REPORT

Protective effect of a protease against Trichomonas vaginalis *infection of the murine genital tract*

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ABSTRACT

Trichomonas vaginalis is a pathogen that infects the squamous epithelium of the human urogenital tract. Although the details of the process through which this microorganism parasites host tissues and ultimately causes disease are largely unknown, most authors agree that a necessary first step for establishing infection is the adhesion of the parasite to vaginal epithelial cells. High levels of proteolytic activity have been found in *T. vaginalis*, and it has been proven that some of these proteases are involved in the colonization of the host. Since there are no effective vaccine candidates against this organism, and the presence of resistance against metronidazole during treatment is not infrequent, there is a pressing need for the development of new strategies for the control of this infection. This paper studies the role of a 62 kDa *T. vaginalis* secreted protease on the adherence of this parasite to epithelial cells, showing that monoclonal antibodies (mAbs) against this enzyme can inhibit this process both *in vitro* and *in vivo*, and that intranasal immunization with this protein, adjuvated with either cholera toxin or CpG-containing oligodeoxynucleotides, can protect mice against an intravaginal challenge. Results suggest that this 62 kDa protease is a potential vaccine candidate against trichomoniasis, a disease for which no vaccines are available.

*I*ntroduction

Infection with *Trichomonas vaginalis* is one of the most prevalent sexually transmitted diseases, with a wide geographical distribution and such a high rate of transmission that it is estimated that 170 million persons are infected each year [1]. *T. vaginalis* is associated to a number of health problems in both men and women, including increasing transmission rate for the Human Immunodeficiency Virus, and increasing the incidence of cervical intraepithelial neoplasias in women and non-gonococcal urethritis and chronic prostatitis in men [2]. This parasite infects the squamous epithelium of the human urogenital tract, and although the details of the process through which this microorganism parasites the host tissues and ultimately causes disease are largely unknown, most authors agree that a necessary first step for establishing infection is the adhesion of the parasite to the vaginal epithelial cells. High levels of proteases have been found in *T. vaginalis*, and it has been proven that some of these proteases are involved in this step [3]. Garber and Lemchuk-Favel have purified and partially characterized a 60 kDa protease which has been found in every *T. vaginalis* isolate examined so far, suggesting that this protein might be useful for diagnostics [4]. Due to the absence of an effective vaccine candidate for this infection and the frequent appearance of resistance to metronidazole, the drug most often used for its treatment, there is a pressing need for developing an effective strategy for the control of this parasitosis. Therefore, we have studied the role of a 62 kDa secreted protease from *T. vaginalis* (which is identical to the 60 kDa protein reported by Garber and Lemchuk-Favel in 1989) on the adherence of this parasite to epithelial cells. We show that monoclonal antibodies (mAbs) against this enzyme inhibit adherence *in vitro* and *in vivo*, and that nasal vaccination with this protease, together with cholera toxin (CT) or CpG-containing oligodeoxynucleotides, elicits an immune response capable of protecting mice against an intravaginal challenge.

*M*aterials and methods

The 60 kDa protease was purified using minor modifications to the procedure described by Garber and Lemchuk-Favel [4], employing ionic exchange and gel filtration FPLC. Its purity was verified by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). The mAbs against the protease were obtained and purified following the technology described by Godin JB [5]. A total of 50 parasites were fixed per reaction zone for indirect immunofluorescence, using 10 µL of the experimental mAbs together with their corresponding controls and developing them with a fluorescein isothiocyanate-anti-mouse conjugate. The cytoadherence assays used the HeLa epithelial cell line, labeling the parasites with tritiated thymidine [3]. Passive protection studies for the mAbs were carried out by injecting them at a concentration of 2 mg/mL in a model of intraperitoneal *T. vaginalis* infection in Balb/c mice. The Griess reaction [6] was used to measure nitric oxide concentration in macrophage cultures containing the parasites and the mAbs, as well as in the sera of mice inoculated intraperitoneally with the mAbs and then challenged with *T. vaginalis*.

Three groups of 10 female BALB/c mice each were immunized by intranasal route with 20 µl (20 µg) of the proteinase without adyuvants, and proteinase plus CT (5 µg) or CpG (10 µg), respectively. Two other groups were immunized with each of theses adjuvants plus saline (0.15 M NaCl). A six group was immunized with saline. Serum and vaginal lavage fluid samples were collected pre- immunization, 10 days after the second immunization and 1-week after the T. vaginalis challenge. All sample were stored at -20º C until assayed by ELISA.

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Results

The purification of this protease yielded 3 fractions with enzymatic activity, containing proteins of 62, 40 and 29 kDa respectively. Three stable hybridomas producing IgG1-subclass mAbs (4D8, 3C11, 1A8) against the protease were obtained, of which only two (4D8 and 1A8) produced antibodies capable of binding the parasite in an immunofluorescence assay. An *in vitro* assay for adhesion to HeLa monolayers showed that all three mAbs (4D8, 1A8 and 3C11) were able to inhibit the adherence of the parasite to the cells (Figure 1). The monoclonal antibody 4D8, administered intraperitoneally 24 hours before a challenge with *T. vaginalis*, was able to protect the majority of the mice. This was also observed for mAb 1A8, although the protective effect was lower. A high level of nitric oxide was found in the cultures and in the sera of mice treated with mAb 4D8 (tables 1 and 2, respectively). Since *T. vaginalis* infects the human genital tract, and in order to determine whether immunization with this protease could protect female mice against a vaginal challenge, 2 groups of mice were vaccinated intranasally with the 62 kDa antigen adjuvated with either cholera toxin or CpG oligodeoxynucleotides, and four control groups were immunized with CT, CpG, protease or saline alone, respectively. After conditioning the animals for infection and challenging with 8 x 10^5 parasites, vaginal samples were collected for assessing the presence or absence of *T. vaginalis*. The rate of infection ranged from 5 to 15% of the mice for the experimental groups (p-62-CT and p-62-CpG) and from 80 to 90% for the control groups (table 3). The levels of antibodies against p-62 in the sera and vaginal washes of the mice were measured by ELISA, finding significant concentrations of IgG ($p<0.05$) and IgA ($p<0.01$) against the antigen in the vaginal washes of the experimental groups.

Discussion

The fundamental role of *T. vaginalis* proteases during the process of cytoadherence has been revealed by the studies of Mendoza-López *et al.* [1] and Arroyo and Alderete [3]. Their observations are coherent with the results of this study, which shows that mAbs against a 62 kDa protease can inhibit this process.

The administration of mAb 4D8, 24 hours before an intraperitoneal challenge, protected 92% of the mice. Significantly, this antibody displayed the highest inhibitory values for adhesion *in vivo*, followed by mAb 1A8; suggesting that the epitopes recognized by these antibodies are important for the events of cellular adhesion in *T. vaginalis*.

Our results prove that these mAbs may exert their protective effect through cellular mechanisms, probably through the interaction with residing macrophages at the peritoneum. The generation of nitric oxide

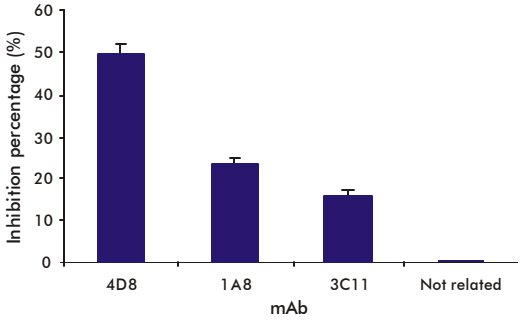

Figure 1. Effect of the mAbs against the 62 kDa protease on the adhesion of *T. vaginalis* to cultured HeLa cells. The thymidine-labeled parasites and the mAb were added to HeLa cells and incubated for 30 min. Results correspond to two separate experiments, and show the mean ± standard deviation of 10 wells. Student t-test.
* Statistically significant differences in comparison to the unrelated mAb.

Table 1. Nitric oxide determined in macrophage cultures treated with mAbs in the presence of the parasite

Incubation	Nitrite (μM) Mean ± SD
1A8 + LPS + IFNg	590.01 ± 32.53 *
4D8 + LPS + IFNg	676.75 ± 26.52 *
4D8 + LPS + IFNg + L-NMMA	96.4 ±7.60
Unrelated mAb + LPS + IFNg	83.20 ±4.27

A total of 6 x 104 parasites together with the mAbs were placed in wells containing 3x105 cultured macrophages. Two hours later, LPS, γIFN and L-NMMA were added, and the supernatants were collected 18 hours later and mixed with Griess reagent to measure the nitric oxide concentration. The values represent the mean and standard deviation from 5 wells (n = 5). Statistical significance was estimated using Student's t-test.
* Represents statistically significant differences compared to the unrelated mAbs

Table 2. Nitric oxide determined in the sera of mice inoculated with mAbs against the T. *vaginalis protease*

Group	mAb	Nitrite (μM) Mean ± SD
1	3C11	80.09 ± 24.03
2	1A8	139.90 ± 39.77 *
3	4 D8	165.82 ± 65.58 *
4	Unrelated	83.20 ± 21.40

Four groups of animals were inoculated intraperitoneally at a concentration of 2 mg/mL with the mAb. After 24 h the animals were challenged intraperitoneally with 8 x 106 parasites per mouse, and serum nitric oxide concentrations were determined 3 days later using the Griess reaction. The values represent the mean and standard deviation from 5 mice (n = 5). Statistical significance was estimated using Student's t-test.
* Represents statistically significant differences compared to the unrelated mAbs

Table 3. Protective effect of nasal immunization against the p-62 protease on the infection with T. vaginalis in Balb/c mice

Pretreatment	Mice infected with T. vaginalis/group[6]		Total	
	A	B	Infected mice/ Total mice	Infection percentage (%)
p-62[1]	8/10	10/10	18/20	90
p-62 + TC[2]	0/10	1/10	1/20	5*
p-62 + CpG[3]	1/10	2/10	3/20	15*
CT Control[4]	10/10	8/10	18/20	90
CpG Control[5]	8/10	8/10	16/20	80
Saline solution	9/10	9/10	18/20	90

20 μg antigen (dose/mouse).[1]
20 μg antigen, 5 μg CT (dose/mouse).[2]
20 μg antigen, 10 μg CpG (dose/mouse).[3]
5 μg CT (dose/mouse).[4]
10 μg CpG (dose/mouse).[5]
The mice received an intravaginal challenge of 8 x 10^5 parasites 2 weeks after the last immunization.[6]
* Represents statistically significant differences compared to the control (saline solution). Statistical significance was estimated using Student's t-test.

is typical of many cells involved in the immune response, such as natural killers (NK), monocytes and macrophages [7]. Previous studies with *Trichomonas foetus* have shown that high levels of nitrite probably inactivate the FeS proteins in the hydrogenosomes, leading to the death of the parasite [8]. The high levels of nitric oxide found in the sera of mice treated with mAbs 4D8 and 1A8, as well as the nitric oxide measurements taken during the *in vitro* experiment, suggest that this is indeed the cause of the toxic effect of the antibodies on the parasites in the *in vitro* model.

The use of intranasal immunization schemes has the advantage that this route can potentially induce both systemic and mucosal responses, with the latter appearing at local or remote effector sites [9]. Our results show that intranasal immunization with the 62 kDa proteinase with CpG or CT is not only capable of inducing specific anti-p-62 antibodies in the serum, but also in vaginal secretions, resulting in protection against the challenge with *T. vaginalis.* Results also suggest that both adjuvants are effective for this immunization route, and that high IgA levels are an important component of a protective immune response against this parasite. New studies are needed to elucidate the immune response mechanisms activated by the immunization against *T. vaginalis* with the 62 kDa protease.

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