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SHORT COMMUNICATION

In vitro haemolytic and cytotoxic activity of soluble extract antigen of *T. vaginalis* isolates from symptomatic and asymptomatic women

Nancy Malla · Praneet Kaul · Rakesh Sehgal · Indu Gupta

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Abstract Human *Trichomonas vaginalis* infection ranges from asymptomatic to mild, moderate or severe clinical manifestations. The reasons for diverse symptomatology have been found to vary in several attributes and both parasite and host factors appear to play a role in the pathogenesis. The present study reports the in vitro haemolytic and cytotoxic activity of crude soluble extract (CSE) antigen of *T. vaginalis* isolates from 15 symptomatic and 15 asymptomatic women. The haemolytic activity following the interaction of CSE antigen with human erythrocytes and cytotoxic activity by adding CSE antigen to normal human vaginal epithelial cells was significantly higher with the use of CSE antigen of isolates from symptomatic women as compared to those from asymptomatic women. Furthermore, cytotoxic effect was found to be pH dependent. The study demonstrates, for the first time, the significant effect of parasite antigen of isolates from symptomatic women as compared to those from asymptomatic women in inducing haemolytic and cytotoxic effect and supports the earlier report that contact-independent mechanism(s) may be playing a role in establishing symptomatic trichomoniasis.

N. Malla (✉) · P. Kaul · R. Sehgal
Department of Parasitology,
Postgraduate Institute of Medical Education and Research,
Chandigarh 160012, India
e-mail: drnancymalla@yahoo.com

I. Gupta
Department of Obstetrics and Gynaecology,
Postgraduate Institute of Medical Education and Research,
Chandigarh 160012, India

Introduction

Human trichomoniasis caused by *Trichomonas vaginalis* is the most common non-viral sexually transmitted disease. The disease is estimated to affect more than 200 million people worldwide annually (Fiori et al. 1999). In India, the incidence of trichomoniasis ranges from 5.7% to 10% (Sharma et al. 1988; Malla et al. 1989; Divekar et al. 2000; Valadkhani et al. 2003; Yadav et al. 2006). The protozoan parasite is a proven pathogen, yet its presence in the genitourinary tract does not always produce symptoms. The clinical presentation ranges from asymptomatic state to several inflammatory manifestations (Muller 1983) and the reasons for diverse symptomatology produced by this parasite have been found to vary in several attributes. Both parasite and host factors seem to play a role in leading to symptomatic infection. The isoenzyme analysis (Soliman et al. 1982; Vohra et al. 1991), antigenic structure of the parasite (Alderete 1983) and restriction fragment length polymorphism analysis (Sapru et al. 1994) have not been able to clearly differentiate isolates from symptomatic and asymptomatic women. However, other multiple factors such as adhesive ability, cell detaching factors, pore-forming proteins and soluble factors like haemolysins and extracellular proteinases are recognised virulence markers (Fiori et al. 1999). Our earlier reports have indicated that reactive nitrogen intermediates (Malla et al. 2004; Yadav et al. 2006) and superoxide radicals (Valadkhani et al. 2006) production may be playing a role in establishing the infection. Furthermore, isolates from symptomatic and asymptomatic women have been found to differ by random amplified polymorphic DNA technique (Kaul et al. 2004) thereby indicating that genetic polymorphism in the parasite may determine the outcome of infection.

Both contact-dependent and contact-independent mechanisms (Pindak et al. 1993) have been reported to play a role in the pathogenesis of this infection and we have observed that adhesion of the parasite to vaginal epithelial cells was time and pH dependent and, by interacting parasite isolates with human erythrocytes, the amount of Hb released was significantly higher by isolates from symptomatic than isolates from asymptomatic women (Valadkhani et al. 2003). The present study was undertaken to assess the in vitro cytotoxic and haemolytic activity of crude soluble extract (CSE) antigen of *T. vaginalis* isolates from symptomatic and asymptomatic women to assess the effect of parasite antigen, if any, in the pathogenesis of this infection.

Materials and methods

Patients One thousand women of childbearing age group who were attended to at the Outpatients Department of Obstetrics and Gynaecology, Nehru Hospital attached to the Postgraduate Institute of Medical Education and Research (PGIMER), Chandigarh, India were enrolled for the study after taking their informed consent.

History and relevant clinical findings of all the subjects were recorded. The patients complaining of vaginal discharge and/or pruritis, dysuria and/or dyspareumia were considered as symptomatic and those who attended at the clinic for routine ante-natal/post-natal check-ups, infertility and family planning advice with no complaints suggestive of trichomoniasis were considered as asymptomatic.

Samples and isolation of T. vaginalis Vaginal swabs and urine samples collected from all the symptomatic and asymptomatic patients were processed for isolation of *T. vaginalis* by wet smear and culture examination (Sharma et al. 1991). *T. vaginalis* isolates were maintained in culture medium (Diamond 1970) axenically, by subculturing at the log phase of growth, which was observed after 48–72 h of incubation. Axenisation of isolates was achieved by adding the antibiotics pencillin (1,000 μ/mol) and streptomycin (1,000 μg/ml) in the first three to five subcultures.

Preparation of crude soluble extract antigen The parasite cultures were chilled in ice water for 10 min and centrifuged at 1,000×*g* for 15 min at 4°C, followed by washing with phosphate-buffered saline (PBS) (pH 7.0) and sonication (SONIPREP 150, MSE) at 8 kc/s for 5 min, and spun at 10,000×*g* for 30 min (Yadav et al. 2005). The same batch of antigen was used for assessing haemolytic/cytotoxicity assay of all the test isolates. Protein concentration of the antigen was determined (Lowry et al. 1951) just before use.

Haemolytic assay The assay was performed as detailed earlier (Vergas-Villarreal et al. 2003) with slight modifications. Briefly, erythrocyte suspension prepared from human blood (group O) was adjusted to optical density of 0.8 at 415 nm, placed in 1.5-ml-capacity eppendorf tube and mixed with 80 μl of CSE antigen. With every experiment for each isolate, two tubes each containing erythrocyte suspension with double distilled water or Hanks' balanced salt solution instead of CSE antigen were used as 100% haemolysis and 0% haemolysis, respectively, as controls. Tubes were incubated at 36.5°C for 2 h, followed by addition of 1 ml PBS and centrifugation at 600×*g* for 5 min at 4°C. The absorbance at 415 nm was measured by a spectrophotometer. One haemolytic unit was defined as the amount of protein from antigen required to produce 50% haemolysis (Vergas-Villarreal et al. 2003). The percentage haemolysis was calculated by the formula

$$\%\ \text{Haemolysis} = \frac{\text{EXHR (Experimental haemoglobin release)} - \text{*SHR (Spontaneous haemoglobin release)}}{\text{**MHR (Maximum haemoglobin release)} - \text{SHR (Spontaneous haemoglobin release)}}$$

with *SHR as in mixture with added Hanks' balanced salt solution instead of crude soluble extract antigen and **MHR as in mixtures treated with double distilled water instead of crude soluble extract antigen.

Cytotoxicity assay The cytotoxicity assay was performed as detailed out earlier (Pindak et al. 1993) with slight modifications. Normal vaginal epithelial cells (VEC) were collected from women who were attended to at the Obstetrics and Gynaecology Department, Nehru hospital, PGIMER, Chandigarh, India for family planning advice and who were found to have healthy vagina and cervix on perspeculum examination and in whom *T. vaginalis* could not be detected either by wet smear or culture of vaginal swab and urine sample.

CSE antigen doubling dilutions (1:2 to 1:32) were made in glucose saline and PBS (pH 7.0) separately and separate experiments were run to assess the effect of pH. Fifty microlitres of diluted antigen was added to 50 μl of VEC suspension (4×10^4/ml); the tubes were incubated at 37°C for 2 h and pH was measured. Cells were washed with respective diluent, stained with trypan blue (0.4%), washed

again and examined under microscope for the dye uptake. The unstained cells were considered as live and the stained cells as dead. In each experiment for each isolate, 100 VEC were counted in various fields. The percent lysis was calculated as follows:

$$\text{Percent lysis} = 100 - \frac{\text{Count in test sample}}{\text{Count in control}} \times 100.$$

Ethical clearance

The study was granted permission by the Institute Ethical Committee, PGIMER, Chandigarh, India.

Results

Out of 1,000 women, 537 were symptomatic and 463 were asymptomatic. Thirty-eight patients were found to be positive for *T. vaginalis,* out of which 22 (4.09%) were symptomatic and 16 (3.45%) were asymptomatic. Isolation percentage from symptomatic versus asymptomatic women was not statistically significant ($P>0.05$). The sensitivity was 97.3% and 78.9% by culture and 76.3% and 52.6% by wet smear examination of vaginal swabs and urine samples, respectively.

Haemolytic assay Percentage of haemoglobin released ranged from 17.3% to 21.8% (mean, 19.9%) and 9% to 18.8% (mean, 15.9%) when human group O red blood cells were interacted with crude soluble extract antigen of *T. vaginalis* isolates from symptomatic and asymptomatic women, respectively. Percentage of haemoglobin release was significantly higher ($P<0.05$) with the use of CSE antigen of isolates from symptomatic women, as compared to those from asymptomatic women (Fig. 1).

Cytotoxicity assay Mean percentage of dead VEC when incubated with 1:2, 1:4, 1:8, 1:16, and 1:32 dilutions of CSE antigens from symptomatic isolates was found to be

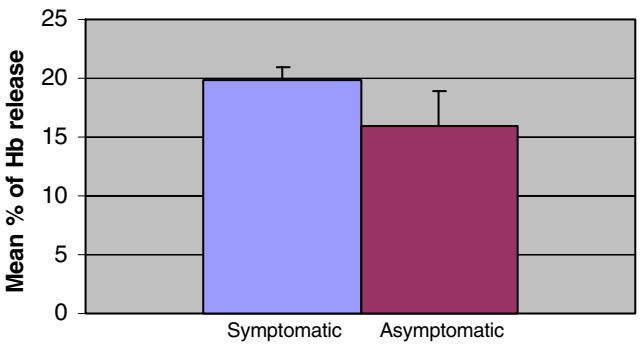

Fig. 1 Haemolytic activity (mean percentage of Hb release) by interacting crude soluble extract antigen of *T. vaginalis* isolates from symptomatic and asymptomatic women with human erythrocytes

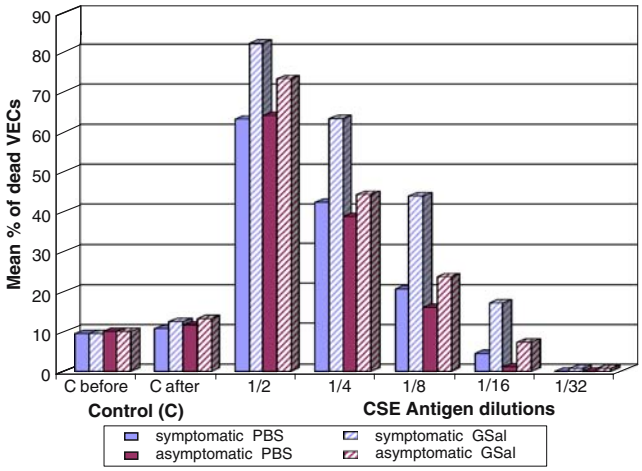

Fig. 2 Cytotoxic activity (mean percentage of dead VEC) of crude soluble extract antigen of *T. vaginalis* isolates from symptomatic and asymptomatic women *c before*—percentage of dead VEC before experiment; *c after*—percentage of dead VEC after experiment (2 h)

63±16, 42±16, 21±17, 4±9 and 0±0 (mean±SD), respectively, when PBS was used as diluent and 82±9, 63±15, 44±17, 17±12 and 1±1 (mean±SD), respectively, when glucose saline was used as diluent.

When VEC were incubated with similar dilutions of CSE antigen of isolates from asymptomatic women, the mean percentage (%) of dead VEC were 64±13, 39±14, 16±11, 1±4 and 0±0 (mean±SD), respectively, when PBS was used as diluent and 73±13, 44±13, 24±9, 7±7 and 1±1 (mean±SD), respectively, when glucose saline was used as diluent.

Maximum cytotoxic effect was found in 1:2 dilution as compared to control ($P<0.05$) with the use of isolates both from symptomatic and asymptomatic women. Cytotoxic effect with CSE antigen of isolates from symptomatic women was significantly higher ($P<0.05$) as compared to isolates from asymptomatic women (Fig. 2).

For both symptomatic and asymptomatic isolates, after 2 h of incubation, final pH was 6.8 (initial pH 7.0) and 5.5 (initial pH 7.0) with the use of PBS and glucose saline as diluent, respectively.

Discussion

In the present study, 38 (3.8%) women were found to harbour *T. vaginalis*, which is in agreement with earlier reports (Sharma et al. 1988; Malla et al. 1989; Divekar et al. 2000; Valadkhani et al. 2003; Yadav et al. 2006). Culture of vaginal swabs has been found to be a significantly sensitive technique as compared to wet smear examination. The present study indicated 97.3% and 78.9% sensitivity by culture and 76.3% and 52.6% by wet smear examination of vaginal swabs and urine samples, respectively, which is in agreement with earlier reports (Sharma et al. 1991; Krieger et al. 1992).

In our earlier study, based on contact-dependent mechanism, the amount of haemoglobin released by isolates from symptomatic patients was significantly higher than isolates from asymptomatic women (Valadkhani et al. 2003). In the present study, contact-independent mechanisms were studied and lytic effect of crude soluble antigen of *T. vaginalis* isolates from symptomatic and asymptomatic women was compared by haemolytic and cytotoxic assays. The haemolytic and cytotoxic potential of CSE antigen of isolates from symptomatic women was significantly higher than from asymptomatic women. These findings are in agreement with the earlier report (Krieger et al. 1983) whereby fresh *T. vaginalis* isolates from four symptomatic women showed a significantly higher level of β-haemolytic activity as compared to isolates from three asymptomatic women. Furthermore, in the present study, cytotoxic effect was higher when glucose saline was used as diluent as compared to PBS. This suggests that the cytotoxic effect is pH dependent and is in agreement with the earlier reports on contact-dependent mechanism whereby adhesive and haemolytic activity was found to be pH dependent (Pindak et al. 1993; Valadkhani et al. 2003). This is of clinical relevance since the normal pH of the vagina is 4.5 and trichomonad haemolytic activity depends on pH. The change in vaginal pH during trichomoniasis may therefore be crucial in the pathogenesis of this infection. The present report is also supported by an earlier report (Vergas-Villareal et al. 2003), whereby trichomonal total extracts or vesicular (P 30) and soluble (S 30) subcellular fractions from three pathogenic strains lysed both human and rat erythrocytes in a time- and dose-dependent manner.

In conclusion, the study supports the earlier observation that contact-independent mechanisms seem also to play a role in establishing symptomatic trichomoniasis and the action is pH dependent. This is the first report indicating the role of *T. vaginalis* parasite antigen in inducing haemolytic and cytotoxic activity and its effect was significantly higher with antigen of isolates from symptomatic as compared to those from asymptomatic women.

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