Heterogeneity of *Trichomonas vaginalis* and Discrimination among Trichomonal Isolates and Subpopulations with Sera of Patients and Experimentally Infected Mice

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The antibody response in trichomoniasis patients was examined with a variety of methodologies including enzyme-linked immunosorbent assays, indirect immunofluorescence, immunoblotting, and radioimmunoprecipitation-electrophoresis-autoradiography. Based on enzyme-linked immunosorbent assay recognition of trichomonal isolates, sera from patients with trichomoniases were categorized into reactive class I (IA, IB, and IC) and nonreactive class II sera. A diminished ability to precipitate antibody-binding trichomonal membrane proteins by the whole cell radioimmunoprecipitation assay was noted from class IA to class II sera. The antigenic distinctions among various *Trichomonas vaginalis* isolates appeared due to high-molecular-weight protein antigens detected by class IA sera in a whole cell radioimmunoprecipitation assay. The heterogeneity in antigenic patterns was confirmed among the isolates with sera from experimental animals. Also, live *T. vaginalis* cells appear to have only a few of the entire repertoire of major immunogenic surface proteins accessible to antibody binding. Immunoblotting demonstrated that the high-molecular-weight proteins responsible for trichomonal isolate heterogeneity are present in all isolates. The data suggest that trichomonads of a given isolate express only a subset of internally synthesized protein antigens on their surface. Importantly, the presence of these protein antigens on *T. vaginalis* membranes correlated with antibody production in subcutaneously challenged mice. Finally, indirect immunofluorescence studies with highly reactive, pooled sera from either patients or mice revealed a subpopulation of nonstaining trichomonads. These data support the view that heterogeneity among *T. vaginalis* is dependent upon the surface disposition of highly immunogenic protein antigens. Strategies may now be developed not only for studying potential vaccine reagents, but also for examining possible antigenic phenotypic variations in this experimental model.

Although it is becoming apparent that a complex host-parasite interrelationship exists during urogenital trichomoniases (1–5, 11, 13, 15, 16, 19–22, 24, 25), the mechanisms by which *Trichomonas vaginalis* mediates host cytopathogenicity are unclear. Little information is available, for example, on specific *T. vaginalis* properties or components that are involved in mediating disease pathogenesis.

Trichomonal strains appear to differ antigenically (1, 3, 12, 26–29) as assessed by various immunological approaches, but the relevance of these differences to parasite virulence and disease is unknown. Complicating matters is the paucity of information regarding specific trichomonal membrane components (2, 17, 18, 30), which may be responsible for any heterogeneity or which may be involved in disease pathogenesis (5, 8, 12, 13). It is clear that the identity and function of *T. vaginalis* surface protein antigens (1, 29) that regulate trichomonal heterogeneity must be determined, as has been accomplished for other pathogenic microorganisms (6, 7, 14).

Antibody to *T. vaginalis* in secretions and serum of infected persons has only recently been reported (3, 24, 25), but no data are available elucidating the parasite antigens recognized by these antibodies. It will be important to determine the role of specific immunoglobulin in either host protection by parasite elimination or in the development of host immunopathological reactions. Also, because no immunodiagnostic test for trichomoniases is available, knowledge of key immunogens common to all isolates will be essential for development of diagnostic assays, perhaps detecting antigen present in all patients with trichomoniases (4).

In this paper we attempt to determine the surface antigens that may mediate trichomonal heterogeneity by using antibody in sera of patients with trichomoniases and of infected animals (3). We show that membrane proteins are highly immunogenic and that the location of these immunogens may contribute to the antigenic diversity among pathogenic human trichomonads.

MATERIALS AND METHODS

Organisms and culture conditions. *T. vaginalis* strains NYH286, ATCC 30236 (JH31A) (13), RU375, and IR78 have been used before (1, 19). Organisms labeled JHH and JHHR were from different patients and were obtained by inoculating Diamond TP-S-1 medium (10) with a swab containing vaginal material from respective patients. After transfer, these fresh isolates exhibited growth properties in Diamond’s Trypticase (BBL Microbiology Systems)-yeast extract-maltose-serum medium (9) as described before (19). Fresh isolates were from asymptomatic women with vulvovaginal irritation and typical signs of vaginitis (purulent discharge, erythema of vaginal walls, and punctate hemorrhages), and cultures were grown in vitro in Trypticase-yeast extract-maltose-serum for no more than one week. Intrinsic and extrinsic radiolabeling of *T. vaginalis* used for radioimmunoprecipitation (RIP) assays was performed as detailed elsewhere (1, 2).

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Patients and antisera. Sera were obtained from patients with trichomoniasis (IHS), and pooled control normal human sera (NHS) were from various female donors with no history of trichomonal vaginitis. Normal and infected mouse sera (NMS and IMS, respectively) have been described elsewhere (1–3). IHS and IMS were nonreactive with medium components. Sera from individual rabbits immunized with T. vaginalis were those used and characterized in earlier studies (1, 2) and possessed antibody to trichomonad surface proteins as seen for IMS (see Fig. 3) (2, 4). Prebleed normal rabbit sera served as controls.

Sol and WC RIP and immunoblotting assays. The identification of immunogenic T. vaginalis proteins was a modification of an earlier published procedure (1, 2). For the soluble antigen (Sol) RIP (1), 100 μl of a Zwittergent 3-12 (Calbiochem-Behring Corp., La Jolla, Calif.) extract of fresh or frozen radiiodinated trichomonads was mixed with 200 μl of either undiluted NHS or IHS or with 100 μl of NMS or IMS. The remainder of the procedure was as described previously (1, 2). The analysis of immunogenic membrane proteins on T. vaginalis accessible to antibody was accomplished by a whole cell (WC) RIP procedure (2). Immunoblotting procedures have been described earlier (20), and rabbit serum reagents were primarily employed because of the larger volumes necessary for these experiments.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis and autoradiography. Solubilized, radiolabeled proteins of RIP samples or total trichloroacetic acid-precipitated trichomonad proteins (1, 20) were electrophoresed as detailed elsewhere (1, 19). Molecular weight (MW) standards were purchased from Bio-Rad Laboratories, Richmond, Calif.

ELISA, indirect immunofluorescence, and quantitation of lysis and agglutination by antibody. The detection of serum antibody from patients with trichomoniasis was by WC enzyme-linked immunosorbent assay (ELISA) (3). Indirect immunofluorescence was accomplished as recently detailed (2). The extent of complement-independent parasite lysis and agglutination resulting from incubation of organisms with heat-inactivated sera was assessed by measuring viable or nonagglutinated cells after a 20-min incubation of samples handled as for indirect immunofluorescence (2). A lysis and agglutination index was determined by using a 1+ to 4+ scale where 1+ represented a 0 to 25% lysis or agglutination of cells and a 4+ was indicative of 75 to 100% lysis or agglutination of trichomonads. Control NHS caused no lysis or agglutination of T. vaginalis, and parasites remained highly motile in NHS throughout the experiment.

RESULTS

T. vaginalis WC ELISA with sera from patients with trichomoniasis. Seven of 15 representative sera from patients with trichomoniasis (IHS) reacted with three long term-grown isolates (NYH286, JH31A, and IR78) and three fresh isolates (JHH, JHHR, and RU375) of T. vaginalis (class IA). Four additional IHS (no. 8 through 11) produced strong reactions with 286, JH31A, JHH, and JHHR (class IB); RU375 and IR78 were not readily detected by the class IB IHS. Class IC IHS (no. 12) recognized only two fresh isolates (JHH and JHHR). Finally, class II sera (no. 13 through 15) were nonreactive with trichomonads and gave values for the ELISA equal to those of pooled NHS controls. These designations of sera on the basis of WC ELISA isolate reactivity are representative of no less than 50 additional sera screened similarly. These initial data indicate a differential host antibody response among patients with trichomoniasis.

Antibody to trichomonad surface proteins in IHS. Experiments were then performed to understand the trichomonad antigenic distinctions among the serum classes. IHS were tested by WC RIP on the isolate routinely used in our laboratory, NYH286, to identify antibody-binding membrane proteins (2). Figure 1A shows the gel pattern of iodinated trichomonad proteins recognized by IHS antibody. Surface antigens were precipitated by all class IA IHS, and the extremely low intensity and number of bands seen with class IB through class II sera appeared due to low levels of antibody to individual immunogens (1). No precipitation of labeled proteins was ever observed with NHS.

We also conducted an Sol RIP with the various IHS to see whether antibody was present to iodinated membrane protein antigens not reactive by WC RIP (Fig. 1A). No new iodinated protein bands were detected, and the extent and nature of the reactions exactly paralleled those seen with the WC RIP (Fig. 1A). These data show that the antibody response among humans, if any, is toward highly immunogenic, exposed surface proteins of T. vaginalis.

We next pooled the class IA IHS and examined by WC RIP for the detection of trichomonad proteins among T. vaginalis isolates. Pronounced differences were observed in the antibody binding of surface proteins among the isolates (Fig. 1B). Only NYH286 and JHH gave strong similar reactions when compared with iodinated IR78, JH31A, and RU375. Similar results were obtained when intrinsically labeled trichomonads were employed in the WC RIP, demonstrating the inaccessibility of epitopes or the absence of the same protein antigens from the membranes of some of these isolates. These data illustrate the dramatic differences in the repertoire of surface antigens among the isolates.
Fluorescence, lysis, and agglutination of cells with class IA IHS. During the course of RIP studies, we performed indirect immunofluorescence with the pooled class IA IHS to correlate with the heterogeneity just presented. Figure 2 shows the fluorescence obtained with a representative long term-grown strain, NYH286 (Fig. 2, panel B1), and fresh isolate JHH (panel B2). Surprisingly, no fluorescence was evident among some trichomonads of each isolate (Fig. 2, arrows). A decreased intensity in fluorescence with heterogeneity was observed for trichomonad isolate JH31A. Both IR78 and RU375 gave very little fluorescence; only less than 1% of organisms gave clearly visible reactions. These data show the different distribution of antigens among isolates and also indicate a heterogeneity within a population of a given isolate. Since some fluorescence was observed in isolates such as IR78 and RU375, which react minimally by WC RIP (Fig. 1B), the data also suggest that fluorescence might principally be due to high-MW trichomonad antigens.

Data on lysis and agglutination of *T. vaginalis* isolates by class IA IHS were also obtained. For example, IHS no. 1 and 5 gave strong ELISA and fluorescence activity with all strains, but produced only 3+ lysis and 3+ agglutination of strain JHHR. Serum no. 4 produced 3+ lysis with no agglutination of only JH31A and JHH isolates, and all class IA sera (no. 1 through 7) failed to recognize isolates NYH286 and RU375 by these assays. Higher serum concentrations yielded no change in the agglutination and lysis patterns. These observations on lysis agglutination differences among isolates with the same or different IHS are consistent with the idea of an antigenic heterogeneity among the strains and subpopulations of each isolate.

Trichomonad antigen analysis with sera of experimentally infected mice. Mice infected subcutaneously with *T. vaginalis* NYH286 and other isolates (3, 13) represent a model for assessing the immune responses to trichomonad antigens (1). We therefore wanted to compare patterns of antibody reactivity of human patients (Fig. 1) with that of infected mice in an attempt to better understand and study antigenic heterogeneity.

Figure 3 shows that pooled mouse anti-NYH286 sera (IMS) possessed high-titered antibody to most or all NYH286 trichomonad membrane proteins as determined by Sol RIP (2). On the other hand, only iodinated protein bands of MW greater than 90,000 were prominent by WC RIP. Minor bands seen are the result of prolonged exposure of X-ray film. Antisera adsorbed against live NYH286 organisms did not precipitate any labeled proteins by either Sol or WC RIP, showing the reaction of antibody with only surface antigens of *T. vaginalis* (2). These data illustrate the highly immunogenic nature of most or all surface protein antigens and indicate that numerous lower-MW proteins on intact *T. vaginalis* are inaccessible or poorly reactive with IMS antibody.

A Sol RIP with individual extracts of 125I-labeled isolates and pooled mouse antiserum (IMS) to NYH286, which contained antibody to all high-MW antigens (Fig. 3), was then performed. IMS precipitated surface proteins to different levels for each isolate (Fig. 4, lanes b through f) when compared with the number and intensity of bands for NYH286 (lane a). No proteins were precipitated with NMS. Similar results were obtained when the iodinated isolates were evaluated by WC RIP with anti-NYH286 serum (data not shown), except that the major protein at about MW 65,000 was not detected (Fig. 3). Rabbit anti-NYH286 sera (IRS) (2, 4) gave identical results, reinforcing the idea that antigenic distinction among the isolates was indeed a property of the trichomonads. These data are consistent with the earlier experiments with pooled class IA patient sera and the different *T. vaginalis* isolates (Fig. 1A).

We next wanted to test whether the high-MW proteins
were indeed absent from isolates such as IR78 or RU375. Immunoblots with IRS revealed bands common to all isolates (Fig. 5B). Some minor differences in MW of major bands were noted (Fig. 5B, arrows) which were not apparent with higher acrylamide concentrations. Use of the antisera adsorbed with live NYH286 resulted in no detection of the high-MW proteins. As expected (4), except for minor differences in the intensity of some bands, similar results were obtained with pooled mouse antisera to NYH286. These data indicate that all T. vaginalis isolates possess the same high-MW immunogens that are present in NYH286 surfaces.

Finally, we tested whether infected mice make antibody to proteins absent from the surface of some T. vaginalis isolates. Figure 6 shows the different recognition of iodinated NYH286 proteins by respective antisera of long-term-grown and fresh isolates of T. vaginalis. Both anti-IR78 and anti-RU375 sera reacted little, if any, to antigens with MW ≥70,000, but did precipitate lower-MW proteins. As expected, no iodinated parasite proteins were recognized by the pooled NMS control. Although the immunogenic, high-MW antigens appear to be common to all isolates (Fig. 5), these data indicate that antibody synthesis during infection of mice is related to the surface disposition of T. vaginalis proteins. This may explain the serological reactivities of patients perhaps based on the surface composition of the infecting trichomonal isolate (Fig. 1A).

**DISCUSSION**

Both cellular and humoral immune responses by patients and infected animals follow T. vaginalis infection (1, 3, 11, 23–25, 27, 28), yet the contribution of host immunological mechanisms to resistance or resolution of trichomoniasis is unknown. Also, other studies with conventional immunological tools indicated an antigenic heterogeneity among the pathogenic human trichomonads (12, 26–28). This paper presents the application of a variety of immunological assays, including the RIP technique, to examine the antibody response among humans and animals to specific trichomonad membrane proteins and to gain insight into any antigenic heterogeneity for this model system.

It is noteworthy that all class IA sera from patients with trichomoniasis and infected mouse antisera (data not shown) discriminated unreactive subpopulations of trichomonads by indirect immunofluorescence (Fig. 2). These antisera also demonstrated a unique agglutination-lysis pattern despite antibody to several surface proteins as determined by RIP (Fig. 1). It may be possible to explain the data on the basis of either poor reactivity or reduced levels of antibody to most surface antigens on live parasites. Thus, small differences in antibody titer to surface antigens already known to be present in low copy number (2) might produce dramatic variations among the strains (Fig. 1). Also, a recent observation showed the release of high-MW membrane protein antigens during growth and multiplication (4); thus, an alternative explanation that accounts for unstained subpopulations of organisms (Fig. 2) may be the shedding or release of key proteins from trichomonal surfaces. Of importance will be future comparisons and characterization of

**FIG. 3.** Comparative autoradiograms after sodium dodecyl sulfate-polyacrylamide gel electrophoresis of [125I]-labeled T. vaginalis NYH286 proteins bound by antibody in NMS and IMS with Sol and WC RIPs. TCA refers to a total stained protein profile of trichloracetic acid-precipitated trichomonad proteins. Serum concentrations were 20%, and numbers refer to MW standards (K = 1,000).

**FIG. 4.** Radioautography after Sol RIP and sodium dodecyl sulfate-polyacrylamide gel electrophoresis of [125I]-labeled Zwittergent 3-12 T. vaginalis extract of various strains as indicated after incubation with antisera from mice infected with NYH286. NMS with iodinated extract of each strain failed to yield any protein bands, and a representative control RIP is shown with an extract of iodinated NYH286. Serum concentrations are 20%, and numbers refer to MW standards (K = 1,000).
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**FIG. 5.** Stained blots (A) and corresponding immunoblots (B) for detection of high-MW *T. vaginalis* antigens after sodium dodecyl sulfate-polyacrylamide gel electrophoresis with 6% acrylamide gels. (A) Bands showing proteins of the various isolates of *T. vaginalis* on stained nitrocellulose blots. (B) Proteins of the various isolates were blotted onto nitrocellulose and incubated with 20% antiserum from rabbits immunized with NYH286. Control refers to immunoblot of NYH286 with prebleed normal rabbit serum. Arrows show major bands common to all isolates, but which appear to vary in MW (K = 1,000).

Both Sol RIP and WC RIP assays showed poor antibody responses among patients to a major 65,000-MW protein and other surface antigens (Fig. 2). This is different from the high levels of antibody to most trichomonad antigens in sera of subcutaneously infected mice (Fig. 3, Sol RIP). All mice antisera, however, also failed to detect the 65,000-MW protein and other lower-MW proteins by WC RIP (Fig. 3: unpublished observations). This may indicate immunodominance and accessibility to antibody among epitopes of the high-MW proteins compared with the other surface antigens.

Major divergence among strains of the pathogenic human trichomonads appeared due to whether most trichomonads of a given isolate possessed the high-MW proteins on their surface. The data indicated that, since all strains synthesized the same reactive high-MW antigens (Fig. 5), the surface disposition of these proteins on *T. vaginalis* isolates was responsible for heterogeneity (Fig. 1A, 4, and 6). Trichomonads without the surface-exposed antigens generated little or no antibody to these molecules (Fig. 6), suggesting that variation in host antibody levels or responses was a function of antigen location. These data help explain past reports regarding antigenic heterogeneity among *T. vaginalis* (12). Ongoing studies in our laboratory show a commonality among isolates based on two-dimensional electrophoretic analysis (unpublished observations) and are in agreement with earlier reports showing an antigen homology among different isolates (1). Clearly, it will be important to develop probes, such as monoclonal antibodies, to these high-MW surface proteins not only to better study their contribution to *T. vaginalis* virulence and disease pathogenesis, but also to possibly fractionate populations of organisms (Fig. 2) with defined surface phenotypes. In addition, studies are aimed at cloning *T. vaginalis* and examining their surface antigenic composition as detailed in this report.

The significance of the presence or absence of a repertoire of high-MW antigens on *T. vaginalis* surfaces remains to be elucidated. A subpopulation of organisms of a given isolate without highly immunogenic proteins on their surface would appear to insure infection of a human host already possessing antibody in urogenital tissues or during menstruation.
when antibody can be made available to the area. Furthermore, it remains to be determined whether this aspect of heterogeneity is a form of phase variation (31) that contributes to trichomonal pathogenicity, possibly through immune evasion. We hope this study will ultimately help define at a molecular biochemical level the factors responsible for virulence among the pathogenic human trichomonads.

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LITERATURE CITED