JOURNAL OF CLINICAL MICROBIOLOGY, Nov. 1986, p. 790–795
0095-1137/86/110790-06$02.00/0

Vol. 24, No. 5

Immunoglobulin Isotypes of Anti-*Trichomonas vaginalis* Antibodies in Patients with Vaginal Trichomoniasis

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Received 30 April 1986/Accepted 12 August 1986

Studies of anti-*Trichomonas vaginalis* antibodies in patients with vaginal trichomoniasis were undertaken in attempts to identify the predominant antibody isotype produced and to delineate clinically significant antigens. The total antibody content of serum samples from 23 patients was determined by an enzyme-linked immunosorbent assay (ELISA) that employed anti-human immunoglobulin and isotype-specific antisera. The immunochemical reactivity of these antibodies was examined by Western blot analysis. The anti-*T. vaginalis* titer of all but two of these serum samples was greater than 200 (range, >200 to 12,800). By using an ELISA titer of at least 200 as a criterion, 21 of the serum samples contained antibodies of the immunoglobulin G (IgG) isotype, 17 contained IgM antibodies, and 6 contained IgA antibodies directed to the protozoan. Western blot analyses of these serum samples revealed approximately 29 antigenic trichomonad polypeptides, with apparent molecular sizes ranging from 14 to >100 kilodaltons and with individual serum samples possessing different patterns of reactivity. These results add to the current understanding of the serological and secretory immune responses to *T. vaginalis*, as well as define potential antigens for use in immunodiagnostics.

Trichomoniasis is a sexually transmitted disease caused by the flagellated protozoan *Trichomonas vaginalis*. An estimated 3 million individuals are infected annually in the United States (19). It is a major cause of vaginitis in women and has been associated with nongonococcal urethritis in men (8, 11, 21). Furthermore, reported sequelae observed with trichomoniasis include increased incidence of endometritis during pregnancy (18) and respiratory infections in infants born to infected mothers (17). Additionally, many individuals may be asymptomatic and thus serve as unsuspecting carriers (5). As diagnostic procedures improve and additional clinical data are accumulated, further morbidity may be associated with this infection.

Infected individuals produce humoral, secretory, and cellular immune responses to the organism, therefore allowing the potential use of immunologically based tests as diagnostic tools. Reactivity to *T. vaginalis* antigens has been demonstrated by passive hemagglutination (10, 28), complement fixation (9), enzyme-linked immunosorbent assays (ELISAs) (1, 2, 22), immunofluorescence (11, 23, 24), and lymphocyte transformation assays (15, 28), specifically with peripheral T lymphocytes (15). Despite the various techniques used to study the immune response to this organism, microscopic examination of genital secretions remains the most widespread method used for the diagnosis of trichomoniasis (19). Although this technique is rapid and inexpensive, under the best of circumstances it is only 50 to 70% sensitive (20). More sensitive, cost-effective procedures need to be developed.

In this study we measured immunoglobulin isotypes produced during the humoral immune response to the organism and attempted to identify key *T. vaginalis* antigens recognized by the individual immunoglobulin classes. In addition to providing a clearer picture of the immune mechanisms that are operative during infection, the identification of clinically significant antigens is a necessary prerequisite for further immunodiagnostic development.

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MATERIALS AND METHODS

Preparation of organisms. *T. vaginalis* ATCC 30236 was cultured under axenic conditions at 37°C in a modified Trypticase (BBL Microbiology System, Cockeysville, Md.) yeast-maltose media (pH 6.0) (4) supplemented with 10% heat-inactivated rabbit serum (Pel-Freeze, Rogers, Ark.). The organisms were routinely transferred every 3 to 4 days after reaching a density of 2×10^6 to 4×10^6 cells per ml and were >90% motile. After the cells were harvested by centrifugation at 1,000 × *g* for 10 min, the trichomonads were washed four times in phosphate-buffered saline (PBS; pH 7.2; Sigma Chemical Co., St. Louis, Mo.), and the final concentration of the organisms was adjusted by using a Neubauer counting chamber. The organisms were stored as pellets at −70°C. No deleterious effects on antigenicity were observed between frozen and freshly harvested organisms.

Patient sera. Serum samples were collected from 23 female patients at the City of Philadelphia Department of Health Sexually Transmitted Disease Clinic No. 1. These patients were diagnosed as having *T. vaginalis* infection by microscopic examination of wet smears. Serum was obtained from a female volunteer with no history of trichomonal vaginitis; this sample served as a parasite control serum sample. All serum samples were stored at −70°C until use.

ELISA. The anti-*T. vaginalis* antibody content of patient sera was assessed in an ELISA; both the total antibody response to the organism and the individual immunoglobulin isotypes were measured. Briefly, wells of a polystyrene microtiter plate (Immulon 2; Dynatech Laboratories, Inc., Alexandria, Va.) were coated with 10^5 trichomonads, and unreactive sites were blocked with 1% bovine serum albumin in PBS. Individual serum samples (prediluted to either 1:10 or 1:100) were added to wells in the first row and serially diluted twofold in 1% bovine serum albumin–PBS. Following an overnight incubation at 4°C, the plates were washed with PBS-0.05% Tween 20, and appropriate pretitered horseradish peroxidase (HRP) goat anti-human immunoglobulin reagents (Cooper Biomedical, Malvern, Pa.) were added to

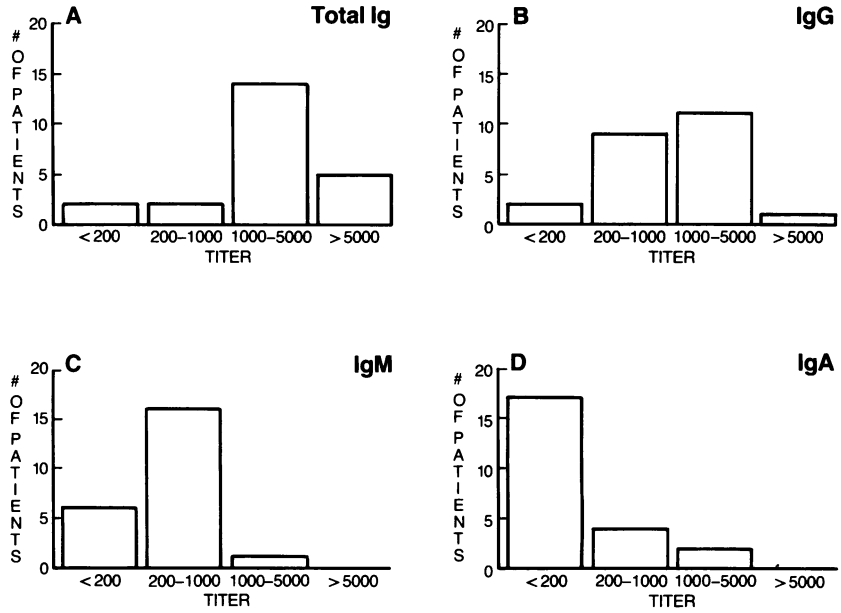

FIG. 1. ELISA titers of anti-*T. vaginalis* antibodies in the serum of 23 individuals infected with the protozoan. The humoral immune response to *T. vaginalis* was measured in an ELISA employing trichomonads immobilized on individual wells of a 96-well polystyrene microtiter plate and HRP-conjugated antibodies either pan-reactive with all human immunoglobulin (Ig) isotypes (A) or specific for IgG (B), IgM (C), or IgA (D).

each well. After a 2-h incubation at room temperature and another cycle of plate washing, reactivity was visualized by using 2,2'-azino-di(3-ethyl-benzthiazoline sulfonate) (Kirkegaard and Perry, Gaithersburg, Md.) as the substrate. The green color produced was measured at 414 nm in a microtiter plate reader. The endpoint titer was operationally defined as the highest dilution of patient sera which resulted in an optical density twice that of the negative control. The experiments described here were repeated three times, and results generally agreed within one dilution.

Secondary HRP-conjugated antibodies used in these studies included goat anti-human immunoglobulins (γ, μ, and α chain specific), goat anti-human immunoglobulin G (IgG) (Fc specific), goat anti-human μ chain, and goat anti-human α chain. These conjugates were pretitered on plates coated with human IgG, IgM, and IgA (Cooper) and were found to exhibit extremely good isotype specificity; that is, they showed only background-level activity on inappropriate isotypes. Additionally, in the absence of a human serum sample, these antibodies were devoid of any demonstrable activity on *T. vaginalis*-coated plates. Appropriate dilutions of these reagents were selected to yield approximately equal assay sensitivity to the three isotypes investigated.

Western blot. Polyacrylamide gel electrophoresis (PAGE), performed in the presence of the anionic detergent sodium dodecyl sulfate (SDS) and utilizing the discontinuous buffer system described by Laemmli (13), was used to separate the proteins of *T. vaginalis*. All electrophoresis reagents were purchased from Bio-Rad Laboratories, Rockville Centre, N.Y. In the studies reported here a 4% stacking gel and 10% separating gel were employed, and electrophoresis was carried out at 20 mA in the stacking gel and 30 mA in the separating gel. *T. vaginalis* antigens as well as marker proteins were boiled at 100°C for 5 min in the presence of the final sample buffer containing 0.0625 M Tris (pH 6.8), 2% SDS, and 5% 2-mercaptoethanol immediately prior to electrophoresis. Samples were loaded at 100 μg of protein per well. The protein concentration of each sample was determined by the procedure of Lowry et al. (14) prior to solubilization in SDS-PAGE sample buffer.

The resolved trichomonad polypeptides were transferred from acrylamide gels onto 0.2-μm-pore-size nitrocellulose paper (Schleicher & Schuell, Inc., Keene, N.H.) for 18 h at 30 V with the Tris-glycine-methanol buffer system described by Towbin et al. (25). Immunological detection of transferred trichomonad polypeptides was performed by the procedure described by Wos and Wicher (27). Briefly, nonspecific binding was blocked with 5% gelatin in Tris-buffered saline. Patient sera were diluted 1/20, overlayed onto the nitrocellulose, and incubated for 1 h at 37°C. The blots were then washed and incubated either with HRP-labeled protein A (Zymed Laboratories, South San Francisco, Calif.) diluted 1/3,000 for use as a general probe for anti-*T. vaginalis* antibodies or with isotype-specific conjugates (Cooper) diluted 1/500. After washing, bound HRP conjugate was detected by a reaction with 4-chloro-1-naphthol (Sigma). Two additional control experiments were performed to ensure that immunologically relevant bands were being developed in these experiments. Control blots (i.e., those in which the primary human serum sample was omitted and only the enzyme-labeled second antibody was added) failed to produce any observable bands. Furthermore, Western blots probed with an enzyme-labeled second antibody specific for rabbit immunoglobulins was also devoid of visible bands, suggesting that no spurious immunochemical reactions resulted from the passive uptake of material from the rabbit serum-supplemented growth medium in which the trichomonads were grown. Molecular weights were interpolated (26) from known molecular weight standards.

Fifteen patient serum samples were probed with HRP-protein A. Five of these serum samples were used for the isotyping experiments. The serum samples were chosen primarily because adequate supplies were available and they

TABLE 1. Antigens of *T. vaginalis* reactive with sera from patients with trichomoniasis[a]

MOLECULAR WEIGHT (10^3)	PATIENT NUMBER 1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
290	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
250	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
230	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
118		+		+		+	+	+		+				+	
86							+			+					
84				+		+	+			+					
76						+	+	+		+					
74	+			+	+			+					+		+
72			+					+			+				
68	+			+		+	+			+		+	+		
64	+	+	+	+	+	+	+		+	+	+		+	+	+
58	+			+		+	+	+	+			+	+		+
56	+	+								+					+
52		+	+	+	+			+	+		+		+	+	
46	+									+			+		
45		+		+	+			+				+			
42	+	+	+						+						+
40	+		+	+	+	+	+		+	+	+	+			
36	+		+	+		+	+		+	+			+	+	
34		+				+				+		+		+	
32		+		+		+	+			+		+		+	
29		+		+	+		+	+		+		+	+		
28							+			+	+		+		
24	+	+		+		+						+	+		
23			+		+	+			+				+	+	
20		+				+	+			+					
18	+	+		+	+	+	+						+		
13	+	+	+	+			+					+			
RECIPROCAL ELISA TITER:	3200	6400	1600	6400	3200	3200	12,800	1600	1600	6400	1600	3200	6400	3200	1600

[a] A plus sign indicates reactivity to a particular polypeptide, regardless of the intensity of the staining.

afforded a good representation of isotype-specific responses, as determined by ELISA.

RESULTS

Antibody titers to *T. vaginalis* in patient sera. An ELISA was performed on the serum samples of 23 patients diagnosed by wet mount as having vaginal trichomoniasis. Total immunoglobulin and isotype-specific antibody titers are shown in Fig. 1. The total antitrichomonad antibody titers of these serum samples (Fig. 1A) was, in general, quite profound. For example, in one titration, a 1/100 dilution of the control serum sample exhibited an optical density value of approximately 0.1, while 16 of the test serum samples had values of >1.0 at this dilution. By using an endpoint titer of >200 as a criterion for positivity, 21 of the 23 serum samples tested had significant antibody titers to the protozoan (range, 800 to 12,800); 19 of these serum samples exhibited titers of 1,000 or greater. Of the three immunoglobulin isotypes investigated, it was clear that the IgG response (Fig. 1B) predominated, with all of the 21 antibody-positive serum samples showing IgG titers of >200 (range, 400 to 12,800). In contrast, 17 of the antibody-positive serum samples had IgM titers of >200 (range, 200 to 1,600) and only 1 serum sample possessed an IgM titer of >1,000 (Fig. 1C). Six of the serum samples tested displayed significant IgA titers to the trichomonad (range, 200 to 1,600), with only two of these having titers greater than 1,000 (Fig. 1D).

Antibody reactivity to *T. vaginalis* polypeptides. The specificity of the antibodies produced by these patients was investigated in Western blot studies. Antibody reactivity to specific *T. vaginalis* polypeptides was detected initially with

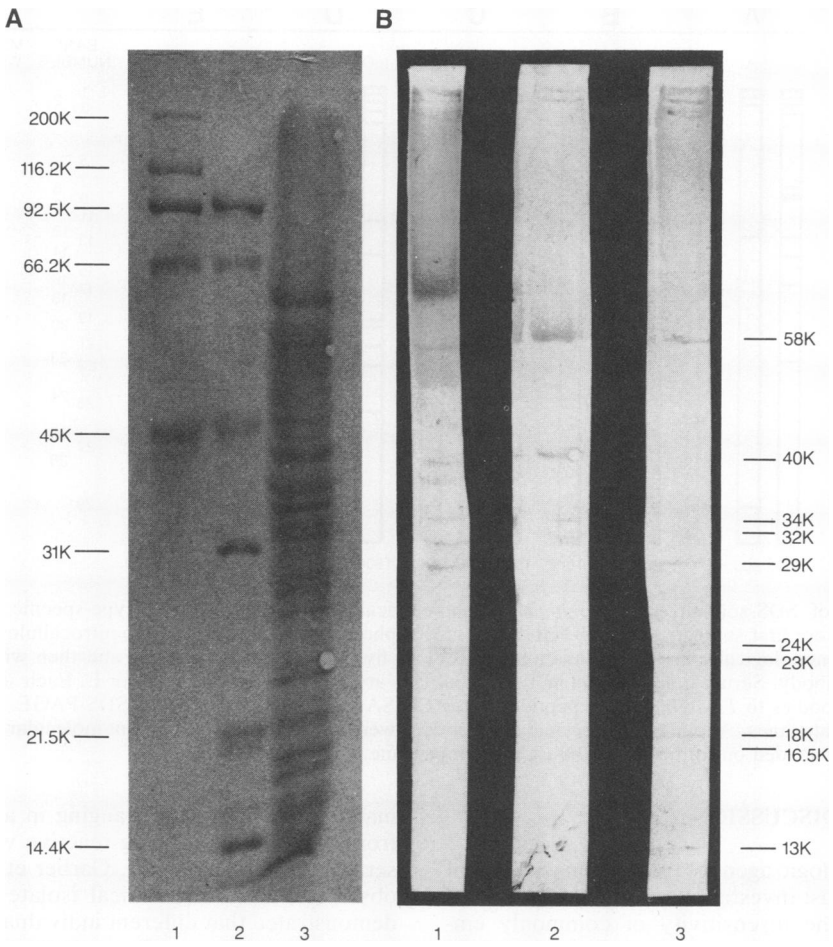

FIG. 2. Western blots of SDS-solubilized *T. vaginalis*. SDS-solubilized trichomonads were first subjected to SDS-PAGE and electrophoretically transferred onto nitrocellulose, and then the separated proteins were detected with either amido black staining (A) or immunologically (B). (A) Amido black staining of solubilized *T. vaginalis*. Lane 1, high-molecular-size standards; lane 2, low-molecular-size standards; lane 3, *T. vaginalis*. (B) Immunological detection of transferred *T. vaginalis* polypeptides. After tranfer to nitrocellulose, separated polypeptides were detected by incubating the blot first with a dilution of an individual patient serum and then, after washing, development of the blot with a HRP-conjugated anti-human immunoglobulin antibody. Lane 1, HRP-anti-IgG; lane 2, HRP-anti-IgM; lane 3, HRP-anti-IgA. Samples were loaded onto the gel at 100 μg of protein per lane. K is molecular size, in kilodaltons.

HRP-protein A. A total of 29 polypeptides of *T. vaginalis*, ranging in apparent molecular size from 13 to >200 kilodaltons (kDa), were found to be antigenic to the panel of 15 serum samples examined (Table 1). Each serum sample displayed a unique pattern of reactivity, both qualitatively and quantitatively. The largest number of polypeptides that were reactive with an individual serum sample was 18 (patients 4, 7, and 10) and the fewest number was just 8 (patients 11 and 15).

The specificity of anti-*T. vaginalis* antibody isotypes (i.e., IgG, IgM, or IgA) produced by five of these patients was also examined by immunodetection of Western blots. Data obtained in these studies are presented in Fig. 2 and 3. The entire profile of *T. vaginalis* polypeptides discernible on an amido-stained Western blot is shown in Fig. 2A. The antigens reactive with a serum sample from one of these patients when the blot was developed with isotype-specific conjugates are shown in Fig. 2B. The IgG antibodies recognized a broad molecular size range of antigens, while the IgM antibodies were reactive with 58-, 40-, 34-, and 32-kDa polypeptides. Furthermore, IgA antibodies also recognized the 58-kDa polypeptide and lower-molecular-size antigens, ranging from 13 to 40 kDa.

A schematic of Western blot patterns obtained from serum samples of all five patients after detection with isotype-specific conjugates is shown in Fig. 3. In general, IgG antibodies were found to be reactive with a broad range of antigens common among the patients. IgM and IgA antibodies, however, presented more selective reactivities. All the patients produced IgM antibodies directed to a 58-kDa polypeptide. In addition, four of the serum samples had reactivity to 40- and 13-kDa polypeptides, three contained IgM antibodies that recognized a 34-kDa protein, and two contained IgM antibodies that recognized a 32-kDa polypeptide. Those patients with IgA titers of >200 produced antibodies that were reactive with lower-molecular-size antigens, ranging from 13 to 28 kDa. Of particular interest were the serum samples from the three patients with high IgA titers that demonstrated reactivity with 16.5- and 18-kDa polypeptides. All five of these serum samples contained antibodies reactive with high-molecular-size polypeptides (>200 kDa) which were represented by all three isotypes.

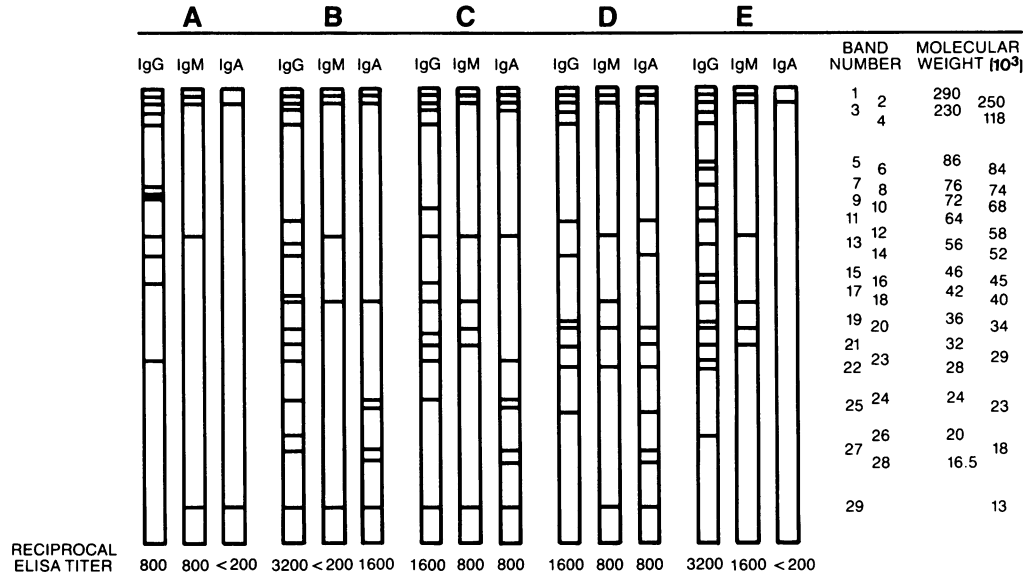

FIG. 3. Western blots of SDS-solubilized *T. vaginalis*: immunological development with isotype-specific second antibodies. SDS-solubilized trichomonads were first subjected to SDS-PAGE and electrophoretically transferred onto nitrocellulose, and then the separated proteins were detected immunologically first by incubation with one of five patient serum samples and then with an enzyme-conjugated, isotype-specific second antibody. Serum samples from individual patients are designated A, B, C, D, or E. Each serum sample was analyzed for IgG, IgM, and IgA antibodies to *T. vaginalis* polypeptides in the ELISA prior to the initiation of SDS-PAGE. The specific titer obtained is listed below the appropriate lanes. A total of 29 antigenic polypeptides were discerned; their apparent molecular sizes are given to the right of the figure. Samples were loaded on to the gel at 100 μg protein per lane.

DISCUSSION

T. vaginalis is the etiologic agent of trichomoniasis, one of the most frequent yet least investigated sexually transmitted diseases. Because of the insensitivity of commonly employed diagnostic methods (20) it is difficult to know its prevalence with any precision. However, it is frequently described as the most prevalent sexually transmitted disease, infecting an estimted 180 million people a year worldwide (8). In addition, pathological conditions ascribed as a consequence of trichomonad infestation such as vaginitis (19) and pulmonary infections (7, 17) substantiate the viewpoint that these protozoans are important human pathogens. Although it is generally accepted that patients mount both a systemic antibody and local secretory immune response, the role of these antibodies during the course of infection and the identity of the antigens to which they are directed are not known. Results of the studies reported here represent attempts to define, utilizing a sensitive ELISA and Western blotting, the immunoglobulin isotypes produced during the human humoral response to *T. vaginalis* and the specific polypeptides to which these antibodies were directed.

ELISA results of serum samples from 23 patients demonstrated that a high percentage of these samples (21, 91%) possessed antibody titers of >200 against *T. vaginalis*. Furthermore, the predominant immunoglobulin in these serum samples was IgG, while 17 (81%) of the samples contained IgM titers and 6 (26%) exhibited IgA titers. These findings are in agreement with those of other reports (12, 16, 22) of seropositivity associated with infection. The possible correlation of the appearance of these isotypes in serum with the course of infection is presently unknown.

An examination of the antigens of *T. vaginalis* reactive with 15 patient serum samples was studied by Western blotting with HRP-protein A as a general probe. Each serum sample had a unique pattern of reactivity, with approximately 29 polypeptides ranging in apparent molecular size from 13 to 290 kDa being reactive with one or more of the serum samples. Recently, Garber et al. (6) reported similar observations. Using clinical isolates of *T. vaginalis*, they demonstrated that different individuals varied in their immunological response to *T. vaginalis* antigens. Delineation of important immunogens requires further study.

We addressed the problem of defining *T. vaginalis* antigens by examining the immunochemical specificity of human IgG, IgM, and IgA antibodies to the protozoan produced by five patients by Western blot analysis using isotype-specific conjugates. The examination of isotype specificity to *T. vaginalis* antigens has not been previously reported. Although many generalities were not clearly discernible by protein A studies, a number of similarities among the isotype reactivities of the serum samples of these patients was observed. First, all the patients produced IgG, IgM, and IgA antibodies which recognized one or more high-molecular-size polypeptides (>200 kDa). Second, the greatest number of polypeptides was recognized by IgG antibodies. This observation was not surprising since it is well established that IgG antibodies are produced during primary and secondary immune responses and is the predominant immunoglobulin subclass present in the circulation. However, antigens were identified which elicited an apparent isotype-specific response. All five serum samples of these patients had IgG antibodies that reacted with a 118-kDa polypeptide and contained IgM antibodies reactive with a 58-kDa polypeptide. Although the data were obtained from a small sample of patients, these antibodies may have been elicited to a surface antigen of the protozoan described by Connelly et al. (3). Using monoclonal antibodies, these investigators delineated a surface antigen of *T. vaginalis* composed of 115- and 58- to 64-kDa polypeptides. Further studies are required to determine whether this antigen is species specific and, thus, may provide utility as a diagnostic marker. In addition,

four serum samples demonstrated reactivity to 40- and 13-kDa polypeptides, suggesting that these antigens, as well as the 58-kDa polypeptide, may be recognized early in the disease process. Furthermore, these four serum samples also contained IgA antibodies reactive to the 13-kDa polypeptide, and all three serum samples with a high titer of IgA recognized two polypeptides of 16.5 and 18 kDa which appeared to be unreactive with IgG and IgM antibodies. This finding may have particular relevance for potential vaccine production, as IgA is known to be the predominant isotype produced in vaginal secretions of infected women (23; R. M. Watt, G. J. Sam, and S. M. Wos, unpublished data).

Currently, a number of investigators are focusing on the dissection of the antigenicity of *T. vaginalis*. Results of the studies reported here, as well as those of others, will aid in the elucidation of the membranous moieties that are important in the pathological process, as well as afford focal points for the targeting of monoclonal antibodies for use in immunodiagnostics.

ACKNOWLEDGMENTS

We thank R. G. Charrar, H. Heineman, and M. Goldberg of the City of Philadelphia Department of Health, Sexually Transmitted Disease Clinic No. 1, for providing the patient sera and Abraham Philip for his assistance as liaison.

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