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Monoclonal Antibody to a Major Surface Glycoprotein Immunogen Differentiates Isolates and Subpopulations of *Trichomonas vaginalis*

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To produce monoclonal antibodies (MAbs) to highly immunogenic membrane proteins of *Trichomonas vaginalis* NYH286, the sera of subcutaneously infected BALB/c mice were first monitored for antibody to trichomonad surface proteins. The sera possessed antibody to one major surface protein by 7 days and antibody to numerous other trichomonad membrane proteins by 4 weeks postinfection. A hybridoma was then generated that synthesized an MAb, designated C20A3, which reacted to a parasite-derived glycoprotein possessing a molecular weight of 267,000 (267K glycoprotein). The immunogen corresponded to the single high-molecular-weight immunogenic surface protein recognized by 7-day mouse antisera. The MAb differentiated *T. vaginalis* isolates by a whole-cell enzyme-linked immunosorbent assay and by indirect immunofluorescence, using either fixed or live organisms. All isolates, however, possessed C20A3-reactive material when tested by enzyme-linked immunosorbent assay, using detergent extracts of the isolates incubated with MAb-coated microtiter well plates. The epitope was accessible to antibody binding on live *T. vaginalis* organisms expressing the major immunogen, and the 267K glycoprotein was readily removed from the parasite membranes by trypsinizing the intact trichomonads. The antigen incorporated radiolabeled glucose, mannose, and acetate. Also, an unlabeled 267K glycoprotein on nitrocellulose blots was detected by ^{125}I-concanavalin A and ^{125}I-wheat germ agglutinin, confirming the glycoprotein nature of the immunogen. Finally, of seven isolates used in this study, one possessed a cross-reactive 170K, rather than 267K, antigen. The data reinforce the idea that antigenic heterogeneity among *T. vaginalis* isolates may be a function of the presence or absence of high-molecular-weight glycoprotein immunogens from trichomonal membranes.

We attempted to understand the mechanism of previously reported antigenic heterogeneity among isolates of *Trichomonas vaginalis* (4, 8, 10, 19, 20). In a recent paper (4), sera from patients with trichomoniasis or from experimentally infected mice discriminated among *T. vaginalis* isolates and subpopulations of organisms from given isolates. The surface location of a group of high-molecular-weight (MW) proteins appeared to be responsible for the antigenic heterogeneity based on the antibody recognition of trichomonads (4). All isolates, however, synthesized the same complement of highly immunogenic proteins.

We felt it was necessary to attempt to generate a monoclonal antibody (MAb) toward key and important immunogens (1, 2, 4) to study in more detail this significant property of the parasite. It was essential to attempt to obtain a MAb that distinguished trichomonal isolates and subpopulations in a manner similar to that of sera from patients with trichomoniasis or sera of experimentally infected mice (4). In this study, we extended our earlier report (4) by examining the reactivity of a MAb to a high-MW, glycoprotein immunogen of *T. vaginalis*. The glycoprotein is one of the antigens contained within the repertoire of the numerous trichomonad surface proteins involved in the antigenic distinctions among *T. vaginalis* isolates (4). Data in this paper, therefore, further support the view that *T. vaginalis* isolates differ in their surface disposition of specific proteinaceous antigens (4) and show the diversity among isolates and subpopulations based on a specific surface marker.

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MATERIALS AND METHODS

Growth and radiolabeling of organisms. *T. vaginalis* strains are listed in Table 1 and have been described elsewhere (1, 4, 17, 18). Organisms were passaged daily in Diamond Trypticase-yeast extract-serum medium (BBL Microbiology Systems, Cockeysville, Md.) (6). Growth kinetics and incubation conditions are detailed elsewhere (14, 16). Intrinsic labeling was with 100 μCi of [^{35}S]methionine per ml (specific activity, 1,500 Ci/mmol; Amersham Corp., Arlington Heights, Ill.) (1, 4) and consistently yielded specific activities of 1.5×10^5 cpm/10^7 parasites (800 μg of protein). Surface radioiodination was performed as described before (1, 2, 14). The radiolabeling of parasites was also done by using individually 10 μCi of either ^{3}H-amino acid mixture (4×10^5 cpm/10^7 trichomonads), D-[U-^{14}C]glucose (200 mCi/mmol) (5×10^5 cpm/10^7 trichomonads), D-[U-^{14}C]mannose (200 mCi/mmol) (3.5×10^5 cpm/10^7 trichomonads), and sodium [U-^{14}C]acetate (50 mCi/mmol) (2×10^5 cpm/10^7 trichomonads) purchased from Amersham Corp.

SDS-PAGE and fluorography. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed by the method of Laemmli (11), using 1.5-mm slab gels consisting of a 3% stacking gel and a 7.5% separating gel (1, 14). After electrophoresis, the gels were fixed and dried, or processed for fluorography before being exposed to X-ray film (1, 14). For these experiments, X-ray film was exposed to radioactive gels for no longer than 3 weeks. MW standards were purchased from Bio-Rad Laboratories, Richmond, Calif.

ELISA and indirect immunofluorescence. The whole-cell enzyme-linked immunosorbent assay (WC ELISA) for the detection of antibody was performed, as recently published

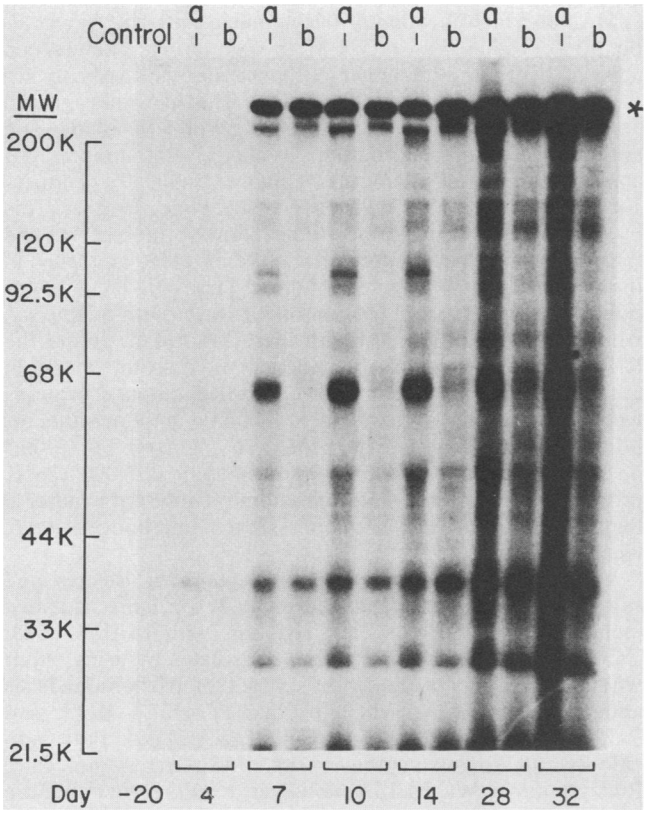

FIG. 1. Autoradiogram showing the time course response to iodinated trichomonad proteins to antibody present in sera of BALB/c mice subcutaneously infected with *T. vaginalis* NYH286. Pooled sera of mice were obtained before and after infection and diluted 1/10 (a) or 1/100 (b) in PBS before use in the Sol RIP assay as described in the text.

(3). A soluble-antigen ELISA (Sol ELISA) was performed as described below by using a detergent extract of *T. vaginalis* organisms obtained by the same method as the radioimmunoprecipitation (RIP) assay (1, 2). Briefly, 1 μg of ascites fluid containing MAb to the parasite antigen (see Fig. 2) was fixed onto individual wells of polyvinyl chloride microtiter plates (3, 22). After an overnight incubation at 4°C, the plates were rinsed three times with phosphate-buffered saline (PBS) bovine serum albumin (3) before use. Ascites containing MAb of the same isotype to *Mycoplasma pneumoniae* were used as a control.

Indirect immunofluorescence microscopy of trichomonads, using supernatants from antibody-producing hybridomas, was performed as recently described (2). When needed, immunoglobulin G (IgG) was purified by protein A-Sepharose (Sigma Chemical Co., St. Louis, Mo.) affinity chromatography and analyzed by SDS-PAGE for purity. MAb to *M. pneumoniae* was used as a control unless otherwise stated.

Sol RIP, WC RIP, and immunoblotting. The immunoprecipitation of trichomonad antigens was accomplished by using a Sol RIP and WC RIP, as recently described (1, 2). For the WC RIP, however, live, motile organisms were washed and suspended in 200 μl of heat-inactivated control or hybridoma supernatant. After a 30-min incubation at 37°C with continuous gentle rocking, the organisms were washed twice with PBS, pelleted, and solubilized with Zwittergent 3-12 (Calbiochem-Behring, LaJolla, Calif.) detergent, as before (1, 2). The remainder of the procedure was as described before (1, 2). The immunoblotting of trichloroacetic acid-precipitated trichomonad proteins (1) after SDS-PAGE (1, 14) was also performed as recently detailed (15). Lectin blotting with iodinated concanavalin A (ConA) and wheat germ agglutinin was done as described by Glass et al. (7).

The adsorption of the radiolabeled detergent extract of the MAb-reactive protein antigen was accomplished by the sequential adsorption of 100 μl of radiolabeled Zwittergent 3-12 trichomonal extract 10 times with *Staphylococcus aureus* pretreated with ascites containing MAb. In this case, 500 μl of washed *S. aureus* was pelleted and suspended with 1 ml of ascites fluid. After incubation for 30 min at RT, the bacteria were divided into 10 microfuge tubes and pelleted. The radiolabeled detergent extract was then incubated with individual microfuge tubes containing IgG-coated *S. aureus*. After the 10 adsorptions, the extract was used for Sol RIP (1, 2).

Trypsin treatment of intact *T. vaginalis*. The radioiodinated trichomonads (2) were suspended in 1 ml of PBS containing 1 mg of trypsin (Type XI; Sigma). The suspensions were incubated at 37°C for 15 min, and the enzymatic reaction was stopped by adding 2 mg of trypsin inhibitor (Type II-0; Sigma). The organisms were then washed with PBS before either the Sol RIP or WC RIP assay.

Antisera for RIP and immunization of mice for generation of primed spleen cells. Pooled sera from BALB/c mice infected subcutaneously with live *T. vaginalis* NYH286 (IMS) were obtained as described elsewhere (1, 3, 9). Parasites were always washed three times with PBS before challenge to remove contaminating medium components from trichomonad surfaces (14). Pooled normal mouse serum was used as a control.

Individual BALB/c female mice (3 to 6 weeks old) were subcutaneously inoculated with 5×10^6 washed *T. vaginalis* NYH286 organisms (1, 3, 9). Mice were challenged twice at 14-day intervals with a final booster inoculation 4 weeks later. The antibody response was monitored to ensure maximal IgG levels (3) to specific trichomonad surface proteins (Fig. 1). Mice were sacrificed 3 days later for hybridization of spleen cells.

Hybridization. The methodology for producing MAbs was based on the procedures of Oi and Herzenberg (13). Nonsecreting SP2/0-Ag14 BALB/c myeloma cells were used (12, 13). Spleen cells from immunized mice and myeloma cells were washed separately in serum-free medium and combined in a 7:1 ratio, respectively. After centrifugation at $400 \times g$ for 10 min, the supernatant was withdrawn, and the residual fluid was carefully removed with sterile swabs. The fusion was accomplished by using the details provided by Morrison-Plummer et al. (12). A total of 10% of the hybridomas produced antibody to *T. vaginalis* surfaces, as determined by WC ELISA (3). Cloning was done by limiting dilution in 96-well microtiter plates (Dynatech Laboratories, Inc., Alexandria, Va.) containing 10^5 BALB/c spleen feeder cells per well. Positive clones were then tested by Sol RIP for reactivity to specific high-MW trichomonad surface proteins. A MAb toward a major immunogen possibly involved in antigenic heterogeneity, as recently reported (4), was used for these studies. The production of ascites was accomplished, also as described elsewhere (13), after the mice were primed at 12 and 3 days before an intraperitoneal injection of 2×10^6 hybridoma cells. All hybridoma supernatants and ascites fluid were kept frozen at −20 or −70°C until use. An isotype analysis was performed by using the

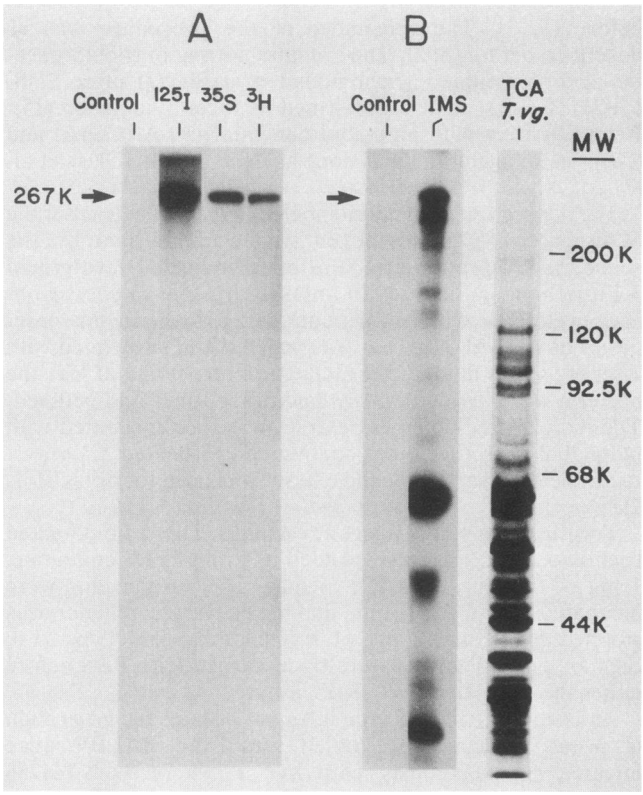

FIG. 2. Radioautography after Sol RIP and SDS-PAGE of [^{35}S]methionine, ^{3}H-amino acid mixture, and ^{125}I-labeled *T. vaginalis* isolate NYH286 Zwittergent 3-12 extract incubated with MAb C20A3. Hybridoma supernatant containing MAb of the same isotype to *M. pneumoniae* served as a control. Patterns obtained with pooled normal mouse sera (control) and sera from subcutaneously infected mice (IMS) in the Sol RIP by using an extract of iodinated trichomonads are also shown. TCA refers to the gel profile after fluorography of total trichloroacetic acid-precipitated proteins corresponding to 40 μg of protein with 15,000 cpm. WM, Molecular weight × 10^3 (K).

WC ELISA (3) with a mouse immunoglobulin subtype identification kit (Boehringer Mannheim Biochemicals, Indianapolis, Ind.).

RESULTS

Time course antibody response to surface protein antigens of *T. vaginalis*. To ensure the generation of a MAb to high-MW proteins (≥90,000 daltons) involved in the antigenic heterogeneity of *T. vaginalis* (4), we first monitored the IgG response to specific trichomonad surface proteins in the sera of BALB/c mice subcutaneously infected with isolate NYH286 (3, 9). Infection with this isolate was important because the key, high-MW proteins reside on NYH286 surfaces, and only those trichomonads with surface-exposed protein antigens elicit antibody during experimental infection (4).

High-titered IgG antibody was detected by 7 days postinfection of mice (Fig. 1) (3, 9). A 1:100 dilution of the antisera still possessed high levels of antibody to a distinct protein band, showing the highly immunogenic nature of certain surface proteins. The level of antibody to other surface protein antigens increased with time after the infection. No proteins were precipitated in the RIP assay with prebled normal mouse serum obtained 20 days before the infection of the same mice used in this experiment.

MAb and identification of trichomonad antigen. A hybridoma that produced an IgG2a MAb was derived from spleen cells of NYH286-infected mice possessing antibody to numerous trichomonad proteins (Fig. 1). This MAb, designated 5bB6 (C20A3), was evaluated by the Sol RIP assay. The autoradiogram and fluorograms of antigen immunoprecipitated from detergent extracts of either extrinsically or intrinsically labeled trichomonads gave a single band (Fig. 2). This protein possessed the same electrophoretic mobility as did the major immunogen precipitated when IMS was used in the RIP assay (Fig. 1 [asterisk] and 2 [arrow]). The adsorption of the radiolabeled trichomonal extract with *S. aureus*, which had been pretreated with the C20A3 MAb before the RIP assay, resulted in no evidence of a protein band in autoradiograms at the same MW. No trichomonad proteins were precipitated by control hybridoma supernatants or normal mouse serum. The protein recognized by C20A3 possessed a mean MW of approximately 267,000 (267K protein) based on five determinations. These data indicate that C20A3 is toward a major surface immunogen of *T. vaginalis*.

MAb C20A3 differentiation of *T. vaginalis* isolates and subpopulations. We next tested eight *T. vaginalis* isolates, including NYH286, for their reaction with MAb C20A3. Table 1 shows the MAb reactions of isolates by using either whole cells (3) or a detergent extract of trichomonads as antigen in an ELISA. Isolates like NYH272, IR78, and RU375 were positive only by Sol ELISA. These data indicate that all isolates synthesize a C20A3-reactive molecule. Recognition by WC ELISA, however, results from either the surface disposition or the quantitative differences among the isolates of the 267K antigen.

The various isolates were then examined further by indirect immunofluorescence. Figure 3 shows dark-field and fluorescence patterns of three representative isolates. For example, *T. vaginalis* NYH286 and JHH were both found to be positive by WC-ELISA, and isolate IR78 was unreactive by WC ELISA for C20A3. Interestingly, strong fluorescence was observed in approximately 90% of the NYH286 (Fig. 3B-1) and in about 70% of the JHH (Fig. 3B-3) trichomonads. Isolate IR78 was mostly nonreactive with

TABLE 1. Recognition of *T. vaginalis* isolates with C20A3 MAb by WC ELISA and Sol ELISA

Isolate designation[a]	Length of in vitro passage	C20A3 reactivity by:	
		WC ELISA[b]	Sol ELISA[c]
NYH286	≧2 yr	+	+
NYH272	≧2 yr	−	+
IR78	≧2 yr	−	+
30001	≧2 yr	+	+
JH31A	≧2 yr	+	+
RU375	≦1 wk	−	+
JHH	≦1 wk	+	+
JHHR	≦1 wk	+	+

[a] Isolates are as described in the text.

[b] *T. vaginalis* organisms (1.25×10^5) were ethanol fixed onto polyvinyl chloride microtiter plates, and ELISA was performed as described in the text. Mean values of triplicate samples, using hybridoma supernatants, exceeded the mean of hypoxanthine-aminopterin-thymidine medium control by no less than two standard deviations. Hypoxanthine-aminopterin-thymidine controls never exceed values of 0.025 ± 0.005.

[c] A 50-μl sample of a detergent extract corresponding to 5×10^5 trichomonads was added to wells coated with 1 μg of ascites of respective MAb or ascites containing an irrelevant antibody. Positive values ranged from 0.100 to 0.300 compared with minimal absorbance values (≦0.030) obtained for the controls. Antibody coating of individual wells is as described in the text.

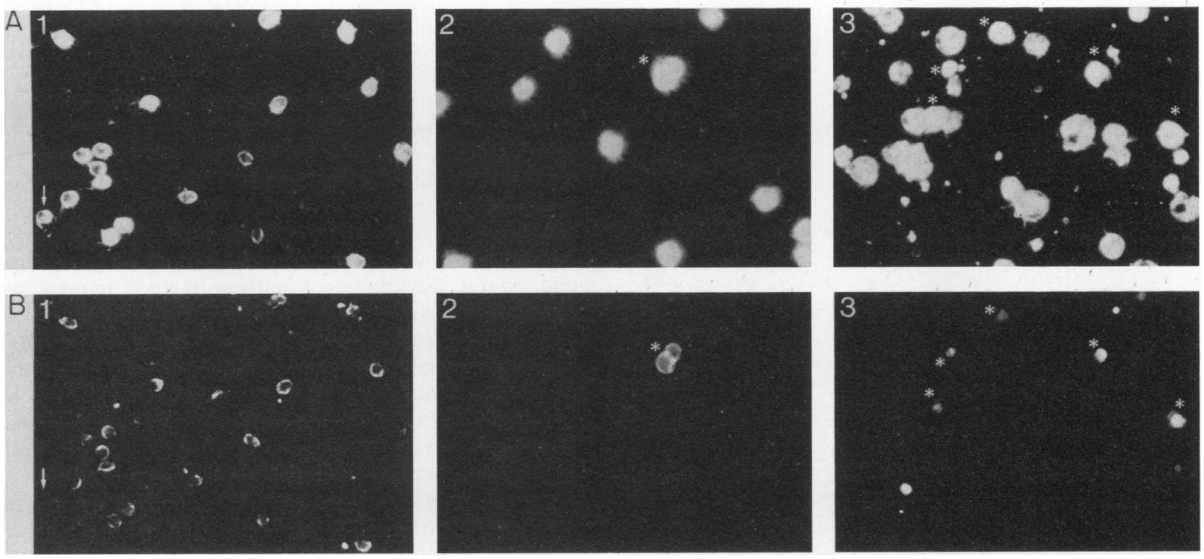

FIG. 3. Dark-field (A) and fluorescence (B) microscopy with hybridoma supernatant containing C20A3 performed with *T. vaginalis* NYH286 (panels 1), IR78 (panels 2), and JHH (panels 3). Arrows or asterisks indicate unstained or stained cells to corresponding dark-field or fluorescence fields. Note the differences in fluorescence patterns among the three isolates. No detectable fluorescence was obtained with a control hybridoma supernatant either with no antibody or with anti-*M. pneumonia* antibody.

C20A3, and in this trichomonad population, only ≤0.1% of the live parasites were fluorescent (Fig. 3B-2, asterisk). The fluorescence patterns were dissimilar among the isolates, perhaps indicating quantitative differences of the 267K antigen or cross-reactive material on trichomonads. Also, the numbers of organisms in a parent population of a given isolate parallel the antigenic distinctions presented in Table 1.

Partial characterization of the major protein immunogen of *T. vaginalis* NYH286. The accessibility to C20A3 binding of the antigen on live trichomonads was demonstrated by a WC RIP assay (2). Figure 4 shows the immunoprecipitation of the 267K protein after live, motile, iodinated trichomonads were incubated with C20A3 (lane b). As expected, the prior adsorption of C20A3 hybridoma supernatant with parasites and the pretreatment of radiolabeled organisms with trypsin resulted in no detectable bands by WC RIP (lanes c and d, respectively). These data indicate that the C20A3-reactive epitope is exposed on trichomonads of the NYH286 parent population.

In addition, the 267K immunogen was radiolabeled with glucose, mannose, and acetate (Fig. 4B, lanes a through c). No additional bands were seen by using these precursors for glycoprotein synthesis. The glycosylated nature of the 267K protein was further supported by the detection of unlabeled antigen blotted onto nitrocellulose with iodinated ConA (Fig. 4C, lane a) (7) and with iodinated wheat germ agglutinin (data not shown). The co-incubation of each ^{125}I-lectin with a respective competing sugar resulted in no detectable lectin recognition of the 267,000-MW band (Fig. 4C, lane b).

Immunoblot and RIP analysis of all isolates. We then performed immunoblots on the total protein preparations of all the isolates to detect the antigen reactive with C20A3. A protein band was readily detectable with C20A3 immunoblotting (Fig. 5A) of all the isolates positive by WC ELISA. Isolate JHH, however, yielded a band with a MW of approximately 170,000. Immunoblots with *T. vaginalis* 30001, JH31A, and JHHR gave patterns equal to NYH286.

Because IR78 and RU375 were negative by immunoblot,

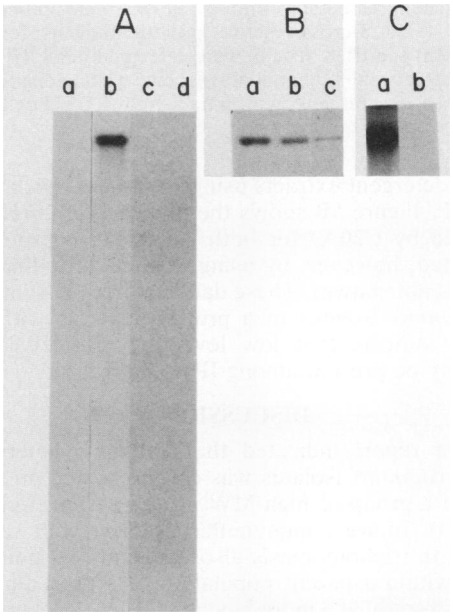

FIG. 4. Partial characterization of P267 of *T. vaginalis* NYH286. (A) WC RIP and SDS-PAGE autoradiography of iodinated trichomonads incubated with control hybridoma supernatant (lane a), C20A3 MAb (lane b), and C20A3 adsorbed with live organisms (lane c). Lane d shows the absence of P267 after trypsin treatment of iodinated parasites before addition of C20A3. (B) Autoradiograms showing P267 after Sol RIP with C20A3 and a detergent extract of trichomonads radiolabeled with glucose (lane a), mannose (lane b), and acetate (lane c) as described in the text. (C) Autoradiograms of nitrocellulose blots of unlabeled P267 after RIP electrophoresis with a detergent extract of unlabeled NYH286 trichomonads reacted with C20A3 and probed with ^{125}I-ConA (lane a) or ^{125}I-ConA in the presence of 1 mg of mannose per ml (lane b).

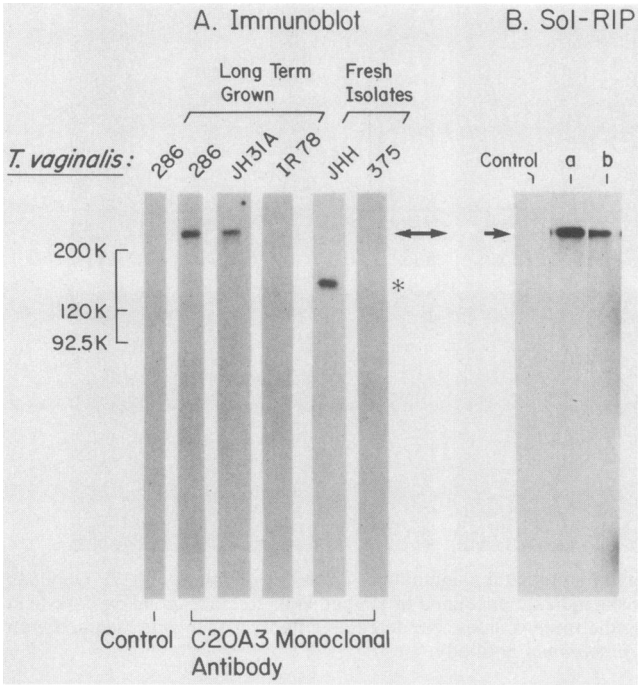

FIG. 5. Identification of C20A3-reactive material among *T. vaginalis* isolates by using the immunoblot (A) or Sol RIP (B) assays. (A) Immunoblot detection was performed by using trichloroacetic acid-precipitated proteins of individual isolates (1, 15). A corresponding NYH286 total protein blot was incubated with a hybridoma supernatant containing anti-*M. pneumoniae* antibody as a control. (B) C20A3-cross-reactive protein antigens for isolates RU375 (lane a) and IR78 (lane b) were detected by Sol RIP by using detergent extracts of [^{35}S]methionine-labeled trichomonads. Control refers to Sol RIP with detergent extract from RU375 or IR78 with anti-*M. pneumoniae* antibody.

we tested detergent extracts using ^{35}S-labeled trichomonads by Sol RIP. Figure 5B shows the presence of 267K antigen precipitated by C20A3 for both isolates. No protein band was detected, however, by using extracts of iodinated parasites (data not shown). These data confirm the synthesis by all *T. vaginalis* isolates of a protein reactive with C20A3 MAb and indicate that low levels of intracellular 267K antigen may be present among IR78 and RU375.

DISCUSSION

A recent report indicated that antigenic heterogeneity among *T. vaginalis* isolates was caused by the presence or absence of a group of high-MW proteins from trichomonal surfaces (4). Indirect immunofluorescence with sera from patients with trichomoniasis also distinguished nonreactive parasites within a parent population of representative isolates (4). To study at a more biochemical-molecular level the antigenic distinctions among trichomonal isolates and subpopulations, we attempted to generate a MAb with reactions similar to those observed for sera of patients with trichomoniasis or sera of experimentally infected aminals (1, 3). In this report, we present data that describe a MAb to a major trichomonad immunogen (Fig. 1) (2, 4) produced by all isolates (Table 1; Fig. 5). The antigenic distinctions of the isolates and subpopulations of a given isolate appear to be a function of the surface disposition of the trichomonad glycoprotein detected by C20A3 MAb (Table 1; Fig. 3 and 5). The results from these experiments support the idea that some *T. vaginalis* isolates synthesize, but do not externalize, a group of major immunogens involved in previously defined antigenic heterogeneity reactions (4, 8, 10, 19, 20, 23, 24).

Experiments (Fig. 4) involving the recognition of the C20A3-binding molecule by lectin blotting (7) and the incorporation of radiolabeled sugars or acetate strongly suggest that the 267K antigen is a glycoprotein. The MAb C20A3 appears directed at the protein moiety, since protease, but not periodate, pretreatment of trichomonads either before or after fixation for WC ELISA, abrogated antibody binding (data not shown). Recently, efforts have been made to understand the chemical nature of trichomonal surfaces through the use of lectins (8, 23, 24). For example, both conA and wheat germ agglutinin cause extensive agglutination of certain organisms of *T. vaginalis* isolates. Therefore, these data, along with a recent report (4), possibly illustrate a mechanism responsible for the pronounced differences in levels of lectin agglutination among *T. vaginalis* isolates (23, 24).

T. vaginalis NYH272, IR78, and RU375, which possess primarily nonreactive trichomonads by WC ELISA and indirect immunofluorescence (Table 1; Fig. 3), appear to have very low intracellular levels of 267K glycoprotein based on immunoblot assays (Fig. 5A). Both isolates, however, synthesize the high-MW immunogen as demonstrated by RIP by using a detergent extract of intrinsically labeled trichomonads (Fig. 5B). It is noteworthy that a few organisms ($\leq$0.1%) were found to be positive by indirect immunofluorescence for isolate IR78 (Fig. 3), but not for RU375. While the significance of this observation remains unknown, trichomonads of both isolates have remained almost totally nonstaining for $\geq$2 years, indicating that the absence of surface-associated 267K immunogen is a stable phenotype among some isolates of *T. vaginalis*.

The fact that JHH possessed a protein lower in MW than 267K, which was seen for all other isolates, illustrates the complexity in the antigenic composition of *T. vaginalis* organisms. While the reason(s) for the altered MW for the JHH antigen remains to be determined, preliminary experiments show no glycosylation of the protein by using those conditions employed for NYH286 (Fig. 4B and C). Therefore, reactions that show antigenic heterogeneity among trichomonads may also result from modifications of individual surface protein immunogens.

This MAb supports the hypothesis that *T. vaginalis* antigenic heterogeneity, previously characterized by a variety of immunologic methodologies (8), is caused at least in part to the presence or absence of key immunogens from the surfaces of trichomonads (4). Recently, others (5, 21) evaluated *T. vaginalis* isolates with MAbs to a trichomonad glycoprotein possessing a MW within the region involved in parasite distinctions (4) and found reactions consistent with those reported here. The absence of parasite antigen within one particular isolate (5) may be a reflection of quantitative differences in specific trichomonad proteins among isolates (Fig. 5), and this possibility requires additional attention. The synthesis of the same group of important immunogens (Fig. 1; Table 1) (4) indicates a need for caution in the designation of serological markers for *T. vaginalis* isolates (5, 21). Finally, of interest will be the stability and dynamics of the trichomonal populations of respective isolates for this and other important antigenic markers.

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