Proteinases of *Trichomonas vaginalis*: antibody response in patients with urogenital trichomoniasis

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SUMMARY

Immunoprecipitation combined with electrophoresis in gelatin-polyacrylamide gels was successfully used for detection of antibodies against numerous proteinases of *Trichomonas vaginalis* in infected patients. The method proved to be highly specific as anti-proteinase antibodies were absent in women with negative cultivation of *T. vaginalis* and no history of trichomoniasis. Sera of 71 % and vaginal washes of 86 % patients with trichomoniasis were positive for these antibodies. In vaginal washes, but not in sera, antibodies were partly complexed with proteinases, possibly of trichomonad origin. It was also shown that serum antibodies as well as local anti-proteinase antibodies persisted for weeks after patients had been cured.

Key words: *Trichomonas vaginalis*, trichomoniasis, proteinases.

INTRODUCTION

Human urogenital trichomoniasis is a common, sexually transmitted disease caused by a parasitic protozoon, *Trichomonas vaginalis*. Our knowledge of the biology of the host–parasite relationship and of the pathogenesis remains deficient.

Currently, attention is being paid to the characterization and the role, if any, of the trichomonad proteinases in *T. vaginalis*–host interaction (Arroyo & Alderete, 1989; North, Mottram & Coombs, 1990). *T. vaginalis* proteinases are now classified into two types. The major type is represented by multiple cysteine proteinases (Lockwood *et al.* 1987; Neale & Alderete, 1990), while the other comprises only two enzymes possibly belonging to the metallo-proteinase class (Bózner & Demeš, 1991).

Cysteine proteinases have been found extracellularly in culture medium (Lockwood *et al.* 1987) and it has been suggested that release of these enzymes from the parasites also occurs *in vivo*. Recently, involvement of trichomonad proteinases in cytadherence as well as cytotoxicity was suggested (Arroyo & Alderete, 1989).

Because the immunogenic nature of the cysteine proteinases of *T. vaginalis* has been reported (Garber & Lemchuk-Favel, 1989; Neale & Alderete, 1989), it was important to address the question as to whether local antibody is made by patients with trichomoniasis. We therefore investigated the presence of antibodies against *T. vaginalis* proteinases in the serum and vaginal secretions of infected women.

MATERIALS AND METHODS

Patients

A total of 75 women (aged 18–56) attending the outpatient clinic of the Department of Parasitology in Bratislava was examined. One group of women comprised those with active trichomoniasis (28) while a second group of 26 trichomoniasis patients were those 2–6 weeks after treatment with a single dose (1·5 g) of ornidazole (Avrazor SPOFA). Women in a third (control) group of 21 had no history of trichomoniasis and were negative for *T. vaginalis* by culture. Diagnosis was done by microscopy and cultivation of trichomoniasis from vaginal swabs in modified Diamond's TYM medium (Diamond, 1957). Women included in this group were negative for *Neisseria gonorrhoeae* and *Candida albicans*.

Sample collection

Sera were obtained from all patients and were stored at -20 °C in 1 ml aliquots. Vaginal washes and subsequent aspiration were performed by instillation of sterile isotonic saline (3 ml) into the posterior fornix of the vagina by a syringe attached to a polyethylene tube (Demeš *et al.* 1988). Insoluble material was removed by centrifugation at 10000 ***g*** for 5 min. Unconcentrated supernatant fractions (1·5 ml) were stored at -20 °C until further use.

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Detection of T. vaginalis *proteinase-binding antibodies in human sera and vaginal washes*

A combination of immunoprecipitation with substrate–SDS–PAGE was used. Human anti-proteinase antibodies in sera and vaginal washes were precipitated with protein A-bearing *Staphylococcus aureus* before reacting with *T. vaginalis* lysate. The immunocomplexed proteinases were then visualized in a gelatin–SDS–PAGE system as described below.

Immunoprecipitation was carried out with modifications as described by Neale & Alderete (1990) and Johnstone & Thorpe (1987). Swine antiserum against human immunoglobulins (SwαHIg) was obtained from the Institute of Sera and Vaccines, Prague, Czechoslovakia. Protein A-bearing *S. aureus* (Cowan I strain) was grown and prepared for immunoprecipitation as described by Kessler (1975).

Unless otherwise stated, 50 μl of SwαHIg was added to 1 ml of 10% (v/v) formalin-fixed *S. aureus* in phosphate-buffered saline (PBS), pH 7·2. The mixture was incubated for 1 h at room temperature (RT) and then centrifuged at 10000 ***g*** for 2 min at 4 °C. The *S. aureus* with bound antibody (*S. aureus*-SwαHIg) were washed twice with PBS and resuspended to 2 ml. One hundred μl of this washed bacterial suspension were added to 200 μl of patient serum or to 300 μl of vaginal wash. This mixture was incubated for 2 h at 4 °C by constant stirring, then centrifuged as described above, and the *S. aureus*–SwαHIg-human Ig complexes were again washed twice with PBS before resuspending in 200 μl of PBS. This suspension was mixed with 200 μl of *T. vaginalis* extract prepared as described below, in order to bind immunogenic trichomonad proteinases, and incubated for 30 min at room temperature. The immune complexes on *S. aureus* were then centrifuged and washed 3 times with PBS. Immune-complexed proteins were removed from the bacteria by the addition of 35 μl of an SDS-containing buffer, with 2% (v/v) 2-mercaptoethanol (Laemmli, 1970) followed by heating at 37 °C for 20 min.

The presence of solubilized trichomonad proteinases released from *S. aureus* was visualized by electrophoresis in gelatin-containing polyacrylamide gels (Lockwood *et al*. 1987; Bózner & Demeš, 1991) using a minigel apparatus. After Triton X-100 treatment, the gels were incubated for 12 h at 37 °C in an acetate buffer, pH 5·5, in the presence of 1 mM dithiothreitol. These conditions allow for the detection of cysteine proteinase activity (Lockwood *et al*. 1987). The gels were then stained for 30 min in 0·275% (w/v) Coomassie brilliant blue R-250 prepared in 40% (v/v) methanol, 10% (v/v) acetic acid and destained. Appearance of gelatinolytic activities on acrylamide gels after staining gave indirect evidence of the presence of anti-proteinase antibodies in the patient samples.

Individual samples of serum and vaginal wash from all three patient groups were each tested for the presence of gelatinolytic activities by examining aliquots of 20 μl by the above described electrophoretic procedure. Vaginal washes of most women with active trichomoniasis (Group 1) contained detectable activity as described below in the Results section.

Preparation of T. vaginalis *extract for immunoprecipitation*

Axenic cultures of *T. vaginalis* NYH 286 were grown in modified agar-free Diamond's TYM medium as described previously (Bózner & Demeš, 1991).

Trichomonad lysates were prepared as described by Alderete (1983). Organisms in late logarithmic phase of growth were washed 3 times in PBS before being resuspended to a density of 1×10^8 cells/ml. Approximately 2×10^7 parasites were solubilized by the addition of 10% (w/v) Zwittergent 3–12 (Calbiochem–Behring Corp., La Jolla, CA, USA), and the volume of the extract was adjusted to 1 ml with PBS. This extract was overlaid on a 200 μl sucrose cushion (10%, w/v) and centrifuged for 10 min at 10000 ***g***. The recovered supernatant fraction was pre-treated with 200 μl of 10% (v/v) *S. aureus*–SwαHIg at 4 °C for 20 min. This mixture was centrifuged, the supernatant diluted to the equivalent of 1×10^7 trichomonads/ml and used in the immunoprecipitation procedure. The pellet of *S. aureus*–SwαHIg was washed in PBS and examined for possible non-specific adsorption of trichomonad proteinases. Gelatin–SDS–PAGE, however, did not confirm this.

RESULTS

Electrophoretic detection of trichomonad proteinases precipitated by S. aureus*-bound human antibodies*

Pooled sera from 6 patients with urogenital trichomoniasis were initially tested for anti-proteinase antibodies. *T. vaginalis* NYH 286 proteinases were found to be precipitated by antibodies present in these pooled sera, as evidenced by the appearance of clear bands on the gels (Fig. 1, Lane S(T)). Pooled vaginal washes not containing soluble gelatinolytic proteases (by gelatin–SDS–PAGE) were obtained from 6 women who received anti-trichomoniasis treatment (Group 2). These vaginal washes were also found to have antibody that immunoprecipitated proteinases from a trichomonal extract (Fig. 1, Lane W(CT). The presence of specific antibodies to *T. vaginalis* proteinases in both sera and vaginal washes was partly indicated by the absence of similar antibodies in samples from 6 patients of the control group (Fig. 1, Lanes S(C) and W(C)).

Proteinase activities were commonly seen as clear regions rather than distinct bands (Fig. 1). The

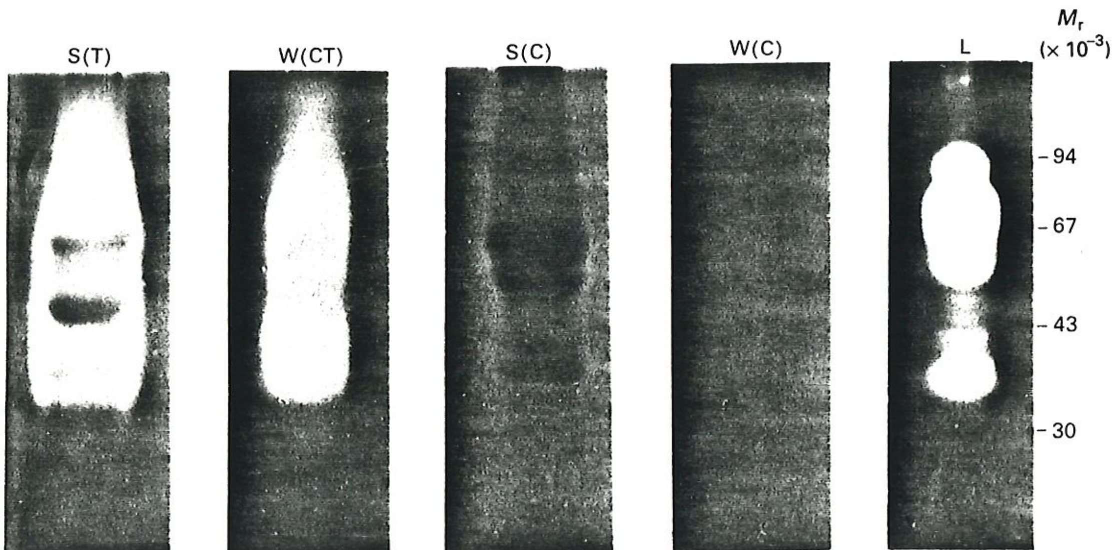

Fig. 1. Gelatin–SDS–PAGE detection of *Trichomonas vaginalis* NYH 286 proteinases immunoprecipitated by antibodies from sera [S(T)] pooled from patients with urogenital trichomoniasis and vaginal washes [W(CT)] pooled from women after successful cure of trichomoniasis. Lanes S(C) and W(C) are sera and vaginal washes, respectively, pooled from patients of the control (3) group. The proteinase profile of *T. vaginalis* NYH 286 cell lysate shown as a control is presented in Lane L.

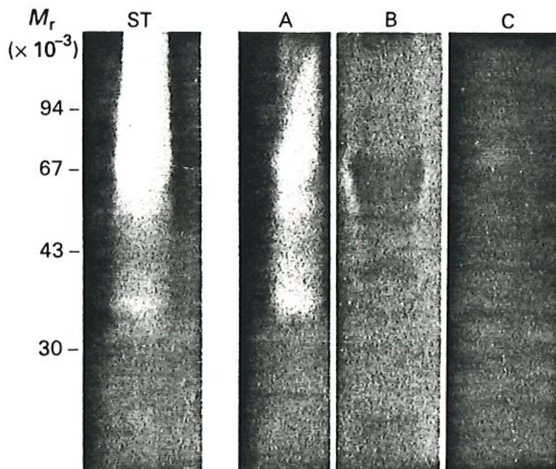

Fig. 2. Gelatin–SDS–PAGE detection of local antibodies complexed with proteinases presumably of *Trichomonas vaginalis* origin. Patients' samples were reacted with *S. aureus*–SwαHIg. The precipitates were not mixed further with *T. vaginalis* NYH 286 lysate as in the standard procedure. Lane A, pooled vaginal washes from patients with active trichomoniasis. Lane B, pooled sera from patients with active trichomoniasis. Lane C, pool of vaginal washes from patients of the control (3) group. Lane ST, the same pool as in lane A, but treated with the standard procedure using *T. vaginalis* NYH 286 lysate.

regions of gelatinolysis appeared in the relative molecular weight range from 40000 to 95000, which is characteristic for the location of the numerous *T. vaginalis* cysteine proteinases (Lockwood *et al.* 1987). These data suggest that antibodies in patients were reactive with several individual *T. vaginalis* proteinases.

A pool of vaginal washes from women with active trichomoniasis contained soluble gelatinolytic proteinases (see Materials and Methods section). These proteinases were probably of trichomonad origin because they occurred in vaginal washes of patients with active trichomoniasis and were absent in samples obtained from women which had been cured of trichomonal infection. We attempted to investigate whether these proteinases were present in a free or in a complexed form with antibodies. The pool was, therefore, reacted with *S. aureus*–SwαHIg which resulted in a precipitation of proteinases in the range 40000–95000 (Fig. 2A). Since *T. vaginalis* NYH 286 proteinases did not bind to *S. aureus*–SwαHIg (data not shown), we suggest that the proteinases demonstrated in Fig. 2A were probably complexed with specific antibodies. The same pool of vaginal washes as in Lane A, but using the standard procedure with *T. vaginalis* lysate, was included for comparative purposes and is presented in Fig. 2ST. A slightly enhanced reaction was invariably observed under these standard conditions, possibly suggesting the presence of antibodies with free binding-sites for *T. vaginalis* proteinases. Some vaginal washes from individual patients with active trichomoniasis did not contain soluble proteinases but were positive for anti-proteinase antibodies (data not shown). The pool of vaginal washes and sera from patients of the control group contained no detectable proteinases (Fig. 2, Lanes B and C).

Pooled positive sera or vaginal washes were also allowed to react with *S. aureus* without SwαHIg. Similar immunoprecipitation reactions were seen, showing that the antibodies against *T. vaginalis* proteinases readily bound to protein A on *S. aureus*. These data indicate that the majority of anti-proteinase antibodies were IgG (Johnstone & Thorpe, 1987).

Table 1. Evaluation of numerous human sera and vaginal washes of patients for the presence of antibodies against *Trichomonas vaginalis* proteinases

Group	Patients	Number examined	Number (%) positive for anti-proteinase antibodies*	
			Serum	Vaginal wash
1	Trichomoniasis	28	20 (71)	24 (86)
2	Cured trichomoniasis†	26	19 (73)	18 (69)
3	Control	21	0	0

* The presence of proteinase activity was evaluated by immunoprecipitation combined with gelatin–SDS–PAGE as shown in the figures.
† Patients in Group 2 were examined 2–6 weeks after successful chemotherapy with ornidazole.

Presence of local and circulating antibodies against trichomonad proteinases in patients with trichomoniasis

A group of 28 women with trichomoniasis was examined for antibodies to *T. vaginalis* proteinases. Twenty sera (71 %) and 24 vaginal washes (86 %) were found to possess anti-proteinase antibodies, as shown in Table 1.

In order to investigate the persistence of anti-proteinase antibodies, a total of 26 trichomoniasis patients was examined 2–6 weeks after successful chemotherapy, as determined by the absence of organisms through cultivation of *T. vaginalis*. Antibody to proteinases was detected in 19 sera (73 %) and 18 vaginal washes (69 %) of patients (Table 1).

None of the 21 women of the control group was found positive for anti-proteinase antibodies either in sera or in vaginal washes (Table 1). These data suggest a high specificity of the method employed.

DISCUSSION

Immunoprecipitation using protein A-bearing *S. aureus*, combined with electrophoresis in gelatin–polyacrylamide gels, provided a specific detection system for demonstration of antibodies against *T. vaginalis* proteinases in patients with active trichomoniasis and in those cured of the infection. Moreover, the method permitted identification of an antibody response against numerous proteinases of the parasite, which ranged in size from 40000 to 95000. This finding could be due to the cross-reaction of an antibody with multiple proteinases. Although this is a possibility, the presence of proteinases at all these relative molecular weights probably means that many of the proteinases are immunogenic, as has been reported (Neale & Alderete, 1990).

Differences observed in representative proteinase band patterns may be due to varying anti-proteinase antibody levels in the patient samples. However, the possibility that the proteinases of the strain NYH 286 used in this study did not bind equally well to antibodies produced by individuals infected with different *T. vaginalis* strains cannot be excluded. Since fresh *T. vaginalis* isolates differ in their proteinase band patterns (Neale & Alderete, 1990; Lockwood *et al.* 1987), the divergent antibody responses may be a reflection of the proteinases being expressed *in vivo* by individual patients.

In comparison with ELISA (Chappell *et al.* 1990) or immunoblotting (Zerda *et al.* 1987), the procedure used in this study has a clear advantage in that it did not require the purification of proteinases as antigens. In contrast with ELISA, however, the method does not allow quantification of the antibody response. Nonetheless, using this method we have demonstrated the presence of local as well as serum anti-proteinase antibodies in women with active trichomoniasis. A plausible explanation for the lack of these antibodies in some patients, positive by cultivation, could be that a fresh infection did not allow enough time for the host to develop a specific antibody response or the antibody concentration was too low to be detected by this method.

According to our results, antibodies against trichomonad proteinases both in sera and vaginal washes were demonstrated 2–6 weeks after treatment and successful cure of trichomoniasis patients, illustrating the immunogenic character of the proteinases. These results are consistent for example with those of Jaakmees *et al.* (1966) who demonstrated a decline of specific anti-*T. vaginalis* antibodies at 3 months, after successful treatment, using complement fixation reaction. Minor modifications of the immunoprecipitation procedure and a different target group in the present study probably explain the previous lack of demonstration of the persistence of anti-proteinase antibodies in post-treatment sera (Alderete *et al.* 1991).

We did not obtain direct evidence for the trichomonad origin of proteinases found in vaginal washes of patients with active trichomoniasis. Nevertheless,

absence of these proteinases in vaginal washes of cured women and in the control group supports this suggestion. Antibodies, already complexed with these proteinases, had no irreversible neutralizing effect on the activity of these enzymes. However, it cannot be ruled out at present that the proteinases might have been neutralized whilst complexed with their antibodies.

Nonetheless, further studies will be needed to determine the immunoglobulin classes which represent these antibodies in serum and in vaginal washes and whether these antibodies have any protective role in urogenital trichomoniasis.

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REFERENCES

ALDERETE, J. F. (1983). Antigen analysis of several pathogenic strains of *Trichomonas vaginalis*. *Infection and Immunity* **39**, 1041–7.

ALDERETE, J. F., NEWTON, E., DENNIS, C. & NEALE, K. A. (1991). Antibody in sera of patients infected with *Trichomonas vaginalis* is to trichomonad proteinases. *Genitourinary Medicine* **67**, 331–4.

ARROYO, R. & ALDERETE, J. F. (1989). *Trichomonas vaginalis* surface proteinase activity is necessary for parasite adherence to epithelial cells. *Infection and Immunity* **57**, 2991–7.

BÓZNER, P. & DEMEŠ, P. (1991). Proteinases of *Trichomonas vaginalis* and *Tritrichomonas mobilensis* are not exclusively of cysteine type. *Parasitology* **102**, 113–15.

CHAPPELL, C. L., DRESDEN, M. H., GRYSEELS, B. & DEELDER, A. M. (1990). Antibody response to *Schistosoma mansoni* adult worm cysteine proteinases in infected individuals. *American Journal of Tropical Medicine and Hygiene* **42**, 335–41.

DEMEŠ, P., GOMBOŠOVÁ, A., VALENT, M., JÁNOŠKA, A., FABUŠOVÁ, H. & PETRENKO, M. (1988). Differential susceptibility of *Trichomonas vaginalis* isolates to complement in menstrual blood and cervical mucus. *Genitourinary Medicine* **64**, 176–9.

DIAMOND, L. S. (1957). The establishment of various trichomonads of animals and man in axenic cultures. *Journal of Parasitology* **43**, 488–90.

GARBER, G. E. & LEMCHUK-FAVEL, L. T. (1989). Characterization and purification of extracellular proteases of *Trichomonas vaginalis*. *Canadian Journal of Microbiology* **35**, 903–9.

JAAKMEES, H. P., TERAS, J. K., ROIGAS, E. M., NIGESEN, U. K. & TOMPEL, H. J. (1966). Complement fixing antibodies in the blood sera of men infested with *Trichomonas vaginalis*. *Wiadomośći Parazytologi* **12**, 378–84.

JOHNSTONE, A. & THORPE, R. (1987). Immunoprecipitation. In *Immunochemistry in Practice*, 2nd Edn, pp. 234–40. Oxford: Blackwell Scientific Publications.

KESSLER, S. W. (1975). Rapid isolation of antigens from cells with a staphylococcal protein A-antibody absorbent. *Journal of Immunology* **115**, 1617.

LAEMMLI, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature, London* **227**, 680–5.

LOCKWOOD, B. C., NORTH, M. J., SCOTT, K. I., BREMNER, A. F. & COOMBS, G. H. (1987). The use of a highly sensitive electrophoretic method to compare the proteinases of trichomonads. *Molecular and Biochemical Parasitology* **24**, 89–95.

NEALE, K. A. & ALDERETE, J. F. (1990). Analysis of the proteinases of representative *Trichomonas vaginalis* isolates. *Infection and Immunity* **58**, 157–62.

NORTH, M. J., MOTTRAM, J. C. & COOMBS, G. H. (1990). Cysteine proteinases of parasitic protozoa. *Parasitology Today* **6**, 270–5.

ZERDA, K. S., DRESDEN, M. H., DAMIAN, R. T. & CHAPPELL, C. L. (1987). *Schistosoma mansoni*: anti-SMw32 proteinase response in vaccinated and challenged baboons. *American Journal of Tropical Medicine and Hygiene* **37**, 320–6.