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The effect of *Ascaris* trypsin inhibitor on mitotic index, chromosome aberrations and sister chromatid exchanges (SCE) frequencies in cultured human lymphocytes

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Summary

The trypsin inhibitor (ATI) isolated from nematode *Ascaris suum* was tested *in vitro* for chromosome aberrations and sister chromatid exchanges (SCE). ATI was obtained from the musculocutaneous sac homogenate of adult *Ascaris* by the modified method of Pudles and Rola. Genotoxicity assessment of ATI was carried out on metaphase plates received from peripheral blood lymphocyte macroculture (48 h-test of structural chromosome aberrations and 72 h-test of SCE) without exogenous metabolic activation. ATI was tested in doses: 25, 50 and 100 μg per ml of culture (continuous exposure). Kinetics of cell divisions was determined by the replication index (RI). The mitotic index (MI) was expressed as a number of metaphases per 1000 nuclei analysed. The chromosome aberration test revealed that ATI did not induce a statistically significant change in chromosome aberrations frequency compared to the control but significantly decreased the MI values in 48 hour test. The 72 hour SCE test demonstrated that ATI in all doses induced a higher number of SCE per cell but less than double frequency as compared to the control. For all doses of ATI, replication index (RI) values were significantly higher than in the control. Thus the *Ascaris* trypsin inhibitor did not show genotoxic properties at these experimental conditions but exhibited influence on division rate of lymphocytes.

Key words: *Ascaris suum;* trypsin inhibitor; chromosome aberrations; human lymphocytes; sister chromatid exchange; replication index; mitotic index

Introduction

Genetic factors of both embryo and pregnant female play major roles in determining the type, frequency, and severity of defects that develop spontaneously or through experimental intervention. It has been commonly accepted that mutagenic chemicals administered into experimental animals may induce preimplantation losses of nonviable zygotes, early fetal deaths, sterility, abortion, and congenital malformations (Epstein *et al.*, 1972).

In the previous studies (Blaszkowska, 1998b; 2001), it was observed that *Ascaris* proteolysis inhibitor disturbed the embryonic development of experimental animals. The *Ascaris* trypsin inhibitor injected during early or late organogenesis produces specific types of congenital malformations in mice (Blaszkowska, 1998a; 1999). The mechanism by which this inhibitor disturbed prenatal development is not clear; its influence on pregnancy is not connected with its antiproteolytic properties but with maternal toxicity (Blaszkowska, 2003).

Recently only few reports have provided information on the influence of helminth metabolites on the host's somatic and generative cells (Shubber & Salin, 1987; Bekish, 2000; 2001). Larvae metabolites of *Ascaris suum* have been found to exert a mutagenic effect on somatic cells of bone marrow, spermatogonies and on the generative cells (spermatides) of infected mice (Bekish & Bekish, 2000; Bekish, 2001). Also, it has been established that different antigens from *Ascaris suum* (whole *Ascaris*, musculocutaneous sac, cavity fluid) change the chromosome apparatus of human blood lymphocytes *in vitro*, increase the number of aneuploid and aberrant cells and increase the number of micronuclei erythrocytes in the bone marrow (Bekish, 1999; Bekish & Bekish, 2000). Another study has shown that the extract from whole worms contains a mitogenic factor, which stimulates human T-lymphocytes (Sasagawa *et al.*, 1987). The *Ascaris* extract (100 μg proteins/ml) induced an increase in [^{3}H] thymidine incorporation into human lymphocytes at a level similar to that obtained with the pokeweed mitogen (11 μg/ml). Lee and Xie (1995) also described the presence of a B cell mitogen in the *Ascaris* body fluid, which stimulated G_0 B lymphocytes to enter the cell cycle. This *Ascaris* fluid did not exert such effect on T

cells. In contrast to the immunostimulatory activity of the *Ascaris* compounds reported above, other investigators have demonstrated immunosuppressive properties of extracts and secretions of this helminth (Soares *et al.*, 1992; Souza *et al.*, 2002; Oshiro *et al.*, 2004).
Having considered previously detected embryotoxic and teratogenic action of the trypsin inhibitor from *Ascaris* we aimed in this study to investigate, by means of cytogenetic tests, whether this chemical exhibits a potential mitostatic and genotoxic activity.

Materials and Methods

Chemicals
The preparation of trypsin inhibitor (protein fraction SF_5) was obtained from the homogenate of *Ascaris suum* tegument using a modified method of Pudles and Rola (1967). The procedure of purification of the inhibitor and its inhibitory activity against crystalline trypsin had been described elsewhere (Blaszkowska, 2003).

Lymphocyte macrocultures
Venous blood from 6 healthy adult male non-smoker donors without a known drug or medical history was used in the experiment. Lymphocyte (whole lymphocyte population) cultures were prepared from heparinised blood according to the method of Moorhead *et al.* (1960). For each assay, 20 ml of aseptically venous blood was allowed to sediment for 2 to 3 h. After red cell sedimentation, the supernatant (1 ml) was added to 8 ml Eagle's fluid 1959 (MEM), 1 ml of fetal serum (Gibco), crystalline penicillin (100 IU/ml) and streptomycin (100 μg/ml). Phytohaemagglutinin - PHA (Difco)- was used for stimulation of cell divisions at the beginning of incubation. The *Ascaris* trypsin inhibitor was added to cultures at the beginning of incubation in doses: 25, 50 and 100 μg/ml (continuous exposure). For every culture of lymphocytes with the tested compound, the negative control was performed.

Chromosome aberrations (CA) assay
The metaphase plates were obtained in the conventional manner from the described above macroculture of peripheral blood lymphocytes after 48 h of incubation. Colcemid (Serva, 0.15 μg/ml) was added 2 h before harvesting. Hypotonic shock was induced by 0.075 M potassium chloride solution. Cell suspension was fixed in a mixture of methanol and glacial acetic acid (v/v 3:1). Preparations were stained with Giemsa.
Chromosome aberrations were classified according to the International Nomenclature (ISCN, 1995). For each dose of the inhibitor, the mitotic index (MI) was calculated. To evaluate the mitotic index, cytogenetic preparations were analysed in a light microscope at 400 × magnification (analysis of 30 fields).
MI was expressed as a number of metaphases per 1000 nuclei analysed. The inhibition of the MI was calculated as 100 -[MI treated x 100/MI control] (Rojas *et al.*, 1993).

Sister chromatid exchanges (SCE) assay
Lymphocyte cultures used for SCE studies were from the same donors and were incubated at the same conditions as described above. *Ascaris* trypsin inhibitor and bromodeoxyuridine (BrdU, 10 μg/ml, Sigma) were added at the beginning incubation. The lymphocyte cultures were incubated for 72 hours. The slides were coded and stained for sister chromatid differentiation according to Perry and Wolff (1974). A minimum of 25 second - division cells were scored for each culture. Only metaphases containing 46 chromosomes were analysed.
Cell division kinetics was determined by replication index (RI) values. One hundred metaphases per donor and concentration were analysed for determination of first, second and third generations of mitotic cells: M_1 (darkly stained chromatids), M_2 (one darkly and one lightly stained chromatids) and M_3 (part of metaphase with darkly [1/3] and lightly [2/3] stained chromatids). RI was calculated according to the formula (Rojas *et al.*, 1992):

$$RI = \frac{1M_1 + 2M_2 + 3M_3}{100}$$

The recorded results were analysed with standard statistical methods. Fisher's exact test was used to compare aberration frequencies and MI values. Mean SCE frequency per cell and RI values were compared by Student's *t*-test and by Mann-Whitney test.

Results

In these experiments two methods for evaluation of genotoxic activity of *Ascaris* trypsin inhibitor were used. In these methods different periods (48 or 72 hours) of incubation of lymphocyte cultures were applied. The SCE test was performed within 72 hours for obtaining two complete cell cycles (two replications). Recently application of structural chromosome aberration test for 48 hours is recommended in order to eliminate possibility of occurring reconstructive mechanisms in next cell cycles.
Preliminary results of the investigations concern genotoxicity assessment of the *Ascaris* trypsin inhibitor without exogenous metabolic activation. Frequencies in structural chromosome aberrations induced by trypsin inhibitor are presented in Table 1. Numbers of structural aberrations per cell for all doses of the inhibitor were not significantly differrent from the negative control.
Mean values of the mitotic index (MI) for all doses of the inhibitor were significantly lower ($P < 0.01$) than in the control group after 48 hours incubation (Fig. 1). Differences between mitotic index values for the three different doses of this inhibitor were insignificant. The reduction in mitotic activity of lymphocytes cultivated in the presence of the inhibitor was demonstrated by the percentage of the MI inhibition (Fig. 1). All doses of the inhibitor reduced the MI value of the control by over 50 %.
Mean SCE frequency per cell (Table 2) for all doses of the inhibitor was higher and differed significantly from the

Table 1. Frequency of structural chromosome aberrations induced by the trypsin inhibitor from *Ascaris*

Dose [μg/ml]	No. of cells scored	No. of aberrations							No. of all aberrations per cell
		G'	G''	B'	B''	AF	DIC	EF	
25	250	0	0	2	0	0	0	0	0.008
50	250	1	0	1	1	1	0	0	0.012
100	200	0	0	1	1	0	0	0	0.010
Control	200	1	0	1	0	0	0	0	0.010

G' – chromatid gap; G" – chromosome gap; B' – chromatid break; B" – chromosome break; AF – acentric fragment; DIC – dicentric; EF – exchange figure

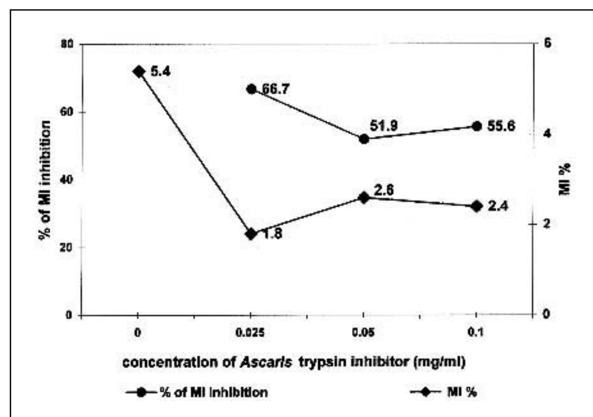

Fig. 1. Effect of the *Ascaris* trypsin inhibitor on the mitotic index (MI) and percentage of MI inhibition of cultured human lymphocytes

Discussion

It has been widely reported that nematodes or nematode products can stimulate or inhibit the generation of lymphocytes response. In our experiment, the test of structural chromosome aberrations showed that the *Ascaris* inhibitor inhibits lymphocyte divisions. The significant decrease in the mitotic index (MI) values of lymphocytes in the presence of the *Ascaris* trypsin inhibitor (after continuous exposure for 48h) suggests that this polypeptide – serine protease inhibitor – delays PHA-induced human lymphocyte transformation *in vitro*. One of the parameters for evaluation of mitotic activity is mitotic index. The MI is interpreted in terms of the cell death or arrest of the cell at any moment during the interphase (Rojas *et al.*, 1992, 1993; Sarli *et al.*, 1998). It is likely that *Ascaris* trypsin inhibitor retards lymphocytes' shift from the rest phase G_0 into the cell

Table 2. Frequency of sister chromatid exchanges (SCE) induced by the trypsin inhibitor from *Ascaris* and corresponding replication index (RI) values

Dose [μg/ml]	No. of cells scored	Frequency of SCE per cell (mean ± SD)	Replication index* (± SD)
25	200	9.49 ± 0.245 [b]	1.97 ± 0.098 [a]
50	250	9.84 ± 0.501 [b]	1.96 ± 0.070 [a]
100	270	10.09 ± 0.342 [b]	1.99 ± 0.098 [a]
Control	250	7.83 ± 0.433	1.71 ± 0.126

* 100 metaphases from each culture were scored; Statistically significant differences in relation to the control: [a] P<0.01; [b] P<0.001;

control after 72 hours incubation (P < 0.001). The inhibitor did not double the SCE rate of the control. No significant differences were observed between the mean values of SCE frequency/cell for the tested doses of the inhibitor. Mean values of the replication index (RI) calculated for all tested inhibitor doses were significantly higher (P < 0.01) than in the control (Table 2). Comparison of mean RI values between three examined doses of the trypsin inhibitor did not reveal any significant differences. The results obtained for the examined doses of the *Ascaris* inhibitor did not show any effect-dose relationship.

The 48-h chromosome aberration test revealed that the trypsin inhibitor does not exhibit genotoxic properties but significantly decreases the mitotic index (MI) values as compared to the control. Moreover, the 72 hour SCE test demonstrated that this inhibitor increases SCE frequency and stimulates the dynamics of lymphocyte divisions.

cycle. The studies concerning the effect of alpha 1-antitrypsin (alpha 1-trypsin inhibitor /1-AT/) on human lymphocyte proliferation demonstrated that *in vitro* 1-AT could inhibit PHA-induced lymphocyte transformation (Bata *et al.*, 1977; Baranova *et al.*, 1980). The investigations conducted by Breit *et al.* (1983) also reported suppression of the PHA response by purified 1-AT. The addition of 1-AT to PHA-stimulated lymphocytes clearly demonstrated a dose-dependent suppression in the presence of 1-AT-deficient sera, but little effect in the presence of normal serum. The addition of increasing amounts of 1-AT into culture medium resulted in an exponential decrease the in response. The results of these studies demonstrated that 1-AT levels of 2g/liter and higher in normal sera would achieve maximal inhibition of lymphocyte activation, but subnormal levels (0.2 – 0.5 g/l) in 1-AT-deficient serum would lead to an increased cellular division. Lipski

et al. (1979) have reported that 1-AT may be expressed on surfaces of concanavalin-A (Con A) transformed human lymphocytes, and evidence has been presented for synthesis of 1-AT by human peripheral blood monocytes (Boldt *et al.*, 1982). Other studies also demonstrated that cultivation of peripheral blood lymphocytes with T-cell mitogens (PHA) leads to the appearance of lymphoblasts with membrane-associated 1-AT (Lohrisch *et al.*, 1981). That experiment showed that unstimulated lymphocytes neither have membrane-associated 1-AT nor possess a binding capacity for it. These data suggest that 1-AT may play important roles in lymphocyte blastogenesis and also immunoregulation.

In this study, we demonstrated a significant increase in the lymphocytes' replication index (RI) values for all tested doses of *Ascaris* trypsin inhibitor as compared to the control. This result indicates that *Ascaris* inhibitor can stimulate the dynamics of cell division (after 72 h of exposure to this inhibitor). However, our results from the CA test demonstrated significant inhibition of the mitotic activity of lymphocytes exposed to *Ascaris* inhibitor for 48 h. Thus, longer exposure time to *Ascaris* trypsin inhibitor may considerably influence cell division kinetics. It is likely that lymphocytes, which entered into a G_1 stage of the cell cycle, proliferate at a higher rate in the presence of this trypsin inhibitor (high percentage of third generation lymphocytes) than in control conditions. In the available literature we have found data on the effect of the *Ascaris* extract on lymphocyte subpopulation proliferation both *in vitro* and *in vivo*. The effect of *Ascaris* compounds on the developing immune response is likely to be complex. For example *Ascaris suum* contains immunomodulatory factors capable of either stimulating (Sasagawa *et al.*, 1987; Lee & Xie, 1995) or inhibiting (Soares *et al.*, 1992; Ferriera *et al.*, 1995) T or B cell responses. Sasagawa *et al.*, (1987) demonstrated that *Ascaris* extract (ASE) stimulates predominantly T lymphocytes. However, the ASE stimulation in the 2-day culture was very weak compared with the other two mitogens of PHA and PWM (pokeweed mitogen). ASE had a marked stimulatory effect, which is equivalent to that of PWM in the 5-day culture. Moreover, the other investigators have shown that infection with *Ascaris* or the use of its soluble products or extracts at different stages of purification potentiates or suppresses the immune responses (Soares *et al.*, 1992; Souza *et al.*, 2002; Oshiro *et al.*, 2004). Some of the discrepancies and apparent inconsistencies in reports regarding the modulation of proliferative response of human lymphocytes to *Ascaris* extract are undoubtedly due to variation in the profile of the helminths' modulatory factors, methods of isolation and different conditions of cell culture.

It has been commonly accepted that mutagenic substances administered into pregnant animals may induce intrauterine death of embryos or fetuses, and also congenital malformations (Epstein *et al.*, 1972). It is worth emphasising that mutagenic activity of antigens from *Ascaris suum* was observed by Bekish (1999). Author demonstrated that the antigens had mutagenic effects resulting in the number of micronuclei erythrocytes in the mice bone marrow. Extracts from *Ascaris suum* changed the chromosome apparatus of human blood lymphocytes *in vitro* having provoked aneuploid and aberrant cells. On the other hand, it has been found that the nematode metabolites of *Trichinella spiralis*, migrate larvae of *Ascaris suum* and *Toxocara canis* caused mutagenic influence on the somatic and the generative cells of experimental animals (Bekish & Bekish, 2000). In the previous studies (Blaszkowska, 1998a; 1999), the *Ascaris* trypsin inhibitor injected during early or late organogenesis produced specified congenital defects. Preliminary results of this study (without exogenous metabolic activation) indicate that trypsin inhibitor isolated from *Ascaris* does not have genotoxic properties. Chromosome aberrations occurred with a low frequency and did not exceed the values from the control group. Also, the SCE test results do not support the genotoxic activity of the trypsin inhibitor from *Ascaris*. This inhibitor increased SCE values but not up to double SCE value of control. This means it is not considered as mutagen.

Considering biochemical properties of the trypsin inhibitor isolated from *Ascaris* (serine inhibitor), and also its *in vitro* effect on dynamics of cell divisions revealed in this study, it is likely that the trypsin inhibitor may influence the activity of protein regulators of the cell cycle.

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