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The dose dependent effect of glucan on worm burden and pathology of mice infected with *Mesocestoides corti (M. vogae)* tetrathyridia

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Summary

The efficacy of three, five and seven doses of glucan and its effect on pathology in mice infected with *Mesocestoides corti* larvae were examined by means of larval counts in the livers and peritoneal cavities and selected pathophysiological and biochemical parameters of the host. Sera and livers of mice were collected on days 32, 42, 50 and 65 p.i. and levels of ALT, AST, cholesterol and hydroxyproline were determined. Administration of glucan significantly ($P < 0.01$) reduced larval counts in the liver of all treated groups. Larval numbers in the peritoneal cavity of mice treated with three glucan doses (15 $mg.kg^{-1}$ of body weight in total) did not markedly change in comparison with control. On days 50 and 65 p.i. in the liver and on day 65 p.i. in the peritoneal cavity the lowest larval counts were recorded in the group treated with five doses of glucan (25 $mg.kg^{-1}$ of body weight in total). In this group of mice the lowest intensity of liver fibrosis was also observed, which was comparable with fibrosis in the control group. The most intense fibrosis was recorded in the group with three glucan doses. With respect to larval burden reduction, administration of seven glucan doses (35 $mg.kg^{-1}$ of body weight in total) was not as effective as administration of five doses. AST activities in all glucan treated groups were markedly ($P < 0.01$) elevated within the experimental period. In the group with three doses of glucan, ALT activities decreased considerably on days 42, 50 and 65 p.i. and in the group treated with five doses they achieved control levels by the end of the experimental period. Cholesterol levels were significantly increased after glucan administration from day 32 up to day 65 p.i., except in three dose-treated mice. The earliest decline of cholesterol in serum was recorded in the control group. The present results indicate that greater glucan efficacy is not proportional to the number of doses, at least in this particular treatment schedule. Administration of five glucan doses appears to be most effective with respect to the highest reduction of parasite burden in the liver and peritoneal cavity of mice and the lowest intensity of liver fibrosis.

Key words: *Mesocestoides*; mice; ALT; AST; hydroxyproline; cholesterol; glucan; liver fibrosis; collagen

Introduction

In metacestode infections invading the liver, such as *Echinococcus multilocularis* or *Mesocestoides vogae* (Etges, 1991), originally described as *M. corti*, fibrosis is an important aspect of the host immune reaction against parasite development (Guerret *et al.*, 1998; Specht and Widmer, 1972). Liver fibrosis is a dynamic process which is involved in tissue repair to hepatocyte injury and which results in net accumulation of extracellular matrix (ECM) proteins. It involves complex interactions between several liver cell populations, which are mediated through release of various cytokines including transforming growth factor (TGF-β), interleukins, tumor necrosis factor (TNF-α) and others (Burt, 1993; Tsukamoto, 1999). Pollacco *et al.* (1978) postulated that encapsulation of *M. corti* tetrathyridia by the collagenous capsules and fibrosis is T-cell dependent and is not developed in hypothymic mice. The development of hepatic fibrosis is associated with a number of biochemical changes, which lead to structural and metabolic abnormalities in the liver.

Similarly to the liver fibrosis, invasion of the host by parasites frequently leads to the tissue damage, which results in the impairment of metabolism in that organ. Extent of tissue damage is often assessed by monitoring enzyme activities, which are released into the blood (White *et al.*, 1982; Velebný and Hrčková, 1995; Soliman *et al.*, 2002). Two enzymes, namely alanine amino transferase (ALT) and aspartate amino transferase (AST) seem to be good markers for monitoring the pathological changes in the liver paren-

chyma, due to their occurrence in the cytoplasm (ALT) or mitochondria and cytoplasm (AST) of hepatocytes (George and Chandrakasan, 2000). Hepatocytes are also the major sites of cholesterol synthesis *de novo*, decreased levels of which in the course of liver fibrosis are manifested as reduced cell volume package and results in liver injury.

There are several studies showing that mononuclear phagocytes play a crucial role in the inflammatory and granulomatous response to cestode parasites (Specht and Widmer, 1972; Voge *et al.*, 1979; Riley *et al.*, 1985; Cook *et al.*, 1988). Jenkins *et al.* (1990) suggested, that macrophages are effector cells with potential larvicidal activity against two metacestode species, *E. granulosus* and *M. corti.* However, this larvicidal activity is down-regulated by the molecules secreted by tetrathyridia *M. corti in vitro* and *in vivo* (Kadian *et al.*, 1994). Macrophage effector functions may also be impaired by TNF-α, which accumulates in the peritoneal cavity, due to the inability of the liver to detoxify endotoxin (Jenkins *et al.*, 1991).

There is a substantial amount of evidence, that host's liver pathology is the result of communication and interactions between immune cells and liver mesenchymal cells. In this respect, administration of an immunomodulatory substance can considerably modulate such balance (Williams *et al.*, 1996) and the dosage of these substances has to be evaluated precisely. Such conclusion was derived, for example, from the study of Liance *et al.* (1998) on experimental infection with *Echinococcus multilocularis* following IFN-γ treatment. Whereas administration of low doses of IFN-γ decreased metacestode growth and liver fibrogenesis, higher doses had the opposite effect, probably due to the overstimulation of certain sub-populations of immune cells.

In the present study we used β-glucan as an immunomodulatory polysaccharide (Mizuno *et al.*, 1995), which like IFN-γ stimulates the immune system of the host. Administration of glucan results in an increased phagocytic activity of macrophages, in activation to Natural Killer cells, T- and B-lymphocytes (Mayell, 2001; Williams *et al.*, 1996); moreover, increased production of cytokines IL-1, IL-2, IL-6, TNF-α and IFN-γ in macrophages was also observed (Rasmussen and Seljelid, 1990; Kidd, 2000). The effects of glucan, either in combination with drugs or alone, were investigated during the therapy of various infectious diseases (Browder *et al.*, 1987; Borošková *et al.*, 1995; Velebný *et al.*, 1997; Hrčková and Velebný, 2001).

Administration of lentinan (a glucan immunomodulator of fungal origin) in experimental *M. corti* infection of mice resulted in a marked reduction in the numbers of parasites (Byram *et al.*, 1978; White *et al.*, 1988). According to the authors, this reduction was mediated by the increasing number of macrophages, enlargement of liver granulomas and higher collagen deposition. In our previous study, we showed that the most intense fibrosis in the livers of mice treated with liposomised glucan and vitamin C did not result in the most extensive parenchymal cell injury, but it resulted in the highest efficacy of treatment (Ditteová *et al.*, 2003).

The effects of different glucan dosage on pathophysiology of parasitic infections of liver has not been investigated. Therefore, the present study aimed to investigate the effects of increasing doses of glucan on the extent of liver fibrosis by measuring hydroxyproline content in the liver. Pathophysiological parameters such as ALT, AST and cholesterol levels, as well as the effect of glucan on larval burdens in the liver and peritoneal cavity of mice infected with *M. corti* tetrathyridia were also monitored.

Materials and Methods

Infection and experimental design

Tetrathyridia of *Mesocestoides vogae* (Etges, 1991) were obtained from the peritoneal cavity of male ICR strain mice and maintained in Hanks Balanced Salt Solution (HBSS, Sigma, USA) prior to the infection of mice. In experiments, male mice of the same strain, aged 6 weeks, were orally inoculated with 57 ± 2 tetrathyridia in 0.5 ml of saline.

Infected mice were divided into four groups comprising 30 individuals. Glucan formulation was administered to mice every 3rd day from day 7 post infection (p.i.) in three, five and seven doses, which corresponded to 15, 25 and 35 mg per kg of body weight in total. Infected and unaffected group served as control. To compare the effect of various doses of glucan, the following parameters were monitored: number of larvae in the liver and peritoneal cavity, hydroxyproline content in the liver, serum levels of ALT, AST and cholesterol. Samples of liver and serum were collected on days 32, 42, 50 and 65 p.i. Six mice per group were sacrificed on experimental days for collection of serum, liver tissue and larvae from the liver and peritoneal cavity. Examinations span a period of 37 days after the last dose. Tetrathyridia were isolated from the liver and peritoneal cavity as described previously (Hrčková and Velebný, 1995).

Glucan formulation

β-glucan (carboxymethylated β-1,3-D-glucan; Mevak, Slovak Republic) was dissolved in saline to a final concentration 1 mg/ml and intramuscularly administered to mice (single dose contained 5 mg per kg of body weight).

Determination of hydroxyproline concentration in the livers

Collagen content in the liver of mice was measured by means of hydroxyproline concentration, which represents the most abundant amino acid of collagen representing approximately 13 % of its mass. It was estimated in hydrolysed liver homogenate (100 mg) using the modified method of Woessner (1961). Data are expressed in μg per 100 mg wet weight of liver tissue.

Determination of ALT, AST and cholesterol in serum

Activities of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels in serum were estimated spectrophotometrically at 525 nm. This determination of aminotransferases is based on the differences of absorbance between hydrazones of 2-oxoglutarate and pyruvate

in an alkaline medium using commercial diagnostic kit (Bio-La-Test Aminotransferase; Lachema, Czech Republic). Activity of enzymes is expressed in μkat/l serum.
Cholesterol levels in serum were determined spectrophotometrically at 575 nm using commercial diagnostic kit (Bio-La-Test Cholesterol; Lachema, Czech Republic). Results are expressed in mmol/l serum.

Statistical evaluation of data
Kruskal-Wallis ANOVA test ($P < 0.01$) was used for statistical evaluation of differences between all groups. All pairwise multiple post-hoc comparison procedures employed the Student-Newman-Keuls method ($P < 0.01$) (Statistica 6.0, Stat Soft Inc., USA).

Results

Larval counts in the liver and peritoneal cavity
Numbers of larvae recovered from the liver and the peritoneal cavity of mice are shown in Tables 1 and 2. In the course of infection the larval counts in the liver were significantly reduced ($P < 0.01$) in all glucan treated groups. The highest larval counts were found in the liver of mice on day 50 p.i.. Up to day 65 p.i. larval numbers in all glucan treated groups markedly decreased. The lowest larvicidal efficiency of glucan was recorded in the group with three doses in the experimental period. The group with five administered doses of glucan appeared to be the most affected with the exception of day 32 p.i.. In this group, larval numbers were significantly reduced ($P < 0.01$) on days 42 and 50 p.i. in comparison with control group and other groups treated with glucan.

Similar results were found in the peritoneal cavity. With the exception of the three-doses group, administration of glucan significantly ($P < 0.01$) reduced larval counts in the peritoneal cavity of mice in comparison with control group during the entire experimental period. The highest larvae numbers were observed in all groups on day 65 p.i.. In the group treated with three doses the lowest efficiency of glucan was observed. Unlike the larvae counts in the liver, in the peritoneal cavity maximal efficacy ($P < 0.01$) of treatment was noted in the group with seven glucan doses on days 32, 42 and 50 p.i.. On day 65 p.i. the lowest larvae numbers ($P < 0.01$) were recovered from mice treated with five doses of glucan, where the larval counts didn't markedly change in comparison with day 50 p.i..

Hydroxyproline content in the liver
Liver fibrosis was monitored during second month post infection, which corresponds to the period of liver regeneration (Specht and Widmer, 1972). Collagen content, measured in terms of hydroxyproline concentration is shown in Fig. 1. Hydroxyproline content in the liver gradually increased up to day 65 p.i., when it achieved maximal levels in all four groups. The most intense fibrosis ($P < 0.01$) was observed in group treated with three glucan doses, whereas the lowest ($P < 0.01$) hydroxyproline concentrations in the livers were recorded in the control group and in the group treated with five glucan doses. Otherwise, no significant differences were noted on days 32, 42 and 65 p.i..

ALT activities in serum
ALT is the enzyme incident in the cytoplasm of liver parenchymal cells, consequently total serum activities of

Table 1. Numbers of larvae recovered from liver (expressed per 1 g of tissue) of mice infected with *M. corti*, treated with three, five and seven doses of glucan

Days post infection	3 doses of glucan	5 doses of glucan	7 doses of glucan	Control
32	$324 \pm 45^{\blacklozenge\square}$	$248 \pm 11^{*\blacklozenge}$	$68 \pm 7^{*\square}$	522 ± 41
42	$450 \pm 36^{\blacklozenge\square}$	$330 \pm 20^{*\blacklozenge}$	$352 \pm 40^{*\square}$	592 ± 63
50	$480 \pm 48^{\blacklozenge\square}$	$346 \pm 39^{*\blacklozenge}$	$387 \pm 22^{*\square}$	704 ± 21
65	$270 \pm 13^{\blacklozenge\square}$	$240 \pm 28^{\blacklozenge}$	$248 \pm 20^{\square}$	672 ± 37

$^{\blacklozenge}$ – significant differences ($P < 0.01$) between groups treated with 3 and 5 doses of glucan; $^{\square}$ – significant differences ($P < 0.01$) between groups treated with 3 and 7 doses of glucan; * – significant differences ($P < 0.01$) between groups treated with 5 and 7 doses of glucan

Table 2. Numbers of larvae recovered from peritoneal cavity of mice infected with *M. corti*, treated with three, five and seven doses of glucan

Days post infection	3 doses of glucan	5 doses of glucan	7 doses of glucan	Control
32	$389 \pm 32^{\blacklozenge\square}$	$293 \pm 27^{*\blacklozenge}$	$51 \pm 4^{*\square}$	533 ± 120
42	$1644 \pm 112^{\blacklozenge\square}$	$1036 \pm 105^{*\blacklozenge}$	$851 \pm 78^{*\square}$	1495 ± 96
50	$2664 \pm 153^{\blacklozenge\square}$	$1712 \pm 165^{*\blacklozenge}$	$1245 \pm 98^{*\square}$	2488 ± 97
65	$3024 \pm 128^{\blacklozenge\square}$	$1887 \pm 56^{*\blacklozenge}$	$2144 \pm 128^{*\square}$	3119 ± 96

$^{\blacklozenge}$– significant differences ($P < 0.01$) between groups treated with 3 and 5 doses of glucan; $^{\square}$ – significant differences ($P < 0.01$) between groups treated with 3 and 7 doses of glucan; * – significant differences ($P < 0.01$) between groups treated with 5 and 7 doses of glucan

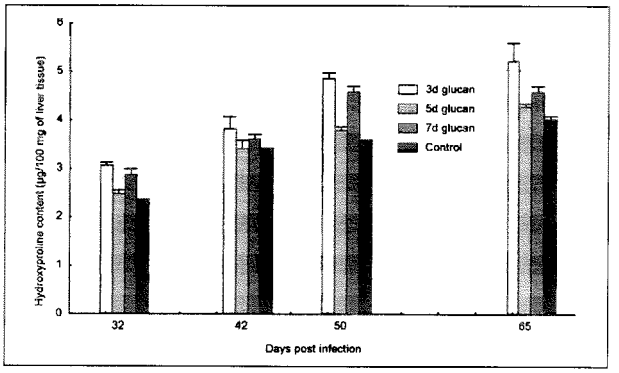

Fig. 1. Effect of three, five and seven doses of glucan on the hydroxyproline content (µg / 100 mg of liver tissue) compared to the control group

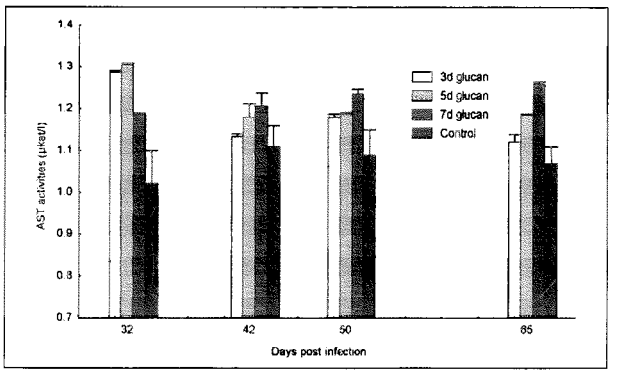

Fig. 3. Effect of three, five and seven doses of glucan on the AST activities (µkat / l of serum) compared to the control group

ALT reflect disruption of cell membranes or changes in their fluidity. Fig. 2 summarises ALT activities. In control mice ALT activities were similar to those in the group given seven glucan doses. On day 32 p.i. the lowest ($P < 0.01$) ALT activities were recorded in the group treated with seven glucan doses (1.267 µkat/l serum), whereas on days 42, 50 and 65 p.i. the lowest ($P < 0.01$) ALT activities were observed in the group treated with three glucan doses. Maximal activities of ALT found on day 50 p.i. after seven glucan doses (1.576 µkat/l serum), considerably decreased (1.484 µkat/l serum) up to day 65 p.i.. In the control group, maximum ALT activity was recorded on day 42. p.i..

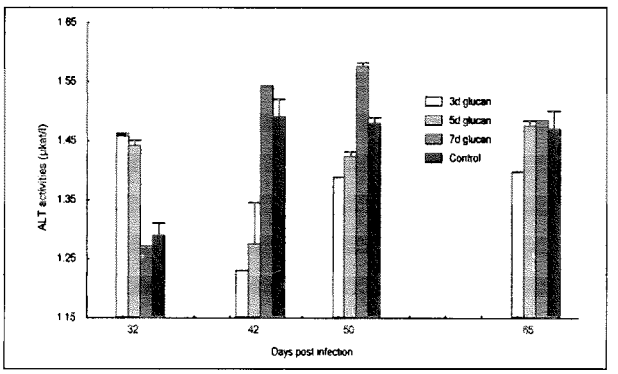

Fig. 2. Effect of three, five and seven doses of glucan on the ALT activities (µkat / l of serum) compared to the control group

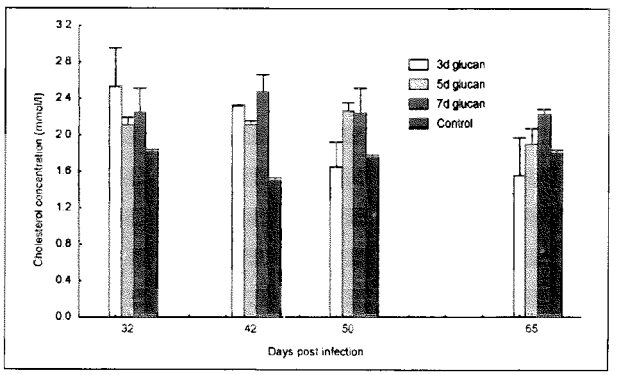

Fig. 4. Effect of three, five and seven doses of glucan on the cholesterol concentration (mmol / l of serum) compared to the control group

AST activities in serum

AST enzyme is present both in the cytoplasm and in mitochondria of hepatocytes, therefore its value in serum is considered as a suitable marker of liver cell damage or proliferation. In entire period the lowest AST activities were observed in controls. AST and ALT activities were quantitatively similar (Fig. 3) in all glucan treated groups. However, the maximal AST levels were observed in groups treated with three (1.288 µkat/l serum) and five (1.305 µkat/l) doses on day 32 p.i., which was followed by a gradual decrease up to day 65 p.i. In contrast, in the group with seven doses of glucan, AST activities moderately increased from day 32 (1.207 µkat/l serum) to day 65 p.i. (1.260 µkat/l serum) and they were significantly higher ($P < 0.01$) in comparison with the other groups.

Cholesterol levels in serum

In contrast with increasing enzyme activities with ongoing infection, cholesterol levels declined gradually up to day 65 p.i. (Fig. 4). The highest ($P < 0.01$) cholesterol level was observed on day 32 p.i. in the group treated with three doses of glucan (2.530 mmol/l serum), then decreased gradually up to day 65 p.i. (1.551 mmol/l serum). Contrary to this observation, cholesterol levels in groups treated with five and seven doses of glucan remained relatively constant during the experiment and were significantly higher than the three glucan dose group on days 50 and 65 p.i.. Cholesterol levels in sera of control mice were significantly lower ($P < 0.01$) compared to groups treated with five and seven doses of glucan during the entire experimental period.

Discussion

It was postulated by Hamuro *et al.* (1980), that each β-1,3-D glucan exhibits a strict dose-response relation, which is manifested by drastic reduction in the cytotoxic activity of peritoneal macrophages at high doses. After optimal dosage of glucan, the cytotoxicity of peritoneal macrophages persisted for more than 23 days (Hamuro *et al.*, 1980). Williams *et al.* (1996) proposed that this phenomenon might be related to the different cytokine pathways, activation of which is selectively dependent upon exposure to different doses of glucan.

Following on from previous reports (Hamuro *et al.*, 1980; White *et al.*, 1988; Hofer *et al.*, 1995; Ditteová *et al.*, 2003), the present work examines the effect of three, five and seven doses of glucan on infection and pathology in mice parasitised by *Mesocestoides corti*. The administration of β-1,3-D-glucan resulted in a significant reduction in larval count in comparison with control group in the course of experimental infection (White *et al.*, 1988, Ditteová *et al.*, 2003). Our results showed, that with regards to the long-term course of infection, the most effective schedule is the administration of five doses of glucan every third day from day 7 p.i. The lowest larval counts were detected in this group of mice from day 42 up to day 65 p.i. in the liver and on day 65 p.i. in the peritoneal cavity. This was observed despite the fact, that at day 32 p.i., the larvae counts were significantly lower ($P < 0,01$) in the liver as well as in the peritoneal cavity of mice administered seven doses of glucan. We therefore presume, that in accordance with the findings of Hamuro *et al.* (1980), the increase in dosage of an immunomodulator with a non-specific effect on host immune system, may leads to initial massive stimulation of cytotoxic functions of immune cells. This stimulation may later be manifested by depletion of the immunocompetent cells of the host and equally by higher intensity of the pathological response, caused by the overproduction of pro-inflammatory cytokines (Hofer *et al.*, 1995; Omer *et al.*, 2000). In this case, where glucan was administered in a fixed dosing regime (5 doses of glucan, 25 $mg.kg^{-1}$ of body weight in total), the cytotoxic activity of macrophages stimulated by glucan, was probably maintained for up to the end of experimental period.

With respect to the highest efficacy of 5 glucan doses, we also found the lowest collagen content in livers of mice and it was comparable with control group. This appears very interesting, as findings to date indicate that the deposition of collagen around the larvae, represents the most important barrier for their multiplication or migration (Pollacco *et al.*, 1978). This assumption, however, cannot be applied generally, as the highest fibrogenesis was observed in the group of mice treated with three doses of glucan, where the highest larvae counts were also detected.

The non-specific immunomodulator β-1,3-D-glucan has been shown to increase the intensity of liver fibrosis in mice infected with *S. mansoni* or *M. corti* (Byram *et al.*, 1979; White *et al.*, 1988; Ditteová *et al.*, 2003) and it was confirmed also in the present study. Moreover, impact of glucan on granuloma formation was also described (Williams *et al.*, 1988; Baker *et al.*, 1992). Hoffmann *et al.* (1998) postulated that the development of fibrotic tissue and granulomas depend on the ratio between two main cytokines, IFN-γ and TNF-α. TNF-α is known by its ability to decrease the larvicidal activity of macrophages and represents the main cytokine responsible for immunosuppression during *M. corti* infection (Jenkins *et al.*, 1991). TNF-α is also one of the main pro-fibrogenetic cytokines (Burt, 1993; Tsukamoto, 1999) and in alveolar echinococcosis serves as a fibrosis mediator produced by macrophages (Bresson-Hadni *et al.*, 1994). We hypothesise that the pro-fibrogenetic and immunossupressive effects of TNF-α were probably responsible for the increased fibrosis and higher larvae counts in mice treated with three doses of glucan. The current knowledge concerning the release of TNF-α by peritoneal macrophages after stimulation with glucan is controversial (Williams *et al.*, 1996). Hoffmann *et al.* (1993) observed decreased release of TNF-α and prostaglandin E_2 by macrophages treated with glucan, while other authors reported elevated release of TNF-α by peritoneal macrophages stimulated with glucan (Sherwood *et al.*, 1987; Steadman *et al.*, 1990). With respect to the administration of glucan, we may presume that the most optimal levels of TNF-α can be achieved by the appropriate glucan dosage. With regard to the highest efficacy in our experimental system, the most suitable dosage may be considered five doses of glucan at a dose of 5 mg per kg of body weight every third day. Additionally, this correlated with the lowest collagen content in the liver tissue. These data indicate a shift in cytokine release related to an increased production of IFN-γ and decrease of TNF-α. Administration of IFN-γ to mice with *E. multilocularis* or *M. corti* infections did not result in total elimination of the parasites (Jenne *et al.*, 1998; Jenkins *et al.*, 1991). IFN-γ is also known for its antiproliferative and antifibrotic effects, therefore it is considered as a potential drug against the development of liver fibrogenesis (Mallat *et al.*, 1995). On the other hand, the extremely increased levels of IFN-γ and TNF-α in mice with *S. mansoni* infection resulted in increased mortality, which correlated with increased serum levels of AST (Hoffmann *et al.*, 2000).

In our study, AST activities were significantly higher in all glucan-treated groups than in control. In contrast, on days 42 and 50 p.i. ALT activities in control group were significantly higher ($P<0.01$) as in groups treated with three and five glucan doses, but significantly lower as in group with seven doses of glucan. The most extensive patophysiological changes in the liver parenchyma, recorded on the basis of AST levels, were seen on day 32 p.i. in mice treated with three and five doses of glucan. From this day, AST activities rapidly decreased, similarly as in the work of White *et al.* (1982). Interestingly, between days 42-65 p.i., the lowest AST and ALT activities were recorded in mice treated with three doses of glucan. This was accompanied with the most intensive fibrosis in this group of mice in

spite of the fact, that liver fibrosis is believed to contribute to liver pathology during infection. Although the levels of AST and ALT were significantly higher ($P < 0.01$) in mice with five doses of glucan that those treated with three doses, much higher AST and ALT levels were recorded following seven doses of glucan. A possible explanation is that the administration of seven doses of glucan resulted in the over-production of the cytokines IFN-γ and TNF-α, release of cytotoxic inflammatory mediators from macrophages, such as oxygen radicals, proteolytic enzymes and arachidonate metabolites (Bowers *et al.*, 1986), that may not result in parasite elimination but can cause significant tissue modulation/damage and hepatic dysfunction (Hofer et al., 1995; Hoffmann *et al.*, 2000). Therefore, overstimulation of the host immune system by non-specific immunostimulators like β-glucan, could have a negative effect on the course of liver infections. Serum activities of ALT and AST recorded in our experiments serve as an indirect evidence supporting this hypothesis.

The serum cholesterol levels decreased significantly from day 32 p.i. to day 65 p.i. in the group treated with three glucan doses. In mice given five and seven doses, the decrease was less pronounced. The majority of liver infections are accompanied with decreased cholesterol levels, that may relate to the rapid decline of biosynthetic liver capacity (George and Chandrakasan, 2000). In our work, where examinations span the chronic phase of infection, differences observed among the glucan-treated groups were not intense. Despite the potential of β-glucans to lower serum cholesterolemia (Safina *et al.*, 1998; Fukushima *et al.*, 2001), the most pronounced ($P < 0.01$) decrease of cholesterol levels was identified in controls during the experimental period.

In conclusion, in our study the most efficient treatment schedule regarding the most efficient parasite reduction in the liver and peritoneal cavity, seems to be the administration of five doses of glucan every 3rd day p.i. in the course of long-term infection. In comparison with other groups, the relatively low level of fibrosis correlated with relatively little liver tissue damage. Also the biosynthetic capacity of liver parenchyma, monitored by cholesterol levels, was increased in this group throughout the experimental period. Finally, for better understanding of glucan effects on liver infections and evaluation of immunological mechanisms which underlay our findings, it would be desirable to monitor the production of cytokines by macrophages and optimal dosing of this immunostimulatory agent.

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