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Degradation of human immunoglobulins and cytotoxicity on HeLa cells by live *Trichomonas vaginalis*

Duk-Young MIN[1], Jae-Sook RYU[1]*, So-Young PARK[1], Myeong-Heon SHIN[2], Whi-Youl CHO[1]

Department of Parasitology[1], College of Medicine, Hanyang University, Seoul 133-791, Department of Parasitology[2], College of Medicine, Ewha Womans University, Seoul 158-050, Korea

Abstract: The present study was undertaken to determine whether live *T. vaginalis* degrades human secretory IgA, serum IgA and IgG molecules. Human immunoglobulins were exposed to live trophozoites, parasite lysate, and excretory-secretory product (ESP) of *T. vaginalis*. To determine the fragmentation of immunoglobulins, the reaction sample was subjected to SDS-PAGE and EITB, and peroxidase conjugated antihuman IgA and IgG were used as probes. Live trophozoites degraded secretory IgA, serum IgA and IgG, and degradation were pressed forward by the prolongation of the incubation time and by increasing the number of trichomonads respectively. Also the lysates and ESP of trichomonads degraded IgA and IgG. The cysteine and serine proteinase inhibitors such as E-64, antipain, iodoacetic acid, iodoacetamide, TLCK reduced the ability of cleaving immunoglobulins. The proteinase activity and cytotoxicity of *T. vaginalis* to HeLa cells were decreased when live *T. vaginalis* was treated with metallo-proteinase inhibitor as well as cysteine and serine proteinase inhibitors. These results suggest that proteinase secreted from live *T. vaginalis* may play a part role in host pathogenesis by *T. vaginalis*, and the cleaving ability of host immunoglobulins by the proteinase may contribute as a one of immune evasion mechanism for parasite survival in the host.

Key words: *Trichomonas vaginalis*, proteinase, degradation of immunoglobulins, cytotoxicity on HeLa cell, proteinase inhibitors

INTRODUCTION

Proteinase probably occurs in all protozoa and proteolysis plays as an important part in many aspects of their life cycle (North, 1982). In *Trichomonas vaginalis*, at least 11 cysteine proteinases have been identified (Coombs and North, 1983; Lockwood *et al.*, 1987). Alderete *et al.* (1986) and Alderete and Neale (1989) reported that proteolytic activity for some trichomonad molecules may be common during normal growth of *T. vaginalis* and one or more proteinase may reside on the surface of this protozoa. Also numerous proteinase and antibody to trichomonad proteinase are present in the vagina of trichomoniasis patients (Alderete *et al.*, 1991a & b).

Several investigators reported about the role of proteinase of *T. vaginalis* that cysteine proteinase of *T. vaginalis* is important in adherence of *T. vaginalis* to HeLa cells or human vaginal epithelial cells (Arroyo and Alderete, 1989), and is related with the haemolytic activity of live parasites (Daily *et al.*, 1990), and cysteine proteinase from *T. vaginalis* lysate degrades human Igs

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*Corresponding author

(Provenzano and Alderete, 1995).

While the proteinases of many parasites, such as *Giardia lamblia, Entamoeba histolytica, Schistosoma mansoni, Dirofilaria immitis, Fasciola hepatica, Spirometra mansoni,* have degraded immunoglobulins of host (Parenti, 1989; Kelsall and Ravdin, 1993; Auriault *et al.*, 1981; Tamashiro *et al.*, 1987; Carmona *et al.*, 1993; Kong *et al.*, 1994). Especially thiol proteinase from *Giardia lamblia* and cysteine proteinase of *Entamoeba histolytica* degraded human IgA (Parenti, 1989; Kelsall and Ravdin, 1993).

In this report, in order to demonstrate the role of parasitic proteinase in host-parasite relationship, we studied whether live *T. vaginalis* degrade human immunoglobulins, and the effect of proteinase inhibitors on HeLa cell cytotoxicity by *T. vaginalis*.

MATERIALS AND METHODS

1. *Trichomonas vaginalis* culture

T. vaginalis KT9 isolate was obtained from the vaginal secretion of a Korean woman with acute vaginitis. Trophozoites were axenically cultured in Diamond's TYM medium (1957).

2. Degradation of human immunoglobulins by *T. vaginalis*

1) Degradation of human immunoglobulins: Human secretory IgA, human serum IgA and human IgG (Chemicon, CA) were used in order to observe the ability of degradation of immunoglobulin by live *T. vaginalis*, *T. vaginalis* lysate and secretory-excretory product. *T. vaginalis* lysate was prepared by sonication and ultracentrifugation at 4°C, 100,000 × g for 1 hr. Excretory-secretory product (ESP) was obtained by which trophozoites were suspended in PBS, cultured in 37°C incubator for 2 hrs and centrifuged at 10,000 × g. In order to observe the ability of degradation by live protozoa, 15 μg immunoglobulin and 5 mM DTT were added to 30 μl phosphate buffer containing mobile *T. vaginalis* 15×10^4 - 500×10^4, and cultured in 37°C incubator for 30 min-22 hrs, and then trophozoites were removed by centrifugation at 10,000 × g and SDS-PAGE sample buffer was added to supernatant to stop the reaction. Amount of *T. vaginalis* lysates and secretory-excretory product used was 50 μg, respectively.

To determine the effect of proteinase inhibitors on the degradation of human immunoglobulins, proteinase inhibitors were added to live trophozoites, incubated at 37°C for 1 hr, and then trophozoites were washed and subsequent steps were done same as above.

2) Sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and enzyme- linked immunoelectrotransfer blot (EITB): SDS-PAGE was carried out on 7.5-15% gradient running gel on 30 mA and 4% stacking gel on 15 mA, under discontinuous buffer system as the method described by Laemmli (1970). Molecular weight was calculated using standard marker [high MW 45,000-200,000 and low MW 14,400-97,400 (Bio-Rad, Rockville Center, NY)], as the method described by Lambin (1978). The gel was stained with Coomassie Blue R-250 or used for immunoblotting. EITB was carried out according to the method of Towbin *et al.* (1979), Tsang *et al.* (1983) and Min *et al.* (1992). For the reaction, 1:1000 diluted peroxidase conjugated anti-human IgG and IgA (Fc fragment, Cappel, Pennsylvania) and diaminobenzidine were used.

3. Cytotoxicity test

Cell culture and cytotoxicity test: HeLa (human cervix epitheloid carcinoma) cell was grown in MEM medium with Earle's salts & L-glutamine (Gibco BRL, NY) supplemented with 10% fetal calf serum at 37°C, 5% CO_2 incubator. Cytotoxicity to HeLa cells mediated by *T. vaginalis* was observed according to modified Alderete and Pearlman (1984)'s method. HeLa cells were grown to confluency in 96 well culture plates by which cells were seeded at a density of 2×10^4 into individual wells and cultivated for overnight. On next day 20×10^4 trichomonads suspended in MEM-TYM complex media (MEM:TYM = 20:1) were adding to monolayer and cultured for 5 hrs by the ratio of HeLa cell/trichomonads 1:10. The non-adherent (floating) cells were removed and adherent cells were washed with PBS, fixed for 10 min by 2% formaldehyde, stained for 15

min by added 100 μl 0.13% crystal violet solution (crystal violet 260 mg, ethyl alcohol 143 ml, formaldehyde 57 ml) to each well. The stained materials were subsequently washed twice with distilled water, the plates air dried, and the remaining stain was solubilised in 1% SDS prepared in 50% ethanol. Individual wells were then scanned using autoreader spectrophotometer at 570 nm.

In order to examine the effect of proteinase inhibitor, some kinds of proteinase inhibitors were added to live *T. vaginalis* at 37°C incubator, after 1 hour, they were washed, and then added to HeLa cell monolayer. Cytotoxicity was calculated as below.

$$\text{Cytotoxicity (\%)} = \left(1 - \frac{\text{O.D. of experimental group}}{\text{O.D. of control group}}\right) \times 100$$

4. Proteinase activity

Proteinase activity was measured by modified methods of North *et al.* (1983), Lockwood *et al.* (1984) and Min *et al.* (1996). Live *T. vaginalis* was treated some kinds of proteinase inhibitor for 1 hr, and washed, and incubated in PBS at 37°C for 2 hrs. Fifteen μg of trichomonads supernatant was reacted with 10 mM DTT 15 μl for 5 min, and then sodium phosphate buffer (pH 6.0) 400 μl and 1 mM benzoyl-proline-phenylalanine-arginine-nitroanilide (Bz-Pro-Phe-Arg-Nan) 20 μl were added to reaction mixture, and enzyme activity was measured by optical density at 405 nm after incubation for 90 min at 37°C.

In order to examine the effect of proteinase inhibitor, some kinds of proteinase inhibitors were added to live *T. vaginalis* at 37°C incubator, at they were washed after 1 hour, incubated in PBS at 37°C for 2 hrs. Proteinase activity of trichomonads were measured as above.

5. Proteinase inhibitors

The cysteine proteinase inhibitors E-64 (N-[N-(L-3-transcarboxyoxirane-2-carbonyl)-L-leucyl]-agmatine), leupeptin (Acetyl-Leu-Leu-Arg-al), antipain, iodoacetic acid (IAA), iodoacetamide, serine proteinase inhibitor such as TLCK (Nα-p-tosyl-L-lysine chloromethyl ketone), metallo-proteinase inhibitor such as EDTA and aminopeptidase inhibitor bestatine[[(2S,3R)-3-amino-2-hydrozy-4-phenylbutanoyl]-L-leucine), were examined for their effect on enzyme activity, degradation of immunoglobulis and cytotoxicity of *T. vaginalis* to HeLa cell. All effectors were prepared in an appropriate stock solutions. All chemicals were purchased from Sigma (St. Louise, MO) except E-64 which were purchased from Boehringer Mannheim (Germany).

RESULTS

Degradation of immunoglobulins by live *T. vaginalis*

Live *T. vaginalis* trophozoites were able to degrade human secretory IgA, serum IgA and IgG at 37°C. These were dose-dependent effect based on the number of trophozoites and time-dependent effect on the reaction time used (Figs. 1, 2 & 3). In case of secretory IgA and serum IgA, live trophozoites digested heavy

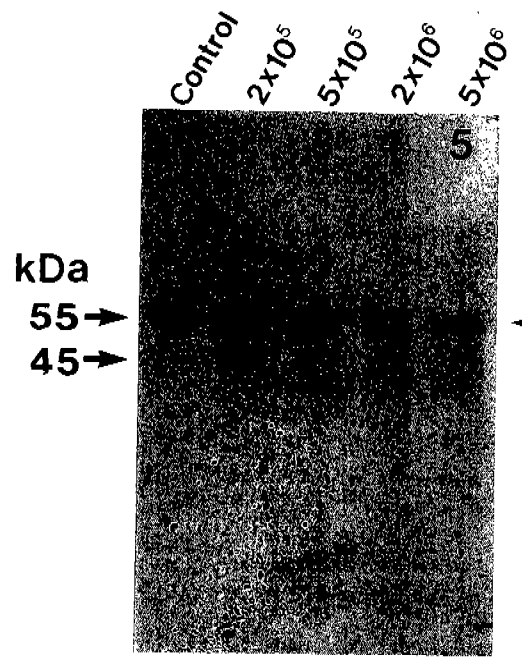

Fig. 1. Degradation of secretory IgA by live *T. vaginalis*. Live *T. vaginalis* digest secretory IgA in a dose-dependent manner. Lane 1, secretory IgA alone; Lane 2-5, secretory IgA incubated with 2 × 10^5, 5 × 10^5, 2 × 10^5 and 5x10^6 of *T. vaginalis*. (▶; heavy chain)

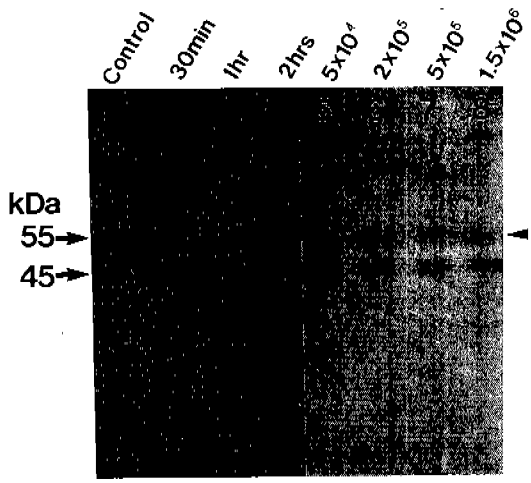

Fig. 2. Degradation of serum IgA by live *T. vaginalis*. Live *T. vaginalis* digests serum IgA in a time-dependent and dose-dependent manner. Lane 1, serum IgA alone; Lane 2-4, serum IgA incubated with *T. vaginalis* (2×10^6) for 30 min, 1 hr and 2 hrs. Lane 5-8, serum IgA incubated for 2 hrs with 5×10^4, 2×10^5, 5×10^5 and 1.5×10^6 of *T. vaginalis*. (▶; heavy chain)

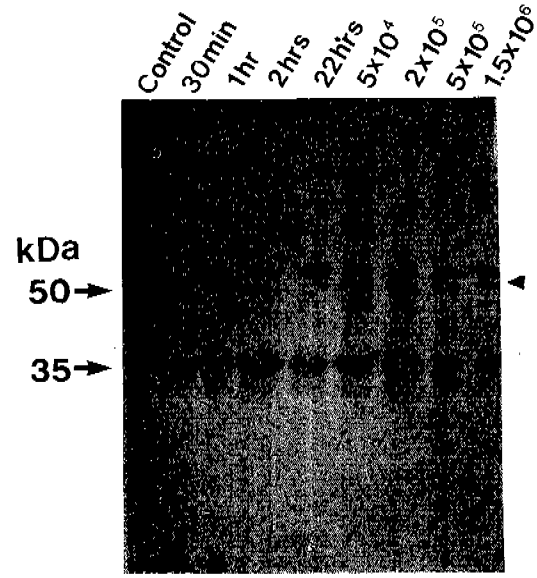

Fig. 3. Degradation of serum IgG by live *T. vaginalis*. Live *T. vaginalis* digests serum IgG in a time-dependent and dose-dependent manner. Lane 1, serum IgG alone; Lane 2-4, serum IgG incubated with *T. vaginalis* (2×10^6) for 30 min, 1 hr, 2 hrs and 22 hrs. Lane 6-9, serum IgG incubated for 2hrs with 5×10^4, 2×10^5, 5×10^5 and 1.5×10^6 of *T. vaginalis*. (▶; heavy chain)

chain of 55 kDa into 45 kDa. Whereas 50 kDa heavy chain of IgG was fragmented into 35 kDa. When 2×10^6 trophozoites incubated with IgG for 2 hrs, 50 kDa heavy chain disappeared and 35 kDa band remained (Fig. 3).

Degradation of immunoglobulins by *T. vaginalis* lysate and excretory-secretory product (ESP)

Immunoglobulins were degraded by 50 μg of lysate and ESP within 2 hrs at 37°C. Especially secretory IgA was completely degraded by ESP. *T. vaginalis* proteinase fragmented IgA heavy chain into 45 kDa molecule, and IgG heavy chain into 35 kDa (Fig. 4).

Effect of proteinase inhibitors on IgA degradation of *T. vaginalis*

Secretory IgA degradation by viable trophozoites was prevented by the serine-specific inhibitor TLCK and the cysteine-specific inhibitors E-64, antipain, IAA, and iodoacetamide. There was a minimal inhibitory effect by leupeptin, and no inhibition by EDTA and bestatin (Fig. 5).

Effect of proteinase inhibitors on the cytotoxicity of *T. vaginalis* to HeLa cells

All of the proteinase inhibitors used in this study significantly reduced the cytotoxicity to HeLa cell by *T. vaginalis* except bestatin ($p < 0.05$, Table 1).

Effect of proteinase inhibitors on the proteinase activity of *T. vaginalis*

Cysteine proteinase such as E-64, antipain, IAA and iodoacetamide, and TLCK, serine proteinase inhibitor, decreased the proteinase activity when Bz-Pro-Phe-Arg-Nan was used as substrate. But their activities were not significantly inhibited comparing with preteinase activity of control group ($p = 0.1$ by Mann-Whitney U test, Table 2).

DISCUSSION

IgA-lysing protease has been described in

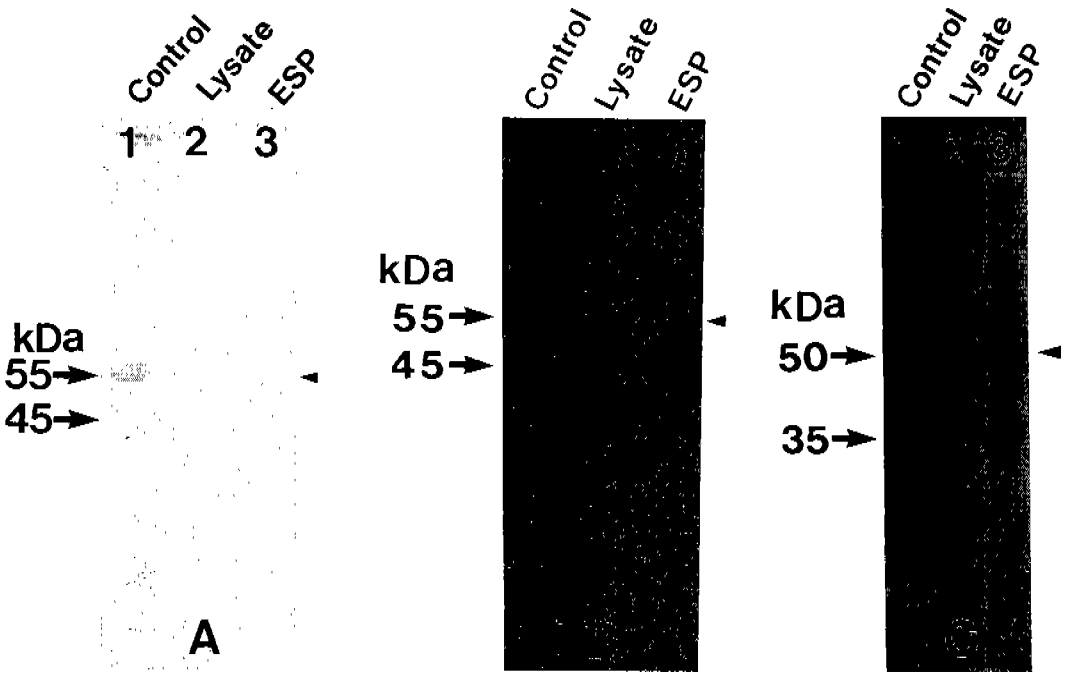

Fig. 4. Degradation of human immunoglobulins by *T. vaginalis* lysate (50 μg) and excretory-secretory product (ESP) (50 μg). **A:** Degradation of secretory IgA by *T. vaginalis* lysate (lane 2) and ESP (lane 3). **B:** Degradation of serum IgA by *T. vaginalis* lysate (lane 2) and ESP (lane 3). **C:** Degradation of IgG by *T. vaginalis* lysate (lane 2) and ESP (lane 3).

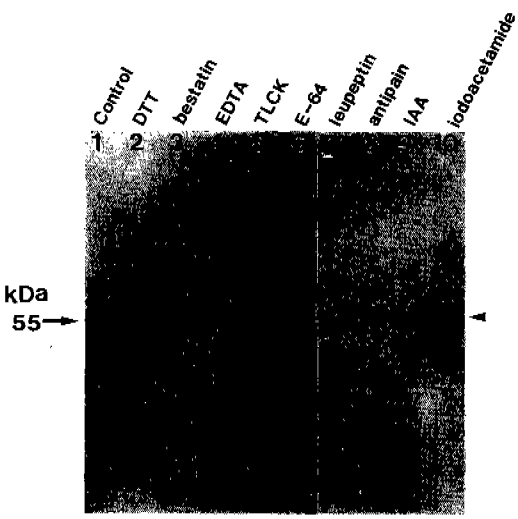

Fig. 5. Degradation of secretory IgA by live *T. vaginalis* treated with following proteinase inhibitors. Lane 1, secretory IgA alone; Lane 2, DTT; Lane 3, bestatin; Lane 4, EDTA; Lane 5, TLCK; Lane 6, E-64; Lane 7, leupeptin; Lane 8, antipain; Lane 9, IAA; Lane 10, iodoacetamide. (▶; heavy chain)

Table 1. Effect of proteinase inhibitor on the cytotoxicity of *Trichomonas vaginalis* to HeLa cells

Inhibitors	Inhibitor conc.	Cytotoxicity (%)
Control		74.9 ± 2.70[a]
Bestatin	100 μM	56.1 ± 16.04
EDTA	100 μM	43.6 ± 10.73[a]
TLCK	100 μM	43.1 ± 20.79[a]
E-64	100 μM	38.9 ± 17.66[a]
Leupeptin	150 μM	61.2 ± 6.35[a]
Antipain	100 μM	45.6 ± 8.96[a]
Iodoacetic acid	25 μM	24.1 ± 12.59[a]
Iodoacetamide	100 μM	7.4 ± 10.17[a]

Ratio of *T. vaginalis* to HeLa cells is 10:1.
Initial number of HeLa cells was 2×10^4.
Incubation time is 5 hrs.
[a]$p < 0.05$, (Mann-Whitney U test)

Giardia lamblia, *Entamoeba histolytica* and *T. vaginalis*. Especially cysteine proteinase of *E. histolytica* and *T. vaginalis*, thiol proteinase of *G. lamblia* are predominantly responsible for the degradation of human IgA (Kelsall and

Table 2. Effect of proteinase inhibitor on the proteinase activity of *Trichomonas vaginalis*

Inhibitors	Inhibitor concentration	Enzyme activity (O.D, at 404 nm)
Control		0.148 ± 0.009
Bestatin	100 μM	0.155 ± 0.002
EDTA	100 μM	0.124 ± 0.012[a)]
TLCK	100 μM	0.005 ± 0.003[a)]
E-64	100 μM	0.007 ± 0.001[a)]
Leupeptin	100 μM	0.072 ± 0.007[a)]
Antipain	100 μM	0.010 ± 0.004[a)]
Iodoacetic acid	100 μM	0.001 ± 0.001[a)]
Iodoacetamide	100 μM	0.008 ± 0.002[a)]

Bz-Pro-Phe-Arg-Nan was used as substrate for enzyme activity. 3×10^6 *T. vaginalis* was suspended in PBS containing proteinase inhibitor and incubated for 1 hr, washed with PBS, resuspended in PBS for 2 hrs.
[a)]$p = 0.1$ (by Mann-Whitney U test)

Ravdin, 1993; Parenti, 1989; Provenzano and Alderete, 1995). In this study live trophozoites of ESP and lysate of *T. vaginalis* degraded secretory IgA and serum IgA antibodies. Cysteine proteinase inhibitors such as E-64, antipain, IAA, iodoacetamide prevented degradation of secretory IgA by live trophozoites but bestatin and EDTA degraded secretory IgA. As like other protozoa, cysteine proteinase of *T. vaginalis* may play a major role in degradation of IgA. Furthermore IgA protease may be one of the factors that allow the organisms to evade host defense mechanisms because numerous proteinases are present in vaginal secretion of trichomoniasis patients (Alderete *et al.*, 1991b).

Several investigators reported the IgG proteinases of helminthes such as *Schistosoma mansoni* (Auriault *et al.*, 1980, 1981), *Dirofilaria immitis* (Tamashiro *et al.*, 1987), *Fasciola hepatica* (Chapman and Mitchell, 1982; Carmona *et al.*, 1993), *Spirometra mansoni* (Song and Chappell 1993; Kong *et al.*, 1994). Provenzano and Alderete (1995) demonstrated the proteolytic activity against IgG in vaginal washes from patients with trichomoniasis. This study showed that trophozoites lysate and ESP of *T. vaginalis* fragmented 50 kDa heavy chain of IgG into 35 kDa in dose-dependent reaction and time-dependent manner.

It is well known that trichomonal cytadherence to epithelial cells is a highly specific key step during initiation of infection and disease pathogenesis (Alderete and Garza, 1985; Alderete *et al.*, 1988). However the role of trichomonad proteinase during parasite binding to epithelial cell surface is not well known. Arroyo and Alderete (1989) evaluated that treatment of *T. vaginalis* with cysteine proteinase inhibitors induced the abrogation of cytadherence and render the trichomonads unable to kill host cells although cysteine proteinase of *T. vaginalis* is not equally inhibited by leupeptin and TLCK. Then they recommended the screening of multiple inhibitors which may be important for future identification of the proteinase involved in cytadherence. In this study, various proteinase inhibitors were screened for *T. vaginalis* cytotoxicity. Except bestatin, all of the proteinase inhibitors used in this study significantly decreased cytotoxicity of *T. vaginalis* to HeLa cells ($p < 0.05$). Therefore several type of proteinase inhibitors may be related with cytotoxicity on HeLa cells although we didn't evaluate effect of cytadherence by proteinase inhibitors. Similarly Keene *et al.* (1990) reported that expression and release of the cysteine proteinase of *E. histolytica* is an important factor in producing the cytopathic effect.

The proteinase activities of live *T. vaginalis*, which were treated with same inhibitors used in the cytotoxicity test, were decreased comparing with that of control trophozoites except bestatin. Various proteinase inhibitors affect the proteinase activity of *T. vaginalis* as well as cytotoxicity. Therefore the cytotoxicity on HeLa cells may be probably associated with proteinase activity of *T. vaginalis* even though it is impossible to statistically compare cytotoxicity with proteinase activity because of different experimental number of each group by various inhibitors.

Through these results, the proteinase secreted from live *T. vaginalis* may act a part role in host pathogenesis by *T. vaginalis*, and cleaving ability of host immunoglobulins by

the proteinase may contribute as one of immune evasion mechanisms for parasite survival in the host.

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=초록=

질편모충 단백질분해효소의 세포독성 및 인체면역글로불린 분해능

민득영[1], 류재숙[1], 박소영[1], 신명헌[2], 조휘율[1]

한양대학교 의과대학 기생충학교실[1], 이화여자대학교 의과대학 기생충학교실[2]

이 연구에서는 살아있는 질편모충의 단백질분해효소가 인체 면역글로불린을 분해하는지 알아보고, 질편모충에 의한 조직세포 독성에 있어서 단백질분해효소의 역할을 시험관내에서 관찰하였다. 실험에 사용한 질편모충은 질염환자로부터 분리한 KT9 분리주이었으며 세포독성을 알아보기 위한 표적세포로는 HeLa세포를 사용하였다. 질편모충 단백질분해효소가 인체 면역글로불린을 분해하는지 관찰하고자 인체의 분비 IgA, 혈청 IgA 및 IgG를 살아있는 원충, 원충의 용출액 및 분비-배설액과 DTT를 넣어 반응시켰다. 여러 계열의 단백질분해효소 저해제(aminopeptidase, serine, metallo, cysteine계열)를 살아있는 질편모충과 미리 반응시킨 후 세척하고 면역글로불린의 분해, 단백질분해효소 활성 및 조직세포독성에 미치는 영향을 관찰하였다. 살아있는 질편모충은 인체의 분비 IgA, 혈청 IgA 및 IgG를 분해하였는데 질편모충 수가 증가할수록, 반응시간이 길수록 분해가 더 잘 이루어졌다. 질편모충의 용출액과 분비-배설액도 분비 IgA, 혈청 IgA 및 IgG를 분해하였다. Cysteine, serine계열의 단백질분해효소 저해제(E-64, antipain, iodoacetic acid, iodoacetamide, TLCK)를 처리한 질편모충은 분비 IgA의 분해를 저해하였으며, 단백질분해효소저해제로 처리한 경우 질편모충의 단백질분해효소 활성은 감소하였고 HeLa세포에 대한 독성이 감소하였다. 이상의 성적을 종합하면 질편모충에서 분비되는 단백질분해효소는 시험관내에서 조직세포에 세포독성을 나타내며 또한 인체면역글로불린을 분해하여 숙주의 방어기전에 대한 도피물질로 작용하는 것으로 생각된다

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