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Immunogenic Proteins of *Trichomonas vaginalis* as Demonstrated by the Immunoblot Technique

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Fifteen clinical isolates of *Trichomonas vaginalis* were evaluated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis with transfer to nitrocellulose and by immunoblots probed with human sera. All *T. vaginalis* isolates showed similar banding patterns by Coomassie brilliant blue and silver staining of the electrophoresis gels and by amido black staining of the nitrocellulose. However, by the immunoblot technique, differences in banding patterns were noted, particularly in the high-molecular-weight zone ($>1.5 \times 10^5$), which were consistent in numerous experiments. A common immunogenic band was noted at a molecular weight of approximately 100,000 in all *T. vaginalis* isolates probed with six sera reactive in an enzyme-linked immunosorbent assay but was not seen or was only faintly visible when isolates were probed with sera considered to be nonreactive by the assay. Many other bands were identified, some of which appeared common to all isolates, but were not recognized by all sera tested. These studies demonstrate the antigenic heterogeneity of *T. vaginalis* and show that different individuals appear to respond immunologically to different *T. vaginalis* antigens.

Trichomonas vaginalis infection is one of the most common sexually transmitted diseases, estimated to annually infect 2.5×10^6 to 3×10^6 women in the United States and 1.8×10^8 women worldwide (5, 6). Despite the high prevalence, little is known about the pathogenesis or immunogenicity of *T. vaginalis*. A humoral and local antibody response has been detected by numerous techniques (1, 4, 11, 13, 16). By using these techniques, it has been possible to show some differences between strains of *T. vaginalis*, and it has been estimated that there are between two and eight serotypes (9, 10, 12, 15, 17, 18). The specific immunogenic differences among strains have not been well characterized, and previous studies with sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (SDS-PAGE) have shown similar banding profiles in different *T. vaginalis* isolates (2, 3, 14). The objective in this study was to evaluate further the humoral immune response to *T. vaginalis* and to identify immunogenic proteins of *T. vaginalis* by using the immunoblot technique with different human sera as probes.

MATERIALS AND METHODS

Strains. Isolates of *T. vaginalis* were obtained from vaginal secretions from women and from urethral and urine specimens from men. *Pentatrichomonas hominis* (ATCC 3000), a human gastrointestinal trichomonad, was purchased from the American Type Culture Collection, Rockville, Md. Organisms were grown in ambient air at 37°C in 13 ml of Diamond TY1-S-33 medium (7) supplemented with 10% heat-inactivated bovine serum in horizontally positioned glass screw-cap tubes (16 by 125 mm). Initial clinical isolates were cultured with penicillin (1,000 U/ml), streptomycin (1,000 μg/ml), and nystatin (100 U/ml). Cultures were passaged every 3 to 4 days, and antimicrobial agents were used only long enough to remove contamination. Purified cultures were mixed with an additional 15% bovine serum–10% dimethyl sulfoxide and stored in liquid nitrogen.

Sera. Blood was collected from patients presenting with vaginitis or urethritis in whom *T. vaginalis* infection was documented either by wet mount or by culture. The University of British Columbia Student Health Service kindly provided control sera, collected from students who had never had any sexual contact. Sera were stored at −20°C.

Isolate preparation and gel electrophoresis. Trichloroacetic acid (TCA) precipitates were prepared by the method reported by Alderete and co-workers (2, 3, 14). The isolates, which had been stored in liquid nitrogen, were quickly warmed to room temperature and inoculated into culture. After 48 h of growth, four culture tubes were centrifuged at $500 \times g$ for 10 min at room temperature. The pellets were combined into one tube and washed in phosphate-buffered saline (PBS; pH 7.2) three times by centrifugation at $500 \times g$. The pellet was then suspended in 10 ml of PBS, and the cells were counted (average, 10^7 trichomonads per ml). The cells were precipitated in 10% (vol/vol) TCA for 4 h at 4°C and then centrifuged at $10,000 \times g$ for 5 min at 4°C. The pellet was washed twice in cold PBS by centrifugation, and the final pellet was suspended in 200 μl of sample buffer containing 62.5 mM Tris hydrochloride (pH 6.8), 10% glycerol, 2% SDS, 2% mercaptoethanol, and 0.05% bromophenol blue as a tracking dye. After the sample was boiled for 3 min in the sample buffer, the supernatant was stored at −20°C (2, 14). Estimates of protein concentrations were made by the Lowry protein assay and were in the range of 26 to 31 mg/ml.

For the SDS-PAGE, stacking and separating gels of 3 and 7.5% acrylamide (Bio-Rad Laboratories, Richmond, Calif.) were prepared. A volume of 15 μl was added to each well. Electrophoresis was carried out by using a Protean Dual 16-cm slab cell apparatus (Bio-Rad) at a constant current of 15 mA (one gel) or 30 mA (two gels) for 1 h which was then increased to 30 mA (one gel) or 60 mA (two gels) for 3 to 4 h until the dye was near the bottom of the gel (2, 14). The gels were then either stained with Coomassie brilliant blue or silver stain (Bio-Rad) or transferred to nitrocellulose (see below).

Transfer to nitrocellulose and immunoblotting. The Bio-Rad technique, which is based upon the method of Towbin et

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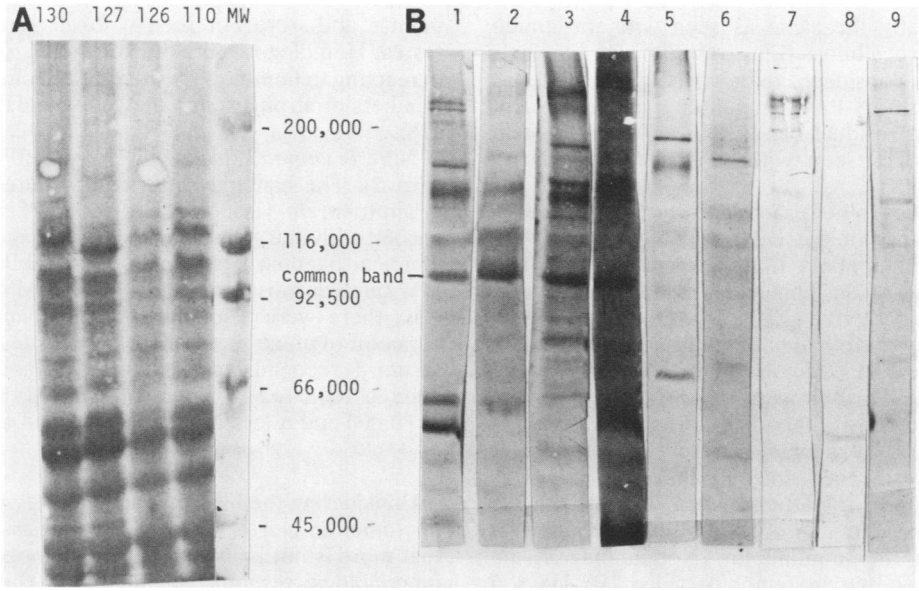

FIG. 1. (A) Amido black stain of four TCA precipitates of *T. vaginalis* after electrophoretic transfer to nitrocellulose MW standards. (B) Immunoblot of a single isolate (no. 146) of *T. vaginalis* probed with sera from four patients infected with *T. vaginalis* and seropositive by ELISA (lanes 1 through 4), sera from two patients infected with *T. vaginalis* but seronegative by ELISA (lanes 5 and 6) and sera from two control patients with no history of any sexual contact (lanes 7 and 8). Lane 9 contains *P. hominis* probed with serum 1.

al. (19), was used for immunoblotting. The nitrocellulose was hydrated in transfer buffer (20% [vol/vol] methanol, 25 mM Tris, 192 mM glycine; pH 8.3) at room temperature, aligned with the gel, and placed into the electroblotting apparatus (Bio-Rad) at 30 V overnight; the voltage was increased to 60 V for 2 h. The nitrocellulose was then placed in a blocking solution of Tris-buffered saline (TBS; pH 7.5)–3% gelatin and incubated for 1 h at 37°C. The first antibody solution, consisting of the probing serum diluted 1:50 with 1% gelatin–TBS, was then added and incubated overnight (approximately 18 h) at 37°C in a shaker incubator. The nitrocellulose was briefly rinsed with 100 ml of deionized distilled water, followed by two 10-min agitated rinses with 100 ml of TBS–0.05% Tween 20 at 37°C. The second antibody solution, which contained rabbit antihuman immunoglobulin G (IgG) conjugated to horseradish peroxidase (Sigma Chemical Co., St. Louis, Mo., or Boehringer GmbH, Mannheim, Federal Republic of Germany) was added at a dilution of 1:500 and incubated at 37°C and agitated for 1 h. After rinsing as above, the nitrocellulose was developed with Bio-Rad HRP developer (containing 4-chloro-1-naphthanol and 0.015% hydrogen peroxide) and photographed as soon as it was dry.

ELISA. An enzyme-linked immunosorbent assay (ELISA) with *T. vaginalis* whole cells was used to screen serum. *T. vaginalis* whole cells were washed three times in PBS as above and then suspended in carbonate buffer (pH 9.6). The cells were counted and diluted in carbonate buffer to a concentration of 2×10^5 cells per ml. A 0.1-ml portion was added to each well of an Immulon 1 microtiter plate (Dynatech Laboratories, Alexandria, Va.), and the plates were covered with aluminum foil, air dried at 37°C, and then stored at −70°C.

When used, the plates were thawed and washed three times with PBS–0.1% Tween 20–0.1% bovine serum albumin. A 0.1-ml portion of serum was added to each well, and the plates were incubated for 1 h at 37°C. The wells were then washed three times with PBS-Tween. A 0.1-ml portion of 1:500 rabbit antihuman IgG conjugated to horseradish peroxidase was added, and the plates were incubated at 37°C for 45 min. After three washes with PBS-Tween, 0.1 ml of 5-aminosalicylic acid–0.005% hydrogen peroxide was added to each well. After 30 min at room temperature, the optical densities (OD) of the samples were read at 449 nm.

RESULTS

ELISA. Ten clinical isolates of *T. vaginalis* were tested against 10 homologous sera. All sera were screened at a dilution of 1:32. Isolate no. 2, when used as the antigen, almost always yielded the highest ELISA OD regardless of the serum tested. Sera with high titers tended to give high ELISA OD readings when tested against all isolates, and sera with low titers gave consistently low readings against all isolates. There was no augmented response seen when an antigen was reacted with its homologous serum. The ELISA was standardized with sera collected from college students who had never had intercourse or any previous vaginal infections. Eight sera were used. The mean OD at a dilution of 1:32 plus 2 standard deviations was 0.104. This was defined as the cutoff between reactive and nonreactive sera. Serum specimens 3 and 4 were highly reactive (high OD; range, 0.700 to 0.900); sera 1 and 2 were also reactive (intermediate OD; range, 0.300 to 0.600). Serum samples 5 and 6, from two males with *T. vaginalis* urethritis, were nonreactive by ELISA (OD < 0.100), and control sera 7 and 8 were also nonreactive by ELISA.

SDS-PAGE and nitrocellulose. Figure 1A shows an amido black stain of TCA precipitates of four *T. vaginalis* isolates after electrophoretic transfer from a polyacrylamide gel to nitrocellulose. These different isolates appear to have virtually identical protein banding patterns, with 15 to 20 bands seen between molecular weights (MW) of 30,000 and 150,000.

Coomassie brilliant blue or silver staining of gels containing different *T. vaginalis* isolates also showed that the different isolates had the same banding patterns. These

patterns were virtually the same as seen with the amido black stain of the nitrocellulose (Fig. 1A). Transfer from gels to nitrocellulose was considered to be virtually complete, as silver staining of the SDS-PAGE gel after transfer to nitrocellulose showed no residual bands. No high-MW protein bands (>200,000) were seen with any of these staining techniques.

Immunoblot results. When samples were probed with human sera, regardless of the ELISA result, bands were seen at 150,000 MW and above that were not evident when amido black, Coomassie brilliant blue, or silver stain was used (Fig. 1 and 2). Reactive (ELISA-positive) sera, however, produced many more bands than did nonreactive sera at all MW ranges and in particular in the high-MW range (Fig. 1B and 2). These bands were reproducible when the same serum was reacted with the same *T. vaginalis* isolate in different immunoblot experiments. Different preparations of TCA precipitates from the same strain of *T. vaginalis* produced identical banding patterns when probed with the same serum. TCA precipitates of the culture medium produced no bands in the immunoblot. Nonimmune serum yielded few bands in the immunoblot (Fig. 1B, lanes 5 through 8). When the first antibody was omitted, no bands were detected with only the second antibody.

A single *T. vaginalis* isolate was probed with different ELISA-positive sera (Fig. 1B, lanes 1 through 4). In fact, all six positive sera tested (only four are shown) produced different banding patterns when probed against the same isolate. All six of the ELISA-positive sera showed a band at approximately 100,000 MW in the *T. vaginalis* isolate tested (one isolate and four sera are shown; Fig. 1B, lanes 1 through 4). The band was not seen on *P. hominis* (Fig. 1B, lane 9; Fig. 2) and was not detected or was only faintly seen when ELISA-negative sera were used (Fig. 1B, lanes 5 through 8). This band was not clearly identified with Coomassie blue, silver stain, or amido black (Fig. 1A). A strongly immunogenic band at approximately 60,000 MW was seen with sera 1, 2, and 4 but not with the highly reactive serum 3. Other common bands were seen but were not as intense and were not seen with all sera or in all isolates tested. Homologous sera did not show an enhanced response in reacting to homologous isolates and did not produce larger numbers or stronger immunogenic bands (Fig. 2, isolate no. 126).

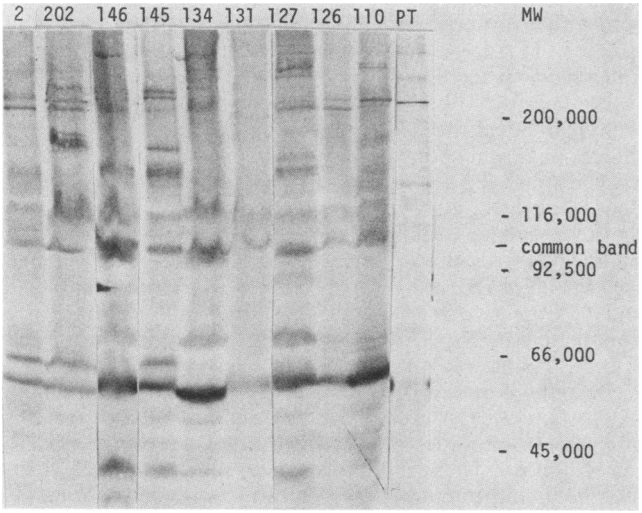

FIG. 2. Immunoblot of nine TCA precipitates of *T. vaginalis* probed with serum 1. Lanes 2, 202, 146, 145, 134, 131, 127, 126, and 110 all represent isolates of *T. vaginalis*. Lane PT represents *P. hominis*. Isolate 126 is the homologous isolate for serum-1. Heavy common immunogens are seen at 100,000 and 60,000 MW.

Nine *T. vaginalis* isolates were probed with a single serum (Fig. 2). The banding patterns of different isolates showed strain-to-strain variability. In total, 15 isolates have been probed with one or more sera, and none of the isolates have identical banding patterns. This variability was seen at all MW but was most evident in the high-MW zones. Nevertheless, there were also immunogenic bands seen that were common to many or all of the isolates tested. In all isolates, serum 1 recognnized the strongly immunogenic common band at 100,000 MW but also a strongly immunogenic band at 60,000 and a less immunogenic band at 116,000.

DISCUSSION

Considering the high prevalence of *T. vaginalis* infections in women in North America and worldwide, it is surprising that more is not known about the pathogenesis and the host immunologic response to infection. The presence of antibody has been previously detected by using a variety of techniques (4, 11, 13, 16), and the presence of serum serotypic heterogeneity has been previously reported (9, 10), but the specific immunogenic differences among strains have not been elucidated.

Alderete evaluated the serologic response in mice to *T. vaginalis* subcutaneous infection and identified possible immunogens. He found that different strains of *T. vaginalis* had similar protein profiles on SDS-PAGE and that there appeared to be a high degree of serologic cross-reactivity among the isolates (2, 3, 14). In this study, we found that the protein profiles of TCA-precipitated *T. vaginalis* seen on SDS-PAGE were similar, as was reported by Alderete. However, we extended this work by transferring the protein bands onto nitrocellulose and probing with human sera. In this way, we have demonstrated a strain-to-strain variability that was not seen with SDS-PAGE alone (Fig. 2). This variability was seen at all MW ranges but particularly in the high-MW zone (greater than 150,000) and was consistent in multiple immunoblot experiments.

All six ELISA-positive sera tested demonstrated a common band in all *T. vaginalis* isolates tested at the 100,000-MW range which was not seen or was only weakly visible when sera that were considered to be nonreactive by ELISA were used or when *P. hominis* was probed. In our work with the ELISA and immunoblot techniques, we have shown that the use of a serum homologous to an antigen does not enhance the ELISA titer or augment the number or the intensity of the bands seen in the immunoblot. This makes the search for common immunogens particularly appealing, and the common band at approximately 100,000 MW may be a common immunogen. Its purification would be particularly useful for a more sensitive serologic test, as a possible vaccinogen, and for studies of pathogenesis.

There were other common bands demonstrated (Fig. 2) which were not recognized by all reactive sera (Fig. 1B). In all isolates tested, sera 1, 2, and 4 yielded a strongly immunogenic band at 60,000 MW which was not seen with the highly reactive serum 3. The presence of common and unique antigens associated with *T. vaginalis* has been previously suggested in work with rabbit sera (8, 9). Our results confirm this finding in a reproducible and visual way. The serum-dependent band pattern variability may be due to individual variations in the immunologic response to this

complex organism. Factors such as multiple or previous exposure to different *T. vaginalis* isolates or the severity or duration of infection may be other possible explanations.

The immunogenic properties of *T. vaginalis* infection need to be further characterized. By using the sensitive technique of immunoblotting, we have been able to detect variability in the banding patterns of different isolates, recognition of common and distinct immunogens by different sera, and a common immunogenic band at 100,000 MW that was identified by all reactive sera tested. These techniques will be valuable for further assessment of the antibody response to *T. vaginalis* infection. The number of serotypes of *T. vaginalis* may be more accurately determined by analysis of additional isolates and sera.

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