Iranian J Publ Health, 2006, Vol. 35, No. 2, pp.76-82

Superoxide Radical Formation in Isolated PMN from Experimental Vaginal Trichomoniasis

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(Received 10 Sept 2005; revised 5 Nov 2005; accepted 24 Feb 2006)

Abstract

Trichomoniasis, the most widespread sexually transmitted disease is caused by *Trichomonas vaginalis*. This parasite is site specific for the genitourinary tract and recruitment of macrophages as well as polymorphonuclear nutrophils (PMN) to the site of infection is the first line of defense as a component of non-specific resistance and immunity. In this study, BALB/c mice were infected with 10 isolates from symptomatic and 10 from asymptomatic patients. Then PMN from vaginal washes, vaginal tissue and blood of infected mice was isolated and the rate of superoxide formation by intact stimulated PMN was measured. Results showed that, mice infected with symptomatic isolates indicated significant increase in polymorphs with increase in days of infection as compared with mice infected with asymptomatic isolate and control (uninfected) animals. Vaginal tissue cells generated maximal amount of superoxide in symptomatic isolates infected animals (5.17 ± 0.36) as compared to asymptomatic isolates (4.54 ± 0.43), which brings out the maximal abnormality in PMN in this localized area. The amount of superoxide radicals generated by cells of vaginal washes and blood of symptomatic isolate infected mice (4.29 ± 0.25 and 2.16 ± 0.35) was less than the asymptomatic isolate (4.94 ± 0.49 and 3.18 ± 0.26), respectively. This study indicates that super oxide radical generation may play role in establishing the infection.

Keywords: *Trichomoniasis, Trichomonas. vaginalis, Super oxide radical, PMN*

Introduction

Trichomoniasis is the vaginitis caused by sexually transmitted agent *Trichomonas vaginalis.* Infection with *T.vaginalis* could have an important role in transmission and acquisition of HIV (1). This parasite is site specific for the genitourinary tract and has been isolated from virtually all genitourinary structures. Asymptomatic disease is common in both men and women, thus screening for disease is important that often evolves into a chronic infection. Random amplified polymorphic DNA (RAPD) technique used to determine genetic differences among isolates of *T.vaginalis* from different clinical presentation (2). They found that these genetic data were correlated with patients' records. Four main groups could be distinguished by RAPD technique and these groups coincide with four different patients categories (asymptomatic and symptomatic: light, moderate and sever infection). On the part of the host the first line of non-specific defense offered, is the activation of alternative pathway of complement, locally increased zinc levels and accumulation of phagocytic cells specially PMN and macrophages (3). Our previous work also has shown

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abundant PMN in vaginal washes of infected mice (4). However, these non-specific defense mechanisms may not be sufficient and the infection either symptomatic or asymptomatic could persist for a long time. Neutrophils and macrophage phagocytosis also stimulates other cellular processes including the respiratory burst where by increased cellular oxygen uptake resulted in the production of the potent bactericidal agents killing *T. vaginalis* by O_2-dependent mechanism (5). It has also been reported that many of the known antiparasitic drugs induce free radicals production. Moreno Do campo (6) found that mechanisms of the trichomonicidal activity of metronidazole are with generation of reactive metabolite (s),which interact with DNA and lead to a subsequent inhibition of nucleic acid and protein synthesis. In recent years, it has become increasingly apparent that free radicals play a critical role in a variety of normal regulatory pathways (7- 8). Thus, oxidant-antioxidant balance is critical for immune cell function because it maintains cell membrane integrity and functionality. Ability of locally collected PMN in eliminating parasite at the site is yet to be defined. Whether similar mechanism is involved in both the group of patients (symptomatic and asymptomatic) is not known. Existing information fails to define/ differentiate between the roles of recognized virulence determinates in the development of symptomatolgy of the disease. Thus, present study planned to differentiate two groups in form of superoxide formation in different samples.

Materials and Methods

Sample Collection. *T. vaginalis* was isolated from vaginal swabs and urine samples from 500 symptomatic and asymptomatic female patients, in Post Graduate Institute of Medical Education and Research (PGIMER), Chandigarh, India. Isolates obtained from patients complaining of vaginal discharge and/or pruritis, dysuria, and dyspareunia were considered as symptomatic patients isolate (Sp). Isolates obtained from patients attending the clinic for routine check-up, infertility or some other gynaecological problems with no complain of above mentioned symptoms were considered as asymptomatic patients isolates (Asp). Clinical samples inoculated in TYIS-33 media and checked by direct smear. Positive cultures were maintained by every 2-3 days subcultures.

T. vaginalis isolates from 10 symptomatic and 10 asymptomatic women were used for experimental induction of infection in female BALB/c mice, 4-6 wk old. (4). For each strain, a group of eight mice in duplicates was used along with uninfected controls. Quantitative analysis of *T. vaginalis* was carried by counting live parasites and PMN in vaginal washes of infected mice.

Sample collection from infected mice On 5th post infection day (pid) which was the day of maximum infection as standardized in earlier experiments of this study (4), vaginal washes, blood and cervico vaginal tissue were collected. Vaginal washes of infected mice were aspirated with the help of autopipette by inoculating 20 µl of PBS (pH 7.2). Samples were taken from all mice in a group and were collected in a sterile micro tube. The samples were processed for isolation of PMN for superoxide formation. Mice were anaesthetized by inhalation of ether (Anaesthetic ether I. P. India) and maximum blood was collected by puncture of heart. Blood was transferred into a micro tube containing 10 iu/ml heparin (Biological E. Ltd., India) and PMN were isolated for measurement of superoxide. Isolation of neutrophils from the blood and vaginal washes was done by the method of Boyum (9) with some modifications.

Isolation of PMN from cervico vaginal tissue After collection of above samples mice were sacrificed by over anaesthetized. Cervico vaginal tissue was removed and method of Davis and Parrott (10) was used to isolate PMN from the tissue.

Superoxide formation assay The rate of superoxide formation by intact stimulated PMN was measured according to the method described earlier (11).

Reagents and solutions PiCM-G buffer (138 mM NaCl, 2.7 mM KCl, 0.6 mM $CaCl_2$, 1.0 mM $MgCl_2$, 5.0 mM glucose and 10 mM $NaH_2pO_4/NaHPO_4$, pH 7.4.), Neutrophil/PMN suspension (1×10^6 cell/ml), Cytochrome c, 10 mM ($Fe^{3+,}$ horse heart, type VI; Sigma) Phorbol myristate acetate (PMA) 10 µg/ml (Sigma chemical USA), superoxide dis-mutase 5 mg/ml (SOD; bovine erythrocyte; Sigma USA).

Procedure For test, 247 µl of PiCM-G buffer was mixed with 45 µl neutrophil/PMN (1×10^6 cells/ml) isolated from infected mice and 4 µl of cytochrome c (0.1 mM) in a microwell plate. Controls were also processed simultaneously in a similar manner using neutrophils from the blood of normal mice. Reference was prepared in the same way as test except that four µl of PiCM-G buffer was replaced with SOD. These were incubated at 37ºC for 10 min with frequent shaking. Absorbance of the samples was recorded by microplate reader (Micro scan MS 5608-India), at 550 nm. Simultaneously four µl of PMA (100 µM/ml) was added to all the wells. After 10 min (as standardized in earlier experiments of this study) following addition of the stimulator (PMA), again absorbance of reduced cytochrome c was recorded at 550 nm. Maximum rate of superoxide formation was calculated by reduction of cytochrome c in the reference, subtracted from the value in the sample. The resulting values gave the rate of SOD inhibitable cytochrome c reduction using the following formula:

$\Delta A/min\times$reaction vol (ml) $\times10^6$ nmol/mmol divided by Specific absorbance (A/mmol/l)$\times$1000 ml/. (absorbance coefficient= 21.1A/ mmol/l)

Statistical analysis Data were analyzed for statistical significance using student's *t*-test, Chi square test and ANOVA.

Results

The parasites were isolated in 22 (4.4 %) patients out of 500 clinical samples examined. Ten isolates from each group were maintained in axenic form in culture and were used for further experiments. Gradual increase up to day 5 followed by decline in all the two parameters i.e. parasites and PMN was observed. Overall, the mice infected with Sp isolates had significantly high number of two parameters in contrast to mice infected with Asp isolates or control animals at all time intervals. The parasite loads in mice infected with Sp isolates were higher as compared with the mice infected with Asp isolates ($P<0.05$). Fifth pid was observed as peak infection day in most experimental animals for both Sp and Asp isolates. Mice infected with Sp isolates indicated significant increase in polymorphs with increase in days of infection corresponding to mice infected with Asp isolate and control (uninfected) animals (Fig. 1). There was significant difference in PMN count on all pid between mice infected with Sp isolates versus control (on 1 pid $P<0.01$ and on 3, 5, 7 pid $P< 0.05$), and Asp isolates versus their control ($P< 0.05$). However, these differences were not significant when PMN count from mice infected with Sp isolates was compared with the counts from mice infected with Asp isolates. Maximum counts were observed on five-pid in both Sp and Asp isolates.

Fig. 2 summarizes the results of superoxide radicals carried out on PMN collected from vaginal tissue, vaginal washes and blood of experimental animals in which, infection was induced by Sp and Asp isolates. Control animals, since were not given any infection failed to give any yield of PMN in vaginal tissue as well as vaginal washes. Vaginal tissue cells generated maximal amount of superoxide in Sp isolates infected animals (5.17 ± 0.36), which brings out the maximal abnormality in PMN in this localized area. The amount of superoxide radicals generated by cells of vaginal washes and blood of Sp isolate infected mice was less than the Asp isolate infected mice ($P> 0.01$ and $P< 0.01$, respectively).

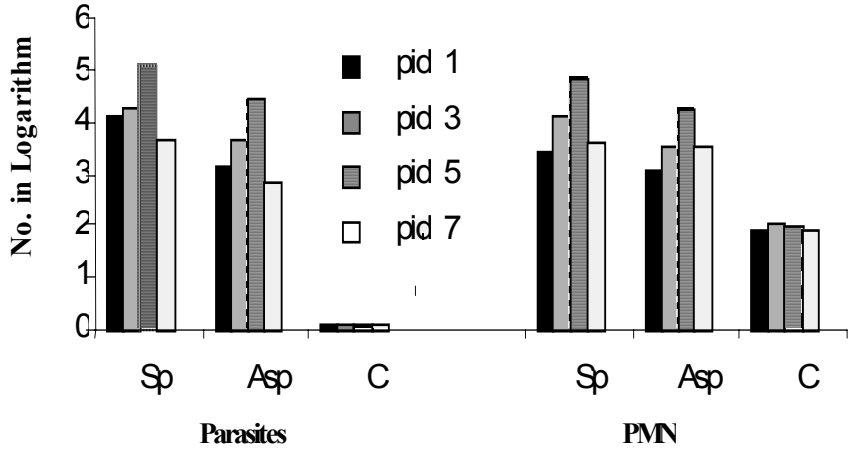

Fig. 1: Comparison No. of parasites and PMN in different post infection days (pid) in vaginal washes of BALB/c mice infected with both groups of isolates.

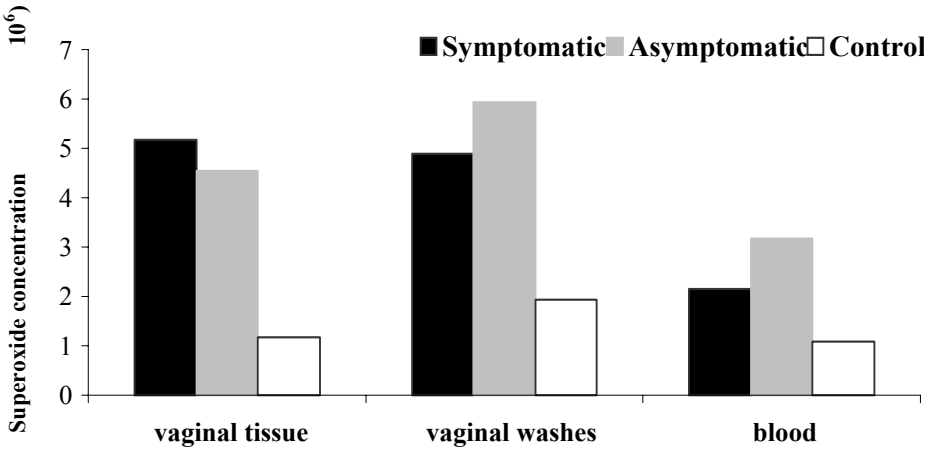

Fig. 2: Concentration of superoxide radicals in PMN from vaginal tissue, vaginal washes and blood of *T. vaginalis* infected mice with symptomatic and asymptomatic isolates

Discussion

Host's immune responses to *T. vaginalis* infection are usually low and variable which may be due to non-invasive interactions between the parasite and human host (12). Although antibodies to *T. vaginalis* may be detected in vaginal secretion or serum of the majority of infected women, it may not be detected due to low titers (13). These circulating antibodies have short life and are not able to provide protection against reinfection (14). It is not known whether the poor response is due to weak antigenicity of the parasite or to active suppression of immunity. Recently, it has been stressed that host factors like extensive layer of mucous, nutrient limiting conditions, antibody responses, constant flow of vaginal fluid and normal flora have important role to play in estab-

lishment of infection by *T. vaginalis* (15). Recruitment of macrophages and PMN to the site of infection is the first line of defense as a component of non-specific resistance and immunity (16). In addition, sensitized T. lymphocytes and the lymphokines produced and parasite-derived factors are directly chemotactic for PMN, which could be important element of protective immunity (17). Neilson and Neilson (18) while examining the vaginal biopsy from trichomoniasis patients, under electron microscope, observed evidence of chronic non-specific inflammation accompanied by sub epithelial infiltration by neutrophils and lymphocytes. Further, in cases of more severe inflammation, neutrophils were observed in deeper layers of the epithelium. Honigberg (19) observed that *T. vaginalis* isolated from Sp were able to stimulate strong chemotactic response towards PMN as compared to those isolated from Asp. This resulted in a higher inflammatory response accompanied by leukorrhea in Sp. In another clinical study, abundant PMN in vaginal smears of patients infected with *T. vaginalis* were observed (20). Malla et al. (21) in an experimental study also observed increase in number of PMN in pap smear from mice infected with Sp isolates as compared to those infected with Asp isolates and maximum number was observed on 7^{th} pid. However our previous work (4) showed that, the maximum number of PMN in vaginal washes of mice was observed on the peak infection day which was 5^{th} pid in mice infected with isolates from symptomatic women although larger number of PMN were observable in vaginal washes throughout the period of study i.e. till 7^{th} pid. Neutrophil-activating factor is released by *T. vaginalis* and characterized as leukotriene B4 (LTB4). This factor was extracted from both the group of isolates i.e. from Sp and Asp (22).

Trichomonads are equipped with several oxygen scavenging systems localized in both cytoplasm and hydrogenosomes. The main systems operating in the cytoplasm consists of NADH and NADPH oxidases (23), which reduce O_2 to H_2O and H_2O_2, respectively. The neutrophil respiratory oxidase (i.e. NADPH-oxidase), responsible for production of reactive oxygen species, is dormant in resting cells. Binding of opsonized particles to the receptors on the neutrophil surface initiates a transduction cascade that leads to activation of the oxidase and generation of highly reactive free radicals e.g. superoxide anion (24). In many situations, PMN initiate oxidative stress by releasing toxic oxidants such as superoxide anion and H_2O_2, which can damage surrounding vaginal epithelial cells (25). In the present study, therefore SOD levels measured from different sources of PMN have been taken as an index of reactive oxygen synthesis generation by the activated cells. Significant higher levels of SOD were only observable in the PMN from vaginal tissue of the mice infected with Sp isolates while in the PMN from blood significantly low level of SOD were observed in the animals infected with Sp isolates. PMN from vaginal washes in contrast did not show significant fall of SOD levels. These results bring out the fact that PMN collected from vaginal washes may not be the ideal source and may failed to project the activation of PMN in the infected individuals. Our previous work also showed reactive nitrogen intermediates is higher in vaginal tissue of infected mice than vaginal washes and blood (8). For this purpose, vaginal tissue although would be desirable, but it involves invasive procedure which may not be suitable for patient situation. Fall of SOD in circulatory PMN observed in the experimental situation needs to be correlated with patient situation to be able to extrapolate these observations to clinical presentation. This also brings out the possibility that the tissue's PMN are in much greater state of activation than the ones present in circulation and vaginal washes. Davis et al. (26) observed inter-strain heterogenecity in the oxidative stress response (OSR). The OSR of *T. vaginalis* and the molecules involved may prove to be important in understanding the relationship between this parasite and the PMN, with which it

interacts in the vaginal canal. Shaio et al. (27) observed that neutrophil could kill *T. vaginalis* only in the presence of 10% normal human serum. This study indicated that trichomonal killing by neutrophils is due to immunoglobulin-enhanced activation of the classical complement pathway. However, the concentration of complement in the vagina is very low and this may explain as to why *T. vaginalis* survives in presence of large number of PMN. Multiple factors therefore might be playing role in the host defense and responsible for clinical presentation with or without symptoms. In spite of large number of PMN secreted in vagina during infection with *T. vaginalis*, especially in symptomatic patients, and release of toxic oxidants such as superoxide anion and H_2O_2 in the vaginal milieu, *T. vaginalis* survives. This suggests that *T. vaginalis* also maybe exhibiting simultaneously some protective mechanisms, which helps as parasite defense mechanism. Reactive oxygen intermediates have been reported to play role in the pathogenesis of other parasitic infections (28).

Further studies will be necessary to ascertain the importance and function of these free radicals in clinical infection.

Acknowledgements

With special thanks to all of colleagues in the Dept. of Obstetrics & Gynaecology and Dept. of Parasitology, Postgraduate Institute of Medical Education and Research, Chandigarh, India.

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