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Specific IgA response, T-cell subtype and cytokine profile in experimental intravaginal trichomoniasis

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Abstract Trichomoniasis caused by *Trichomonas vaginalis* may lead to either a complete absence of symptoms or to severe inflammatory manifestations in infected women. Studies of the role of immune responses in the pathogenesis and varied symptomatology of this disease are lacking. Mice may prove useful as an experimental model for intravaginal trichomoniasis in developing an understanding of the role of local immune responses in the pathogenesis and varied symptomatology of this disease. The present study reports the levels of anti-Trichomonas IgA antibodies in serum and vaginal washes, and T-cell subtype and cytokine profile in vaginal cervical tissues of mice infected intravaginally with *T. vaginalis* isolates from 15 symptomatic and 15 asymptomatic women. It also correlates the responses with symptomatology of the patients. Successful intravaginal infection was established by inoculating *T. vaginalis* in BALB/c mice preinoculated with *Lactobacillus acidophilus* and pretreated with oestradiol. A significant increase in specific IgA antibody levels was detected with enzyme-linked immunosorbent assay in vaginal secretions and serum samples collected on the 7th post-infection day from animals infected with isolates from asymptomatic women when compared with mice infected with isolates from symptomatic women. T-cell subset analysis showed significant differences, with increased $CD4^+$ T-cell count in animals infected with isolates from asymptomatic women compared with animals infected using isolates from symptomatic women. No difference in $CD8^+$ T cells was observed between the two groups. Cytokine profile revealed significantly higher ($P < 0.001$) production of γ-IFN and IL-2 in mice infected with asymptomatic isolates compared with animals infected with symptomatic isolates, using *T. vaginalis* crude antigen extract and nonspecific mitogen (ConA) as stimulants for vaginal cervical lymphocytes. However, no difference in IL-4 levels was observed in the two groups of animals. In contrast, significant increase in tumour necrosis factor (TNF-α) levels was observed in animals infected with asymptomatic isolates compared with those infected with isolates from symptomatic women and controls, thereby indicating that TNF-α may play an important role in the inflammatory response to trichomoniasis. The study further suggests that specific IgA antibodies might help to protect asymptomatic individuals from severe infection and T-lymphocytes may play an important function in the eradication of the parasite. The cytokine profile indicated the involvement of Th-1 like responses in mice infected with asymptomatic isolates, compared with those infected with symptomatic isolates.

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Introduction

Trichomoniasis caused by the protozoan parasite, *Trichomonas vaginalis*, is one of the most common sexually transmitted disease. Recent data have shown that the annual incidence of trichomoniasis is more than 170 million cases worldwide (WHO 1995). In India, the incidence varies from 6.8% to 10% in different geographical areas (Sharma et al. 1988; Malla et al.1989). Clinical presentation of trichomoniasis ranges from complete absence of symptoms to severe inflammatory manifestations. The host immune responses to *T.vaginalis* infection are reported to be usually low and

variable. Further, low levels of host humoral responses might be due to noninvasive interactions between the parasite and human host (Muller 1983). However, the role played by the immune responses in the disease's pathogenesis and varied symptomatology is not well understood.

T-cell subsets and cytokines serve a central function as key factors in the regulation of mucosal immune responses in various parasitic infections. T helper (Th) cells are important for regulation of immune and inflammatory responses through their ability to secrete different cytokines (Mosmann and Coffman 1989). However, studies regarding the role of T-cell subsets and cytokine profile in the pathogenesis of trichomoniasis in a clinical and/or experimental model are lacking. The present study was planned to assess the specific IgA antibody responses in serum and vaginal washes of mice, and T-cell subset and cytokine responses in the vaginal tissues of mice infected intravaginally with *T. vaginalis* isolated from symptomatic and asymptomatic women. The study also assessed the role of local immune responses, if any, in the pathogenesis of this disease.

Materials and methods

The study included 350 women in the reproductive age group attending the Obstetrics and Gynaecology out-patients department (OPD) of Nehru Hospital, attached to the Postgraduate Institute of Medical Education and Research, Chandigarh, India. Vaginal swabs and urine samples collected from symptomatic patients presenting with vaginal discharge, itching, dysuria and dyspareunia, and from asymptomatic subjects attending OPD for either antenatal or postnatal checkup, were examined by wet-smear examination and culture technique (TPS-I medium) (Sharma et al. 1991a). *T.vaginalis* isolates were maintained in TPS-1 medium and axenised. *T. vaginalis* isolated from patients complaining of vaginal discharge and/or pruritis, dysuria and dyspreunia were considered as symptomatic isolates. Eighteen such isolates were obtained. Strains isolated from patients coming for postnatal checkup, infertility, or other gynaecological disorders with no complaint of vaginal discharge and/or pruritus, dysuria and dyspareunia, were considered asymptomatic isolates Seventeen such isolates were obtained. Fifteen isolates from symptomatic and 15 from asymptomatic women were used to induce experimental infection in batches of female BALB/c mice weighing 22–25 g and being maintained in the Central Animal House Facility of the institute. Trichomoniasis was induced in oestradiol-pretreated and lactobacillus (20 µl of 2×10^8 cells) inoculated animals, by inoculating predetermined optimum dose of *T. vaginalis* (20 µl of 10^7 trophozoites per ml). *Lactobacillus acidophilus* infection was confirmed by inoculating vaginal washes in MRS supplemented with 5 µl of ciprofloxacin/ml and 180 µg of cefoxitin (McGrory and Garber 1992). *T. vaginalis* infection was confirmed by wet-smear examination of vaginal washes collected from mice and by culture techniques (inoculating vaginal washes in TPS-1 medium for culture). Animals were checked daily for 15 days for the presence of *T. vaginalis* and quantitative analysis of *T. vaginalis* was carried out by counting live parasites. Peak infection was observed on the 7th day postinfection (d.p.i). For further experiments, animals were divided into three groups. Groups A and B included animals infected with strains isolated from symptomatic women and asymptomatic women respectively, while group C included uninfected animals that served as controls.

Samples were collected on the 7th d.p.i (predetermined peak infection day) (Malla et al. 1999) for estimation of specific IgA anti-trichomonad antibodies in vaginal secretion and serum samples of group A, B and C mice. Crude soluble extract antigen was prepared from *T. vaginalis* trophozoites maintained in culture (Sharma et al. 1991b). Protein content was determined by the Lowry method (Lowry et al. 1951). Anti-trichomonad IgA antibody response to crude soluble extract antigen of *T. vaginalis* was detected by enzyme-linked immunosorbent assay (ELISA technique), as detailed earlier (Sharma et al. 1991a). Optical-density (OD) values were recorded at 490 nm in a Dynatech ELISA reader. Cut-off OD values were taken at mean ± 2 standard deviations (SD) values of pooled samples from six normal, uninfected mice.

Lymphocytes and macrophages were separated by sacrificing mice on the 7th d.p.i by the method of Davies and Parrot (1981) and Iglesias et al. (1989), with slight modifications. *T. vaginalis* crude antigen was prepared by the method of Mason and Gwanzara (1988). For mitogenic stimulation, ConA (20 µl of 6 µg/ml) / LPS (20 µl of 2.5 µg/ml) were used and, for specific antigen stimulation, *T. vaginalis* crude antigen (20 µl of 10 µg/ml) was used. For determination of lymphocyte subpopulations by flow cytometry (Becton Dickinson), the vaginal cervical tissue lymphocytes were analysed using a panel of monoclonal antibodies (mAbs) (Table 1), following the manufacturers' instructions (Becton Dickinson) (Serotec). The results were expressed in percentages. IL-2 and IL-4 determination were performed using the HT-2 cell-line (ATCC Rockville, MD). For IL-2 assay, anti IL-2R mAbs (a cocktail of TIB222, HB8794 and CRC-1698) were used. As a negative control, 100 µl of tissue culture medium was added. Mouse IL-2 and IL-4 were used as standards. The gamma-interferon (γ-IFN) bioassay was carried out using the WEHI-279 cell line (IMTECH, Chandigarh, India). The rest of the procedure was the same as that used for the IL-2 and IL-4 assays. Mouse recombinant γ-IFN was used as standard. Tumour necrosis factor (TNF-α) activity was measured by its ability to inhibit the proliferation of WEHI-164 cells (Espevik and Nissen Meyer 1986). The results are expressed as pg/ml. Data were analysed for statistical significance using a chi square test and ANOVA.

Results

Specific anti-Trichomonas IgA response in vaginal washes

Mean OD values for detection of IgA response in vaginal secretions in mice infected with 15 symptomatic isolates (group A), mice infected with 15 asymptomatic isolates (group B), and control mice (group C) were 0.14 ± 0.02; 0.36 ± 0.04 and 0.03 ± 0.01 respectively on the 7th d.p.i. Significant differences were found in the OD values in vaginal secretions of group A and C mice ($P<0.001$) and group B and C mice ($P<0.001$). The mean OD value of specific IgA in vaginal secretions was higher in group B compared with group A mice (Table 2) and this result was found to be statistically significant ($P<0.001$).

Specific anti-Trichomonas IgA response in serum

The mean OD value of specific anti-Trichomonas IgA antibody was found to be 0.30 ± 0.05, 0.45 ± 0.03; and 0.04 ± 0.02 in mice infected with symptomatic, asymptomatic isolates and controls respectively on the 7th d.p.i. (Table 2). The difference in the mean OD values of

Table 1 Immunophenotyping of lymphocytes obtained from vaginal cervical tissue of mice infected with isolates from symptomatic women (group A); isolates from asymptomatic women (group B) and uninfected control mice (group C) by flow cytometry. *n* Number of isolates

Cell type	Percentage of positive cells		
	Group A $n=15$	Group B $n=15$	Group C –
CD3+	41.56 ± 4.90	52.53 ± 5.41*	29.41 ± 2.61
CD4+	27.66 ± 6.64	38.53 ± 3.22*	18.08 ± 3.22
CD8+	11.98 ± 2.38	13.2 ± 5.28	14.13 ± 2.12
CD4+/CD8+	2.11 ± 0.57	3.15 ± 1.54*	1.66 ± 0.30
NK cells	8.29 ± 0.74	9.45 ± 0.78*	8.38 ± 0.77

*$P < 0.001$ versus (group A) and control (group C) ANOVA

specific IgA in serum was significant between group A and C, and group B and C mice ($P < 0.001$). A significant difference was also observed in the OD values between groups A and B mice ($P < 0.001$).

$CD3^+$

On the 7th d.p.i $CD3^+$ T-cells were found to be increased in mice infected with asymptomatic isolates (group B) (52.53 ± 5.41%) compared with mice infected with symptomatic isolates (group A) (41.56 ± 4.90%) and controls (group C) (29.41 ± 2.61%). The difference between groups A and C was found to be significant ($P < 0.001$). (Table 1).

$CD4^+$ and $CD8^+$ T-cell profile

The $CD4^+$ (helper/inducer) cells on 7 d.p.i. were found to be present in a signficant percentage ($P < 0.01$) in group B (38.53 ± 3.22%) compared with groups A (27.66 ± 6.64%) and C (18.08 ± 3.22%). The differences between group B when compared with group A and group B compared with group C were found to be significant ($P < 0.001$) (Table 1). There was a percentage decrease in the suppressor/cytotoxic cells ($CD8^+$) in group B (13.2 ± 5.28%), group A (11.98 ± 2.38%) and group C mice (14.13 ± 2.12%). This decline was not significant between the three groups.

$CD4^+$:$CD8^+$ T-cell ratio

$CD4^+$:CD8 T-cell ratio on the 7th d.p.i. in group B was found to be higher (3.15 ± 1.54%) than in group A mice (2.11 ± 0.57%), while in control mice (group C) the ratio was 1.66 ± 0.30. This difference was found to be significant when group B was compared with group A and group C ($P < 0.01$). But there was no significant difference when group A was compared with group C.

Table 2 Anti-Trichomonad IgA antibodies in vaginal washes and serum of female BALB/c mice infected with isolates from symptomatic women (group A); isolates from asymptomatic women (group B) and uninfected control mice (group C) (ELISA Mean OD value)

Group	No. of isolates	IgA antibody level in vaginal secretion and serum	
		Vaginal secretion	Serum
A	15	0.14 ± 0.02	0.30 ± 0.05
B	15	0.36 ± 0.04*	0.45 ± 0.03*
C	–	0.03 ± 0.01	0.04 ± 0.02

*$P < 0.001$ versus (group A) and control (group C) ANOVA

Natural killer cells

Natural killer (NK) cells showed significant differences between two groups of animals (A versus B). In group B, the percentage of cells was found to be higher (9.45 ± 0.78) compared with groups A (8.29 ± 0.74) and C (8.38 ± 0.77) on the 7th d.p.i. (Table 1).

IL-2 and IL-4 assay

IL-2 production in mice infected with *T.vaginalis* isolated from symptomatic women (group A) in response to *T. vaginalis* crude antigen and nonspecific mitogen ConA was 138.44 ± 2.25 pg/ml and 433.44 ± 49.70 pg/ml, in mice infected with *T.vaginalis* isolated from asymptomatic women (group B) 148.87 ± 3.75 pg/ml and 517.38 ± 43.01 pg/ml, and in control mice (group C) 18.64 ± 4.3 pg/ml and 116.07 ± 11.00 pg/ml respectively on the 7th d.p.i. Significantly higher ($P < 0.001$) IL-2 production was observed in group B mice compared with groups A and C mice. IL-4 production in response to crude antigen and nonspecific mitogens was 42.22 ± 4.44 pg/ml and 133.02 ± 7.30 pg/ml in group A and 14.91 ± 4.99 pg/ml and 100.24 ± 14.25 pg/ml in group C mice. There was no difference in IL-4 production on the 7th d.p.i. in groups A and B mice with both crude antigen and mitogen ($P > 0.05$); however, levels were found to be significant when results from group A and B were compared with controls (group C) ($P < 0.01$) (Table 3).

IFN-γ profile

IFN-γ production in response to *T.vaginalis* crude antigen and ConA on the 7th d.p.i in mice infected with symptomatic isolate (group A) was 80.93 ± 5.15 pg/ml and 451.89 ± 53.89 pg/ml respectively and in mice infected with asymptomatic isolates (group B) it was 96.09 ± 8.83 pg/ml and 532.81 ± 35.26 pg/ml, while in control mice (group C) it was 21.02 ± 3.80 pg/ml and 119.78 ± 15.45 pg/ml. There was a highly significant increase ($P < 0.001$) in IFN-γ in group B mice in response to crude antigen and nonspecific mitogen when compared with group A and group C mice (Table 3).

Table 3 Cytokine profile of mice infected with isolates from symptomatic (group A), isolates from asymptomatic woman (group B) and uninfected control (group C) in response to *Trichomonas vaginalis* crude antigen. *ConA* Concanavalin A, *LPS* lipopolysaccharide, *n* number of isolates

Cytokine (pg/ml)	Stimulatant	Group A $n=15$	Group B $n=15$	Group C –
IL-2	ConA	433.44 ± 49.70	517.38 ± 43.01*	116.07 ± 11.00
	Ag	138.44 ± 2.25	148.87 ± 3.75*	18.64 ± 4.31
IL-4	ConA	133.02 ± 7.30	135.42 ± 10.33	100.24 ± 14.25
	Ag	42.22 ± 4.44	43.40 ± 9.66	14.91 ± 4.99
γ-IFN	ConA	451.89 ± 53.89	532.81 ± 35.26*	119.78 ± 15.45
	Ag	80.93 ± 5.15	96.09 ± 8.83*	21.02 ± 3.80
TNF-α	ConA	256 ± 36.04	430 ± 40.4**	114.5 ± 11.26
	Ag	129.33 ± 2.76	149.66 ± 3.76**	16.13 ± 6.10

*$P < 0.001$ versus (group A) and control (group C) ANOVA
**$P < 0.001$ versus (group B) and control (group C) ANOVA

Tumour necrosis factor alpha

TNF-α was significantly higher in mice infected with asymptomatic isolates (group B) on the 7th d.p.i. in response to crude antigen (149.66 ± 3.76 pg/ml) and mitogen (LPS) (430 ± 40.4 pg/ml) compared with mice infected with symptomatic isolates (group A) (129.33 ± 2.76 pg/ml), (256 ± 36.0 pg/ml) and control mice (group C) (16.13 ± 6.10 pg/ml), (114.5 ± 11.26 pg/ml) $P < 0.001$ (Table 3).

Discussion

Little evidence regarding the role of immune responses in inducing protection in human trichomoniasis is available in the literature. Specific local antibodies, both IgG and IgA, have been identified in women only (Ackers et al. 1975 and Alderete et al. 1991a, b). It has been suggested that natural antibodies to the parasite, generated by cross-reacting antigens with the normal vaginal flora, may enhance the complement-mediated lysis (Shaio et al. 1991). Specific antibody responses to *T. vaginalis* antigens in serum have also been reported (Alderete et al. 1991c); however, similar to their local counterparts, these circulating antibody levels also differ and appear to have no function in helping the host to get rid of the infection (Honigberg 1990). Although an association between the presence of local antibody and low parasite counts has been postulated, there is no conclusive evidence to suggest that the presence of IgA antibodies is specifically related to the immune response to *T. vaginalis*.

In the present study, an attempt was made to assess the local immune response in experimental intravaginal trichomoniasis (mouse model) to define the role of these responses in the pathogenesis of this disease. The study showed a significant increase in IgA levels in mice infected with *T.vaginalis* isolates from asymptomatic women (group B) ($P < 0.001$) compared with mice infected with isolates from symptomatic women (group A) and control mice (group C). A further significant response was also observed in group A and group B mice as compared with group C (control) ($P < 0.001$). These results correlated well with the earlier study conducted in clinical patients, where significant levels of anti-Trichomonas IgA antibodies in serum and vaginal secretions of patients harbouring *T. vaginalis* have been reported (Sharma et al. 1991a). In *Giardia lamblia*, IgA antibodies have been shown to act as a first line of defence for immune exclusion of the parasite at the mucosal surface, i.e blocking the colonisation of the mucosal surface by the parasite (Kaur et al. 1999). In contrast, other reports indicate that the presence of circulating anti-giardial antibodies in clinical giardiasis patients, and specific serum and IgA antibodies in experimental giardiasis in mice, might stimulate the host humoral immune system.

Studies regarding cellular responses in *Giardia lamblia* (the parasite colonising the gut) infected mice have indicated that, during the declining phase of infection, there is induction of helper T-cell response, which might be playing an important role in parasite elimination from the gut (Vinayak et al. 1989). The present study in trichomoniasis revealed a significant increase in the population of total T cells, as well as $CD4^+$ T cells in mice infected with isolates from asymptomatic women (group B) compared with mice infected with symptomatic isolates (group A). However, no significant difference was observed in $CD8^+$ cells, whereas a significant difference between group B and group A mice was noticed in the vital ratio of $CD4^+$:$CD8^+$. NK cells are thought to participate in host defence against malignancy and viral infections and there is evidence that they may regulate humoral immunity in haematopoiesis.

In the present study, a significant increase in NK cells was observed in animals infected with isolates from asymptomatic women compared with mice infected with isolates from symptomatic women and control uninfected mice, indicating that NK cells might be playing a role in the pathogenesis of this disease. An increase in $CD4^+$ T cells in mice infected with isolates from asymptomatic women appears to be beneficial; it suggests these cells have a role in the elimination of *T. vaginalis* from their mucosal surface, as has been observed earlier in experimental *G. lamblia* infection (Kaur et al. 1999). Clearance of *T. vaginalis* in these animals correlated well with development of anti-IgA antibodies.

Production of IL-2 and γ-IFN by vaginal–cervical lymphocytes of mice infected with isolates from asymptomatic women (group B) was increased in this study,compared with mice infected with isolates from symptomatic women (group A) and the control uninfected group (group C), suggesting that the Th1 type of response might play a role in the elimination of *T.vaginalis*. However, increased production of TNF-α observed in mice infected with symptomatic isolates indicates that it might play a role in the inflammatory process.

Strain variation in *T.vaginalis* has been well documented. Humoral and local antibody responses have indicated strain differences and 2–8 serotypes of *T.vaginalis* have been demonstrated (Soliman et al. 1982), but specific immunogenic differences among strains have not been well characterised. MAbs were also used to classify strains on the basis of surface antigen profile, but this has not met with success (Alderete et al. 1987), probably owing to the ability of the parasite to undergo protein and epitope phenotypic variation. Soliman et al. (1982) divided 32 strains of *T.vaginalis* into five groups based on four enzymes and Vohra et al. (1991) divided 11 strains into three groups based on four enzymes, but no zymodeme pattern could be assigned to symptomatic versus asymptomatic strains according to phenotypic variation of the parasite (Alderete et al. 1987).

Restriction fragment length polymorphism, using a standard probe, may provide a better means of differentiating strains of *T.vaginalis* from patients with varying clinical pictures or from different geographical areas. However, it has been reported that the variation in pathogenic potential of *T.vaginalis* may be expressed phenotypically, rather than genotypically. Furthermore, host factors may also be important in the pathogenesis of trichomoniasis, influencing the biology of the parasite. This has been suggested by the fact that different strains of *T.vaginalis* could not be differentiated by banding patterns following restriction endonuclease digestion (Sapru et al. 1994).

In conclusion, the present study suggests that both the humoral and cellular immune responses might contribute to the varied symptomatology in trichomoniasis-infected patients, thereby reducing symptoms to asymptomatic/subclinical levels. Periodic changes in immune competence, perhaps induced by parasite-derived products, might be responsible for an increase in parasite numbers leading to increased epithelial damage and inflammatory response. The parasite-derived antigens inducing humoral and cellular immune responses need to be identified and could be used further to develop a target mucosal vaccine for prevention of trichomoniasis.

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