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Antibodies to *Trichomonas vaginalis* surface glycolipid

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Abstract

Background—Human trichomoniasis is the most common non-viral sexually transmitted disease, yet immune responses are not well studied.

Methods—Since the *Trichomonas vaginalis* lipophosphoglycan (TvLPG) is an important virulence factor, a bank of eight monoclonal antibodies was generated to define the antigen in clinical isolates. The TvLPG-specific antibody response of women who were culture positive (n=33) or negative (n=33) for *T vaginalis* infection was determined by isotype-specific ELISA.

Results—The bank of monoclonal antibodies reacted with conserved surface TvLPG epitopes in 27 isolates from pregnant women at their first prenatal visit. Conserved TvLPG epitopes were shown to be surface exposed by immunofluorescence. Sera collected from the same patients at the same time were assayed for specific antibodies. Serum and vaginal secretions from 33 *T vaginalis*-

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positive women had statistically higher IgG anti-TvLPG levels than age-matched and race-matched negative controls in the same clinical study ($p<0.01$). Vaginal IgA anti-TvLPG levels of the women with trichomoniasis were almost significantly higher than controls ($p=0.055$). Infected women with normal pregnancies had significantly higher vaginal IgG anti-TvLPG values than infected women with adverse outcomes of pregnancy.

Conclusions—These antibody responses show that infected women can respond to the conserved TvLPG antigen. Since antibodies to trichomonad surface LPG protect in a bovine model of trichomoniasis, the role of these antibodies in the human disease should be investigated.

INTRODUCTION

Trichomoniasis is a very common sexually transmitted disease (STD) of worldwide distribution.^{1–3} It is usually characterised by vaginal discharge,² although the spectrum ranges from asymptomatic carriage in women and men to cervicitis or endometritis in women or urethral discharge in men.¹² Trichomoniasis is also associated with adverse outcome of pregnancy (AOP) including preterm birth, premature rupture of membranes and low birthweight infants.²⁴ Others have shown that trichomoniasis is a risk factor for HIV transmission^{23,56} and prostate cancer.⁷ To understand the pathogenesis of *Trichomonas vaginalis* infection, elucidation of pathogen virulence factors and host responses is critical. Several mechanisms have been identified for human and/or bovine trichomoniasis. Innate immune mechanisms, such as antimicrobial peptides⁸ and acquired immunity,^{9–14} are clearly important in host defense. Mechanisms of pathogenesis include: adhesion to epithelial cells,^{13,15,16} vaginal epithelial cell cytopathology,¹⁷ secretion of extracellular proteinases,^{9,18} cell detaching factor¹⁹ and other putative virulence factors deduced from genome sequencing and proteomic studies.^{20,21} A predominant surface glycosylated molecule of *T vaginalis*, lipophosphoglycan (TvLPG), mediates adhesion to host cells and regulation of the host inflammatory response.^{15,17,22,23} TvLPG has also been characterised as *T vaginalis* lipoglycan.²³ To investigate the importance of antibodies to TvLPG in immunity, we prepared a bank of monoclonal antibodies (mAbs) to *T vaginalis*, characterised their specificity for TvLPG antigen and determined antigen conservation in primary isolates collected from pregnant women at the first prenatal visit. Further, we assessed the isotypic antibody responses to TvLPG of the infected women and uninfected controls.

MATERIALS AND METHODS

Parasites

Strains of *T vaginalis* and *Trichomonas foetus* were grown in Diamonds TYI-S-33 media with 10% fetal bovine serum after treating primary isolates with antibiotics to remove normal flora. Strain D1 of *T foetus* was the challenge strain in our studies of bovine trichomoniasis.^{10–12,14} All *T vaginalis* strains, except for UR-1 and T1, were clinical isolates as described below (under ‘Patient Samples’). UR-1 was a clinical isolate from a woman with a severe case of vaginitis, from the University of Rochester (NY), STD clinic; T1—was a clinical isolate from Taiwan. Initial isolation of *T vaginalis* from the patients in this study was by the ‘In Pouch’ TV method (BioMed Diagnostics, Santa Clara, California, USA).

Parasites were passed in TYI-S-33 a minimum number of times and frozen in liquid nitrogen until used.

Production of monoclonal antibodies

BALB/c mice were immunised four times intraperitoneally with formalin-fixed *T vaginalis* strain 10492 (10^6 in 0.5 mL phosphate buffered saline (PBS)) as in our previous studies with *T foetus* mAbs.¹³ Subsequently, mice were boosted subcutaneously with 10 µg crude shed TvLPG (also called soluble glycosylated antigen—SGA), prepared from strain 10492, as described previously²⁵ (mixed with 10 µg Quil-A Saponin adjuvant (Accurate Chemical and Scientific Corporation, Westbury, New York, USA) in 100 µL PBS). When strong antibody reactivity with *T vaginalis* cells was detected by ELISA and immunoblotting, an intraperitoneal boost with 10^6 live *T vaginalis* parasites and subcutaneous boost with soluble glycosylated antigen were administered 3 days before sacrifice. Splenocytes were then fused with myeloma cells (P3-X63 Ag 8) and polyethylene glycol following our standard methods.¹³ Hybridoma cells were selected with hypoxanthine, aminopterin and thymidine (Gibco Invitrogen, Grand Island, New York, USA) and grown in Dulbecco's Minimal Essential Medium (DMEM) with 20% fetal bovine serum, 100 U/mL penicillin, 100 µg/mL streptomycin, 2 mM L-glutamine, 1 mM sodium pyruvate, 10 mM nonessential amino acids (Gibco Invitrogen), 0.5 mM β -mercaptoethanol, 500 pg/mL recombinant mouse IL-6 (Genzyme, Cambridge, Massachusetts, USA) and 5% conditioned medium from the T24 cell line (ATCC #HTB-4) from human bladder transitional cell carcinoma. Supernatants were screened by ELISA against *T vaginalis* 10492 cells followed by purified TvLPG and soluble glycosylated antigen (see below). Positive cells were cloned twice by limiting dilution. The isotype of each selected mAb was determined with a Mouse Monoclonal Antibody Subtyping Kit (American Qualex, San Clemente, California, USA).

ELISA

ELISA plates (Costar half-well high-binding EIA/RIA plates, Corning Incorporated, Corning, New York, USA) were coated with 50 µL/well of early/mid log phase *T vaginalis* or *T foetus* parasites suspended at 5×10^6 parasites per mL. After vortexing and centrifuging plates, cells were fixed by gently adding 50 µL of 0.1% glutaraldehyde down the side of each well (0.05% final concentration). For assays with TvLPG on the solid phase, antigen was prepared as previously described^{24–26} and half-wells were coated with 1 µg of TvLPG in carbonate buffer (pH 9.6), blocked with 3% gelatin and washed with PBS containing 0.05% Tween 20 (PBS-T). Human antibody samples, mAbs or mouse sera were incubated with antigen for 1 h at 37°C, followed by goat antihuman IgG or IgA or rabbit antimouse IgG, IgM and IgA (H&L) horseradish peroxidase (HRP) conjugate (all from Zymed Labs, San Francisco, California, USA) at 1:4000 for 1 h at 37°C. Plates were developed with TMB substrate (3,3', 5,5'-tetramethylbenzidine; Microwell Peroxidase Substrate; Kirkegaard and Perry Labs (KPL), Gaithersburg, Maryland, USA) for 15 min, the reaction was stopped with 0.18 M sulfuric acid and plates were read at 450/650 nm on a microplate reader (Molecular Device Corp, Menlo Park, California, USA).

ELISA of mAbs against the ceramide-phosphoinositol glycan core

Ceramide-phosphoinositol glycan core (CPI-GC) was prepared from strain UR-1 as previously described²⁶ and dissolved in 80% ethanol, before adding 0.25, 0.5 or 1.0 µg/well to 96-well Immulon plates in triplicate and allowed to dry. Wells were washed with Tris-buffered saline (TBS) and blocked with 3% bovine serum albumin (BSA) in TBS for 2 h. After blocking, wells were washed three times with TBS-T and incubated with mAbs (dilution 1:70) for 2 h followed by washing and incubation with appropriate HRP-conjugated antimouse IgG H and L (KPL). After washing, colour was developed with TMB peroxidase substrate (KPL). The reaction was stopped with 1N HCl and the absorbance was read at 450 nm. For controls, wells were coated with related parasite *T. foetus* LPG or no LPG. Other controls included wells with no secondary antibody. Experiments were performed in the same 96-well plate and repeated three times. Values for controls were always ~0.04–0.08.

Analysis of TvLPG epitope conservation

To determine dilutions of mAbs to be used in ELISAs for conservation of LPG epitopes, twofold dilutions of each mAb supernatant were tested in ELISA three times against *T. vaginalis* strain 10492 (a patient isolate from this study). The dilution required to reach a given absorbance of approximately 1.0 was determined. Then each strain was coated on ELISA plates with strain 10492 included for standardisation. Each strain was reacted with each mAb in duplicate at the dilution required for an absorbance of about 1.0 with strain 10492. Mouse antiserum to *T. vaginalis* was the positive control. Preimmune mouse serum and PBS-T were negative controls. Data from ELISAs run on different days was corrected (based on the reaction of the positive control serum with strain 10492), after subtracting the background optical density (OD) with PBS-T instead of primary antibody. The following formula was used:

$$\text{Corrected absorbance} = \frac{(\text{Mean OD of all test day replicates}) \times (\text{mean OD of the positive control from all assays})}{\text{mean test day OD of the positive control}}$$

Sodium dodecyl sulfate polyacrylamide gel electrophoresis, periodic acid Schiff staining and western blotting

Strain 10492 was boiled in SDS sample buffer and loaded on polyacrylamide minigels. Periodic acid Schiff (PAS) staining was done as previously described.²⁴ After electrophoresis, antigens were electrotransferred to polyvinylidene fluoride membranes. Strips were blocked in 25 mM Tris—HCl (pH 7.5), 150 mM NaCl, 0.05% Tween 20 (TSB-T) containing 5% skim milk for 90 min at 37°C and washed with TBS-T. The strips were probed with a 1:10 dilution of hybridoma culture supernatants followed by goat anti-mouse IgG, IgM (H&L) alkaline phosphatase conjugate (KPL). Blots were developed in 5-bromo-4-chloro-3-indolyl phosphate and nitroblue tetrazolium substrate (Sigma-Aldrich, St Louis, Missouri, USA).

Immunofluorescence

Strain T1 parasites were allowed to attach to coverslips (Thermo Fisher Scientific, Pittsburgh, Pennsylvania, USA) in 24-well plates (Costar, Corning, New York, USA) in a humidified chamber (37°C, 30 min). Cells were fixed with 3.5% methanol-free formaldehyde (prepared from a 16% paraformaldehyde solution, Thermo Fisher Scientific, Rockford, Illinois, USA) in PBS (pH 7.2) (20 min, room temperature (RT)) and reactive groups were quenched with 100 mM glycine in PBS (15 min RT). Coverslips were blocked with 3% BSA in PBS (2 h, RT) and then incubated with LPG mAb supernatants (14D1, 14B11 or 2C6) or mAb 3G9 (specific for a *Histophius somni* antigen) as negative control, all at a 1:4 dilution in PBS with 3% BSA (1 h, RT). After washes, parasites were incubated with anti-mouse IgG (H+L) Alexa Fluor-594 (red) conjugate (Molecular Probes, Invitrogen) at 1:5000 in PBS with 3% BSA. Coverslips were washed three times and mounted on slides with ProLong Gold Antifade Reagent with 4',6-diamidino-2-phenylindole (DAPI) (Molecular Probes, Invitrogen). Parasites were examined with Axioscop 2 epifluorescent Zeiss microscope, and images were recorded with an Axiocam camera and processed with Axio Vision 3.2 program, as previously reported.¹⁵

Patient samples

Isolates of *T vaginalis*, vaginal secretions and serum were obtained in a prospective study of 3701 pregnant women enrolled on their first prenatal visit at the US Naval Medical Center, San Diego during 1996 through 1998. One vaginal swab was cultured for *T vaginalis* by the 'In Pouch' method and another was expressed in 1 mL of PBS after streaking on agar plates for culture of bacterial pathogens. Pregnancy outcome for each woman was recorded. Pregnancy loss at <22 weeks was defined as spontaneous abortion, and preterm birth at <37 weeks (WHO definition). Samples were available from 33 women with trichomoniasis at the first prenatal visit. Thirty-three control women with no trichomonads at the first prenatal visit (within the limits of diagnostic sensitivity) or history of STDs were matched for race and age. Sera and secretions from the matched sets of 33 women were stored frozen until assayed. Trichomonad isolates from each patient were frozen in liquid nitrogen and 27 isolates were resuscitated together for studies of antigen/epitope conservation. This study was approved by US Department of Defense institutional review boards and the use of stored samples was approved by the UCSD institutional review board.

Statistical analysis

Pregnancy outcomes of women with and without trichomoniasis at the first prenatal visit were analysed by Fisher's exact test for 2×2 tables. The ELISA absorbance data for human serum and secretory antibodies was submitted to Student's t-tests for independent samples. Because the data was not normally distributed and the sample variances were heterogeneous a log transformation was applied. Means were compared and probabilities calculated on the transformed data. However, the original means are reported in table 1. ELISA absorbance means for *T vaginalis*-positive patients with or without AOP were compared using t tests for independent samples with unequal variances.

RESULTS

The mAb library was first screened by ELISA against whole *T vaginalis* cells. Positive supernatants were tested against *T vaginalis* soluble glycosylated antigen and TvLPG in ELISA. Eight hybridomas producing antibody reacting with whole cells, soluble glycosylated antigen and TvLPG were cloned by limiting dilution twice. The mAbs were of the IgM class. In western blots, all eight mAbs reacted with whole trichomonads as two polydisperse bands (figure 1A). The lower band migrated similar to TvLPG (~70–80 kDa) and was PAS positive in sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS PAGE) (data not shown). The mAbs reacted with fixed *T vaginalis* parasites and TvLPG but not with fixed *T foetus* parasites or TfLPG in ELISA (figure 1B). All eight mAbs reacted strongly with *T vaginalis* and TvLPG. Furthermore, these mAbs reacted in a concentration-dependent manner with a biologically active component of TvLPG, the CPI-GC²⁶ with some variability (figure 1C,D). Reactivity of mAbs 2C6, 14D1, 14B11 and 8F7 with CPI-GC appeared to be stronger than with other mAbs. The mAbs reacted with the surface of *T vaginalis* as determined by immunofluorescence (figure 2). Conjugate controls were negative. Since antibodies to TvLPG may be protective, conservation in clinical isolates was determined. As in our previous TfLPG antigen conservation studies,²⁷ reactions above an absorbance of 0.2 were considered positive. All eight mAbs reacted with the 27 clinical isolates above this cut-off but there was considerable variability among isolates (data not shown).

The investigation of TvLPG antibody responses of pregnant women with trichomoniasis was coupled with observations on pregnancy outcome. The incidence of trichomoniasis in this prospective study was less than 1%. This was comparable with the incidence of other STDs in this prenatal patient population. With only 33 positive cases and only 30 of these patients in the study until parturition, the number of AOPs was small but the percentage was high (8 of 30 or 26.7%), with slightly more spontaneous abortions (5 or 8 AOPs) than preterm births (3 of 8 AOPs). Although the proportion of AOP was higher than that in women without trichomoniasis (710 of 3668 or 19.4%), there was no significant difference between the two groups. To assess immune responses in women with or without trichomoniasis, antibodies in sera and secretions were measured by ELISA. The difference between IgG-specific anti-TvLPG ELISA values of culturally positive and matched negative groups of women was statistically significant for serum ($p=0.000$) and vaginal secretions ($p=0.007$; table 1). There was no statistical difference between groups in serum IgA antibody, but vaginal secretion IgA anti-TvLPG values between groups approached a statistical difference ($p=0.055$). Finally, IgG and IgA anti-TvLPG absorbance means were compared in trichomoniasis-positive patients with and without AOP. There were no differences in serum and vaginal IgA or serum IgG values for these two groups of *T vaginalis*-positive patients. However, vaginal IgG anti-TvLPG values in *T vaginalis*-positive patients with AOP were significantly lower ($p=0.019$) than the vaginal IgG antibody levels to TvLPG in patients with trichomoniasis without AOP (figure 3).

DISCUSSION

This study demonstrates that the eight mAbs reacting with fixed *T vaginalis* parasites were specific for TvLPG and the biologically active TvLPG CPI-GC molecule derived from LPG. Essentially no reaction to *T foetus* or TfLPG was detected. The two broad western blot bands recognised by the eight mAbs specific for *T vaginalis* LPG were similar to those of *T foetus* reported by Shaia *et al*,²⁸ but of somewhat higher molecular weight. This is consistent with an earlier report showing that TfLPG runs faster than TvLPG on SDS PAGE.²⁴ These mAbs to TvLPG demonstrated several characteristics which may be important in immune defense. Immunofluorescence assays showed that mAbs recognised surface epitopes, which is critical for antibody-mediated protection. Additionally, to be considered as a vaccine candidate, an antigen should be conserved in many strains. The 27 tested clinical isolates from this study reacted with all eight mAbs. The degree of reactivity of each mAb with each isolate varied but the antigenic epitopes appear to be conserved among strains. In vitro expression of TvLPG may not reflect in vivo expression because some of the low passage clinical isolates grew poorly in culture. The 27 strains isolated in San Diego from navy wives and active duty navy women may indicate broad geographical distribution because navy personnel from all over the US are stationed in San Diego and have varying deployment histories. The study of pregnancy outcomes showed slightly higher numbers of AOPs in women who were culturally positive for *T vaginalis* at the first prenatal visit than in culturally negative matched controls. The low number of women with positive trichomonad cultures in this prenatal patient population may account for the lack of statistical significance. However, it is interesting that most of the AOPs in patients with trichomoniasis were spontaneous abortions at 9–14 weeks gestation. Spontaneous abortion is not normally considered in human trichomoniasis but few, if any, studies of AOP due to trichomoniasis include the first half of pregnancy. It would be useful to study spontaneous abortions in a patient population with a higher incidence of trichomoniasis, such as those with incidences up to 56% in STD clinics.¹ The higher levels of TvLPG antibodies in patients with trichomoniasis than in culturally negative women are relevant diagnostically and for potential immunotherapy or immunoprophylaxis. Others have detected antibody responses to protein antigens of *T vaginalis*.⁹²⁹ Addis *et al*²⁹ show that α -actinin is the most common protein antigen recognised by sera of women exposed to *T vaginalis*. Our results indicate that the glycolipid, TvLPG, is also commonly recognised. The human response was variable but this is probably due to the fact that the results were based on one sample per patient taken at the first prenatal visit. Some of the patients with positive *T vaginalis* cultures may have acquired the infection too recently to have developed an immune response whereas some of the culturally negative women may have recently cleared the infection and still have vaginal antibody. The fact that a greater IgG anti-LPG response overall was detected in the infected group signifies that women do respond to the antigen and that it might be possible to enhance such a response by immunisation, as in bovine trichomoniasis. Cattle experimentally infected intravaginally with *T foetus* have local vaginal IgG and IgA antibody responses to *T foetus*^{10–12} and specifically to the TfLPG/protein complex.¹¹¹²¹⁴²⁵²⁷ Systemic immunisation with the immunoaffinity-purified native TfLPG/protein antigen (but not purified TfLPG alone) results in a high level of IgG1 anti-TfLPG in serum and genital secretions.¹¹²⁵ These antibody responses protect against intravaginal

challenge with *T foetus*,^{10–12} and a commercial vaccine is now available for bovine trichomoniasis.³⁰ Perhaps human antibodies to surface TvLPG may also be protective. The higher IgG anti-TvLPG levels in patients with trichomoniasis with normal pregnancy outcomes compared with patients with trichomoniasis with AOP is intriguing. Might it be possible vaginal IgG antibodies to TvLPG may have protected against AOP and even decreased the risk for transmission of HIV?

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Key messages

- ▶ Immunity in trichomoniasis, a common human sexually transmitted disease, needs to be further elucidated.
- ▶ Monoclonal antibodies to *Trichomonas vaginalis* lipophosphoglycan (TvLPG) identified conserved surface antigenic epitopes of *T vaginalis*.
- ▶ Women with trichomoniasis demonstrate higher serum and vaginal IgG antibody levels to TvLPG than women with negative *T vaginalis* cultures.
- ▶ Since immune responses to trichomonad LPG are protective in an animal model, these human immune responses merit further study.

Figure 1.

Reactivity of monoclonal antibodies. (A) Western blot of reactivity of monoclonal antibodies with *Trichomonas vaginalis*. Note the two broad bands, characteristic of highly glycosylated antigens. The conjugate control with no primary antibody was negative. Molecular weight markers are on the left. (B) Reactivity of monoclonal antibodies with *T vaginalis* and *Trichomonas foetus* fixed parasites or with purified LPG. Reactivity with mAb 14B11 is shown. Other antibodies gave similar results. Note that there is strong reactivity with *T vaginalis* and TvLPG but little reactivity (below the 0.2 absorbance cut-off) with *T foetus* or TfLPG. (C and D) Reactivity of monoclonal antibodies with the TvLPG ceramide-phosphoinositol-glycan core (CPI-GC) in ELISA.

Figure 2. Immunofluorescence reactivity of monoclonal antibody 5G6 with *Trichomonas vaginalis* strain T1. Note surface reactivity. Incubation with an unrelated bacterial surface mAb (3G9) gave no reactivity. See the online version for colours. Key: Red=Alexa 564 antimouse immunoglobulin; Blue=DAPI nuclear stain.

Figure 3.

Comparison of vaginal IgG and IgA anti-*Trichomonas vaginalis* lipophosphoglycan (TvLPG) antibody ELISA absorbances in patients with positive *T vaginalis* cultures at the first prenatal visit who had adverse outcomes of pregnancy (AOP) or normal outcomes of pregnancy (NOP). Note that there were no differences in vaginal IgA anti-TvLPG antibodies in these two subgroups but that patients with trichomoniasis with AOP had significantly lower IgG anti-LPG values than those with normal pregnancy outcomes ($p=0.019$).

Table 1

Antibodies to *Trichomonas vaginalis* lipophosphoglycan (TvLPG) in serum and vaginal secretions of women with trichomoniasis and of matched controls

T vaginalis culture results	Mean TvLPG ELISA * absorbances			
	Serum		Vaginal secretions	
	IgG	IgA	IgG	IgA
Positive	2.575	0.695	0.542	0.444
Negative	1.887	0.657	0.267	0.214
p Value [†]	0.000	0.136	0.007	0.055

* Serum was diluted 1:100 and vaginal secretions were undiluted after extracting swabs in 1 mL of PBS.

[†] Values of samples from 33 culturally positive women and 33 matched controls were analysed using logarithmic transformed data but the data is presented in its original scale.