyzed overnight at 4°C against distilled, deionized water. Fractions enriched for membrane material, as determined by following the method for duplicate samples of iodinated organisms (1), were used for immunization as described for L64 above.

HeLa cell cytotoxicity assay. The quantitative colorimetric microassay used here for measuring the ability of *T. vaginalis* isolates to kill HeLa cells in monolayer cultures was as described previously (3).

RESULTS

IF assay for phenotypes of fresh isolates. MAb C20A3 (Table 1) was chosen for study because its reactions to *T. vaginalis* isolates are consistent with those of pooled sera from patients with trichomoniasis (7, 8). Also, the phenotypic variation of the specific trichomonad immunogen has been recently described (6). MAb DM126 (Table 1) was used because it reacts with another high-M_r protein immunogen which may also be involved in phenotypic variation.

Table 2 shows the reaction types of 100 fresh isolates examined. Both the homogeneous Neg phenotype and the heterogeneous Neg and Pos phenotypes were observed for trichomonads of fresh isolates when tested with either C20A3 or DM126. The presence of heterogeneous isolates, as determined by DM126 reactivity, was independent of the phenotypes of *T. vaginalis* isolates with C20A3. With both MAbs, ~70% of fresh isolates were homogeneous Neg phenotypes. Interestingly, for the isolates which were heterogeneous with C20A3, the fluorescent subpopulations ranged from 1 to 20%; however, the range of fluorescent parasites with DM126 was from 20 to 100% of the parent population. Fluorescence was not observed when the

TABLE 2. IF phenotypes of 100 fresh isolates of *T. vaginalis*[a]

Reaction type[b]	Phenotypes[c] with:		No. (%) of isolates with reaction type
	C20A3	DM126	
1a	−	−	69 (69)
1b	−	+/−	12 (12)
2a	+/−	−	11 (11)
2b	+/−	+/−	8 (8)

[a] Isolates of *T. vaginalis* from patients with trichomoniasis were tested for the presence of specific antigens. IF assay was performed on live organisms as described in the text.

[b] Reaction type designations assigned were based on the presence of homogeneous Neg phenotype (type 1) and heterogeneous Pos and Neg phenotype (type 2) populations of parasites for C20A3 MAb as described earlier (6). The DM126 MAb yielded subpopulations within type 1 and type 2 designations.

[c] −, Homogeneous Neg phenotype trichomonads; +/−, heterogeneous Pos and Neg phenotype organisms in the parent populations.

DM116, C55, and L64 MAbs were tested with the patient isolates, a finding which was consistent with results obtained with the laboratory isolate NYH286 (Table 1). Finally, no relationship between the trichomonad phenotypes with C20A3 MAb and the symptomology of the patients was found.

IB and RIP analyses of representative fresh isolates. Table 3 summarizes results from IB and RIP assays performed with select type 1 and type 2 isolates (Table 2). All trichomonad antigens, except the 65K protein that was reactive with C55 MAb, were detected by the IB assay. This protein band, however, was present on the surfaces of all isolates as determined by the RIP assay.

No iodinated trichomonad immunogens were detected by the RIP assay using C20A3 with Neg phenotype parasites, indicating that these molecules were absent from the trichomonal membranes. On the other hand, all representative isolates which were also Neg phenotype with DM126 by IF assay readily gave a protein band by RIP assay. A protein was also precipitated from detergent extracts of iodinated *T. vaginalis* with DM116 and with C55. These findings suggest that IF assay reactions with DM126, DM116, and C55 MAbs were due to the inaccessibility of epitopes to antibody binding rather than the absence of the immunogens from the trichomonal surfaces.

TABLE 3. Analysis by IB and RIP assays of representative fresh *T. vaginalis* isolates

Assay	Reaction type (no. tested)	Presence (+) or absence (−) of protein bands with:				
		C20A3	DM126	DM116	C55	L64[b]
IB	1a (19)	+[c]	+[c]	+	−	+
	1b (9)	+[c]	+	+	−	+
	2a (8)	+	+[c]	+	−	+
	2b (8)	+	+	+	−	+
RIP	1a (9)	−	+	+	+	+
	1b (0)	ND[d]	ND	ND	ND	ND
	2a (1)	+	+	+	+	+
	2b (6)	+	+	+	+	+

[a] Isolates having reaction patterns with C20A3 were used (Table 2, footnote *b*).

[b] For RIP, L64 did not precipitate any protein antigen from extracts of iodinated organisms. Therefore, the RIP assay was performed with [^{35}S]methionine-labeled parasites (1, 8).

[c] Protein bands detected by respective MAbs were apparent only after extended exposure of blots to substrate as described in the text. This was necessary because of the lower levels of specific immunogens synthesized by the Neg phenotype trichomonads (7).

[d] ND, Not done.

TABLE 4. Protein and epitope phenotypic variation for 8 of 24 paired isolates of *T. vaginalis* taken from the same patients at two different times[a]

Isolate pair	Days between isolation of organisms	Phenotype[b] with:	
		C20A3	DM126
417Cz-1		−	−
417Cz-2	6	−	+/− (20)
418Cz-1		+/− (1)	+/− (50)
418Cz-2	6	−	+/− (90)
AL10Cz-1		+/− (10)	+/− (10)
AL10Cz-2	5	−	−
AL20Cz-1		+/− (10)	+ (100)
AL20Cz-2	5	−	+/− (90)
AL32Cz-1		+/− (20)	+/− (20)
AL32Cz-2	5	−	+/− (20)
AL34Cz-1		−	+/− (90)
AL34Cz-2	5	−	−
AL35Cz-1		−	+ (100)
AL35Cz-2	5	−	−
AL37Cz-1		−	+/− (90)
AL37Cz-2	5	−	−

[a] Vaginal wash material at the two time points was used to inoculate medium for growth of *T. vaginalis* or was used directly in the IF assay depending upon the density of trichomonads in the vaginal washes. If needed, in vitro cultivation was performed only until a sufficient number of organisms were available for experiments.

[b] Phenotype designations based on fluorescence reactions: +, homogeneous population of Pos phenotype trichomonads; −, homogeneous Neg phenotype organisms; +/−, heterogeneous population with Pos and Neg phenotype parasites. Numbers in parentheses show percentage of Pos phenotype organisms in the + or +/− populations.

Protein and epitope phenotypic variation among fresh isolates. During this study it was possible to examine isolates obtained at two different times from 24 patients. Only 4 of 24 paired isolates demonstrated changes with C20A3 in the proportions of fluorescent parasites among the trichomonal parent populations (Table 4). In these four paired isolates, the heterogeneous populations, which constituted 1 to 20% of the fluorescent organisms, changed to homogeneous Neg phenotype trichomonads.

Table 4 also shows that DM126 MAb caused dramatic changes in the overall fluorescence patterns of 6 of the 24 fresh isolates. With DM126, the numbers of fluorescent organisms in the parent populations ranged from as high as 100% (Pos phenotype) to zero (no fluorescent parasites). Initial Neg phenotype isolates also yielded heterogeneous mixtures of parasites.

Lastly, we examined 16 fresh isolates by flow cytofluorometry with C20A3 (Fig. 1). The isolates were type 1 and type 2 (Table 2) and were passaged daily in complex medium. Six of seven type 1 isolates remained homogeneous Neg phenotype for C20A3 for several months of in vitro growth. Six of nine heterogeneous isolates became completely Neg phenotype after 7 days of in vitro growth and remained Neg phenotype for 6 months. The remaining four isolates had phenotypic variation during the 3-month time course.

Presence of antibodies to C20A3-reactive immunogens in

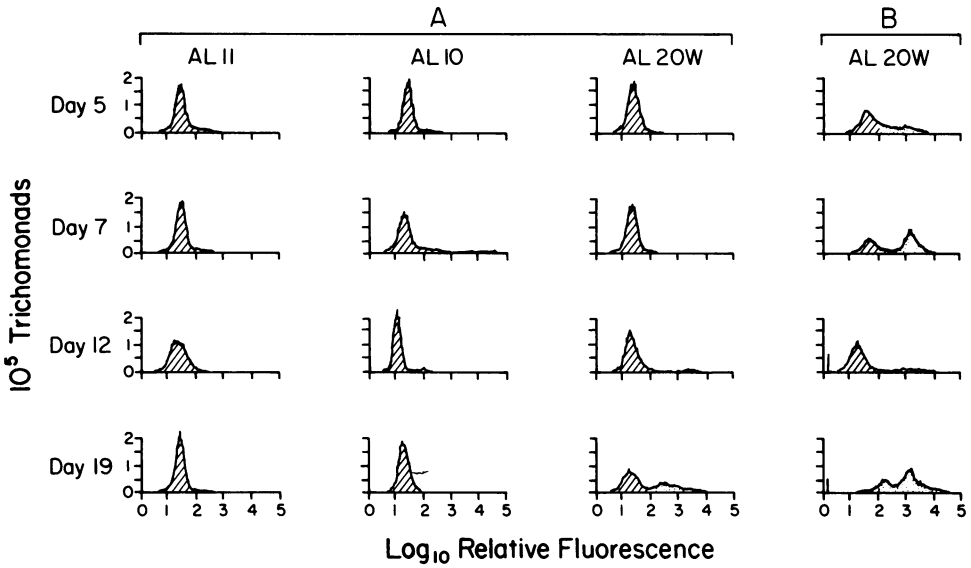

FIG. 1. Results of flow cytofluorometry with MAbs C20A3 (A) and DM126 (B) of representative fresh isolates from patients with trichomoniasis. The original phenotypes of trichomonads from vaginal washes were determined by IF assay. Originally, isolates AL11 and AL10 were homogeneous Neg phenotype and isolate AL20W was heterogeneous Pos and Neg phenotype. The absence of fluorescence or Neg phenotype as determined by the irrelevant MAbs of the same isotype but nonreactive with *T. vaginalis* is shown (▨) (6). Pos phenotype organisms were seen for AL20W with both MAbs C20A3 (day 19) and DM126 (days 5, 7, and 19) (▩). The number of days of in vitro culture are indicated on the left and are the same for all isolates.

sera of patients with trichomoniasis. We screened for antibody to immunogen in the sera of 55 patients with trichomoniasis. More than 90% of the serum samples yielded positive reactions when purified antigen (6) was tested by IB assay (Fig. 2). No bands were detected under the same conditions with control sera from uninfected humans.

HeLa cell cytoxicity. A total of 20 fresh isolates representative of type 1 and type 2 phenotypes (Table 2) were examined for their ability to kill HeLa cells. All of the isolates were maximally cytotoxic to HeLa cell monolayers. These results are in agreement with the ability of parasite populations which have Neg phenotype trichomonads to kill HeLa cells (6).

DISCUSSION

The phenotypes of trichomonal isolates, as defined by the surface disposition of specific immunogens (6–8), appear to be an important aspect of the biology of *T. vaginalis*. The ability to undergo phenotypic variation is also associated with well-defined virulence attributes, such as cytadherence-dependent cytotoxicity (6) and resistance to antibody killing (5). It was necessary to evaluate many fresh isolates in order to confirm the results of these earlier studies performed primarily with isolates grown long term (7, 8). Not unexpectedly, the representative fresh isolates were all cytotoxic to HeLa cells, since type 1 and type 2 isolates (Table 2) had Neg phenotype organisms. In fact, the type 2 heterogeneous populations were ≤20% Pos phenotype, and the Neg phenotype populations have been shown to possess increased rates of HeLa cell killing compared to pure Pos phenotype populations (6). Throughout this study, no relationship was apparent between type 1 and type 2 phenotypes of fresh isolates and host symptomology.

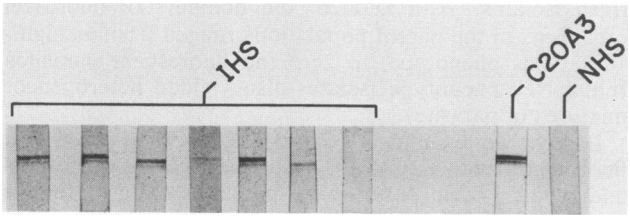

FIG. 2. Immunoblots of sera from trichomoniasis patients (IHS) and uninfected controls (NHS) and the C20A3-reactive immunogen. Purified trichomonad immunogen (1 μg) was electrophoresed and blotted onto nitrocellulose. Blots were then probed with sera from patients and from uninfected controls and with C20A3 MAb. Test procedures were the same as described elsewhere (7), except that peroxidase-conjugated goat anti-human IgG or anti-mouse IgG were used as needed for the second type of antibody.

Data presented here with C20A3 MAb (Table 2) confirm our earlier work (6, 7) concerning the existence of two types of parent trichomonal populations, i.e., homogeneous Neg phenotype isolates and heterogeneous Pos and Neg phenotype isolates. The phenotypic distinctions result from the surface disposition of the specific immunogen (6). The changes in phenotype seen for 4 of 24 paired isolates obtained at two different times of infection at least show, albeit in a minor way, the capability of *T. vaginalis* in humans to undergo phenotypic variation.

Epitope phenotypic variation (Table 2) was observed for the protein detected by DM126 MAb. This finding differed from the phenotypic distinction recorded with C20A3 MAb in that RIP analysis of trichomonads gave a protein band for all isolates regardless of reaction type (Table 3). The fluorescence patterns observed for DM126 were, therefore, due to the accessibility or inaccessibility of the reactive epitope on the surface protein. This observation illustrates the possible dynamic conformational changes of proteins occurring on the membranes of trichomonads.

Unlike DM126, MAbs DM116 and C55 have not yet produced fluorescence to live trichomonads (Table 1). The distinct proteins recognized by these two MAbs appear to be stable and present in all isolates. These MAbs, however, do

appear to be reactive toward inaccessible epitopes of the membrane proteins. The recognition by DM126, DM116, and C55 of surface proteins is supported by the removal by trypsinization of the protein from intact, motile parasites (3, 4) and also by the ability to radioiodinate the proteins on intact organisms (1, 7; unpublished observations). The fact that C55 cannot detect its respective antigen during IB assay may be indicative of the lability of the epitope under the denaturing conditions used in the preparation of proteins for electrophoresis and blotting. Finally, DM116 and C55 MAbs cross-react little, if at all, with their respective heterologous proteins, thus indicating that the MAbs are too different peptides of the same size (preliminary observations).

Most of the 16 fresh isolates examined by flow cytofluorometric analysis remained Neg phenotype for extended periods (Fig. 1), including 9 of 16 isolates originally heterogeneous for C20A3. Placement in complex medium or the loss of Pos phenotype organisms during in vitro cultivation may be responsible for these observations. Clearly, it is necessary to monitor the isolate populations for extended periods to better appreciate whether dramatic shifts from Neg to Pos phenotype will occur.

Examination of paired isolates taken from the same patients at two different times revealed the predominance of Neg phenotype in trichomonal isolates. The size of Pos phenotype subpopulations seen for C20A3 MAb never exceeded 20% of the total number of organisms. It is likely that the 5- to 6-day interval was not long enough for dramatic phenotypic changes in parasites to occur in these patients. On the other hand, trichomonads from 4 of 24 patients in which protein phenotypic variation was observed did not show enhanced ability to undergo phenotypic variation during in vitro cultivation.

The presence of antibody in the sera of trichomoniasis patients to the immunogen seen with C20A3 MAb is noteworthy (Fig. 2). C20A3 appears to be able to kill Pos phenotype trichomonads in vitro by a complement-independent mechanism (5). Thus, the presence of antibody to the molecule in patients suggests that host (immunologic) pressures, in addition to other considerations (13–16), influence the phenotype of infecting *T. vaginalis* populations. Clearly, the study of antibodies and other factors in the mucosal fluids of patients is important in studying mechanisms which may govern the in vivo phenotype of the pathogenic human trichomonads.

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