

Modulation of liver fibrosis and pathophysiological changes in mice infected with *Mesocestoides corti* (*M. vogae*) after administration of glucan and liposomized glucan in combination with vitamin C

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Abstract

The effects of glucan and liposomized glucan, alone or co-administered with vitamin C, and empty liposomes on hepatic fibrosis in mice infected with *Mesocestoides corti* (*M. vogae*) tetrathyridia were studied. Preparations were administered every third day from day 7 to day 31 post-infection (p.i.), nine doses in total. Activities of alanine aminotransferase (ALT), aspartate aminotransferase (AST) and cholesterol levels were measured in sera collected on days 11, 15, 21, 28, 32, 42, 50 and 65 p.i. Liver fibrosis was studied on the same days by measuring hydroxyproline concentration, which is considered a marker for collagen content. Larvicidal effects of the glucan and liposome preparations were estimated on day 65 p.i. in the liver and peritoneal cavity. Glucan formulations significantly enhanced collagen content, most prominently after administration of liposomized glucan in combination with vitamin C. Activities of both enzymes and cholesterol levels were slightly modified after administration of glucan alone. Liposomized glucan with vitamin C significantly increased ALT and AST activity and cholesterol levels up to days 28–32 p.i., after which they plateaued or declined. The most pronounced decrease was after administration of liposomized glucan and vitamin C. The same pattern of biochemical parameters in serum was observed after administration of empty liposomes, however, collagen content was not modified significantly. Larval counts in the liver and the peritoneal cavity were significantly reduced after treatment with either glucan formulation, but were unaffected following treatment with empty liposomes. In summary, intense fibrosis in the liver of mice treated with liposomized glucan and vitamin C did not result in the most extensive parenchymal cell injury but, rather in the highest efficacy of treatment. Liposomal lipids were probably utilized in the reparation of the damaged parenchymal cells, while glucan stimulated phagocytic cells.

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Introduction

Non-specific immunosuppression is a common feature of several helminthic diseases. *Mesocostoides corti* infection has been shown to inhibit progressively the accessory activity of murine macrophages by means of T-cells (Jenkins *et al.*, 1990), activity of which is modified by the antigen produced by larvae to promote their survival (Kadian *et al.*, 1994). *Mesocostoides corti* tetrathyridia proliferate rapidly in the liver and the peritoneal cavity of the hosts and could serve as a suitable model in immunological and pharmacological studies for slowly developing metacestodes of *Echinococcus granulosus* and *E. multilocularis* (WHO, 1984). In the liver, during the reparative phase of infection (days 10–21 p. i.), extensive fibrosis is initiated, followed by the formation of capsules around the parasites (Specht & Widmer, 1972). Hepatic fibrosis is a feature of many chronic liver diseases. It is a dynamic process involving complex interactions between several cell types mediated by various cytokines, including transforming growth factor (TGF)- α , interleukin (IL)-1, tumour necrosis factor (TNF)- α and others (Burt & Oakley, 1993; Tsukamoto, 1999). Most of the growth factors involved are derived from activated Kupffer cells, and there is evidence of a complex interplay between mediators (Burt & Oakley, 1993). Pollacco *et al.* (1978) reported that encapsulation of *M. corti* tetrathyridia by collagenous capsules is a T-cell dependent process that does not occur in hypothyroid mice. On the other hand, the number of larvae recovered from hypothyroid mice was considerably higher than from control mice, indicating that the formation of capsules around the larvae restricts multiplication of the larvae in the liver (Pollacco *et al.*, 1978).

β -glucan extracted from cell walls of the fungus *Saccharomyces cerevisiae* is a polysaccharide with a β -1,3-linkage and molecular weight $\sim 1 \times 10^6$ D (Mizuno *et al.*, 1995). To date, several immunomodulatory activities of β -glucan have been shown. Williams *et al.* (1996), Mayell (2001) and others reported that glucan is able to stimulate the phagocytic activity of macrophages, to activate natural killer cells and T- and B-lymphocytes. Moreover, administration of glucan resulted in an increased production of cytokines IL-1, IL-2, IL-6, TNF- α and interferon (IFN)- γ in macrophages (Rasmussen & Seljelid, 1991; Kidd, 2000) and generation of nitric oxide (Sakurai *et al.*, 1997). Therefore the potential of immunomodulatory compounds, such as glucans is investigated for the therapy of various infections in combination with drugs or alone in many laboratories. However, most of immunomodulators are water-soluble compounds, which represents the drawback in terms of their half-life in the circulation. In this regard their entrapment in a suitable carrier such as liposomes considerably extended their presence in the circulation (Fidler & Kleinerman, 1994).

Liposomes are spherical vesicles formed of one or several phospholipid bilayers which, since their discovery by Bangham *et al.* (1965), have found numerous applications as carriers of both lipophilic and hydrophilic drugs (Gregoriadis, 1988). *In vivo* liposomes are gradually cleared by the mononuclear phagocytic system (MPS), which could be an advantage when liposomes serve as carriers of adjuvants and antigens (Alving, 1983).

Previously we showed that administration of liposome-encapsulated glucan resulted in some reduction of larval counts in mice infected with the nematode *Toxocara canis*, and also that anthelmintics co-administered with liposomized glucan were much more effective than drug alone (Velebný *et al.*, 1997).

Invasion of the host by the parasites frequently leads to tissue damage and its extent may be assessed by monitoring of enzyme levels abundant in the insulted organs, which are released into the blood (White *et al.*, 1982). Two enzymes, alanine aminotransferase (ALT) and aspartate aminotransferase (AST) seem to be good markers of pathological changes in the liver parenchyma (George & Chandrakasan, 2000).

In the present study we evaluated the effect of glucan, liposomized glucan alone or in combination with vitamin C and empty liposomes in mice infected with *Mesocostoides corti* tetrathyridia originally isolated and described by Specht & Voge (1965) and renamed as *M. vogae* by Etges (1991). Larval counts in the liver and peritoneal cavity, extent of liver fibrosis, level of cholesterol and activity of hepatocyte enzymes ALT and AST in serum were examined.

Materials and methods

Parasite and host

Tetrathyridia of *Mesocostoides corti* were obtained from the body cavity of male mice (ICR strain) and maintained in Hanks balanced salt solution (HBSS, Sigma, USA) until infection. In the experiments male mice of the same strain, aged 6–7 weeks, were orally inoculated with 55–60 tetrathyridia in 0.5 ml saline.

Drugs and liposomes

β -glucan (carboxymethylated β -1,3-D-glucan; Mevak, Slovak Republic) was dissolved in saline (1 mg ml⁻¹) and intramuscularly administered (single dose contained 5 mg per kg body weight). Vitamin C was dissolved in water (2 mg ml⁻¹) and perorally administered at 8 mg kg⁻¹.

Glucan was entrapped into liposomes according to the method of Bangham *et al.* (1965). Briefly, chloroform-methanol (2:1, v/v) solution of the mixture consisting of 0.056 mmol Phospholipon 100H (Natterman Phospholipid; Koln, Germany), 0.016 mmol cholesterol and 0.008 mmol dicetylphosphate (Serva, Heidelberg, Germany) was dried under the reduced pressure forming the lipid film. This film was hydrated by 40 ml of 0.1% glucan solution (in saline), yielding a 1 mg ml⁻¹ preparation, which was administered subcutaneously at 5 mg glucan per kg body weight.

Empty liposomes were prepared by the same method and contained Phospholipon 100H and cholesterol at the concentration 1.1 and 0.15 mg ml⁻¹, respectively. They were administered subcutaneously at 5.5 mg Phospholipon 100H and 0.8 mg cholesterol per kg body weight.

Experimental design

Infected mice were divided into five groups comprising 40 individuals each. Mice without treatment served as

control (group 1). The remaining groups of mice were treated with the following preparations: glucan (group 2), liposomized glucan (group 3), liposomized glucan co-administered with vitamin C (group 4) and empty liposomes (group 5). Preparations were administered every third day from day 7 to day 31 p.i., for a total of nine doses.

Sera from five mice in each group were prepared from blood samples collected before infection and on days 11, 15, 21, 28, 32, 42, 50 and 65 post-infection (p.i.) and were used for the determination of enzyme activity (ALT and AST) and cholesterol levels. Hydroxyproline levels were determined in the liver of five mice sacrificed on the same days p.i. (5 mice each day of examination). This schedule allowed us to monitor changes both during the course of administration and up to a month after termination of therapy. Larvicidal effects of the preparations were evaluated after isolation of tetrathyridia from the liver and peritoneal cavity on day 65 p.i. as described previously (Hrčková & Velebný, 1995). The experiment was repeated twice.

Determination of collagen by hydroxyproline concentration in the livers

Collagen content in the liver of mice was estimated by hydroxyproline concentration, which is the most abundant collagen aminoacid forming approximately 13% of the total. Hydroxyproline concentration in liver homogenates was determined by the method of Woessner (1961) and expressed as $\mu\text{g g}^{-1}$ wet weight of liver tissue.

Determination of aminotransferases and cholesterol in serum

The activities of ALT and AST in serum were estimated spectrophotometrically at 525 nm after reaction with dinitrophenyl hydrazine using commercial diagnostic kits (Bio-La-Test Aminotransferase; Lachema, Czech Republic). The activity of enzymes is expressed in $\mu\text{kat l}^{-1}$ serum (according to the Kit's manual) and represents the amount of enzyme which converts 1 mole of substrate per 1 second.

Cholesterol levels in serum were determined spectrophotometrically at 575 nm using a commercial diagnostic kit (Bio-La-Test Cholesterol; Lachema, Czech Republic). Results are expressed in mmol l^{-1} serum.

Statistical evaluation of data

Numerical results were expressed as means \pm SD ($n = 10$). Statistical evaluation of data (larval counts, hydroxyproline, ALT, AST and cholesterol) used one-way Kruskal-Wallis ANOVA ($P < 0.001$). The Scheffé post-hoc ANOVA test was used for the multiple comparison procedure (P level indicated in text) (Statistica 6.0, Stat Soft Inc., Tulsa, USA). Only differences between control and treated groups were evaluated.

Results

Larval counts in the liver and peritoneal cavity

The numbers of larvae recovered from the liver (1 g) and peritoneal cavity of mice in all five groups on day 65 p.i. are shown in fig. 1. After nine doses of either glucan formulation, a significant reduction of larvae ($P < 0.01$) relative to controls was found in both tissues. In the liver the efficacy of glucan formulations was nearly the same, whereas in the peritoneal cavity, treatment with liposomized glucan in combination with vitamin C resulted in lower larval counts than treatment with glucan only. Interestingly, the administration of empty liposomes resulted in more larvae occurring in peritoneal cavity ($P < 0.05$).

Collagen content in the liver

The process of fibrosis in the liver was followed during and after treatment up to 65 days p.i. The collagen content, measured by means of hydroxyproline concentration, is shown in table 1. In the infected untreated group it increased gradually up to day 50 p.i. In the group treated with glucan and liposomized glucan, the hydroxyproline concentration was significantly higher ($P < 0.001$) on most days than in the control group. The administration of empty liposomes had no effect on the hydroxyproline content. The most pronounced increase of total collagen in the liver was recorded in the group treated with liposomized glucan in combination with vitamin C. This group also showed the most intense fibrosis and the highest hydroxyproline concentration ($P < 0.001$) relative to the glucan-treated group. The most prominent increase of hydroxyproline content in this group was observed between days 50 and 65 p.i.

Serum activities of AST

Aspartate aminotransferase is present in the cytoplasm and mitochondria of cells, therefore it is considered a suitable marker to monitor cell damage or proliferation. Parasitization by *M. corti* larvae in the liver resulted in the

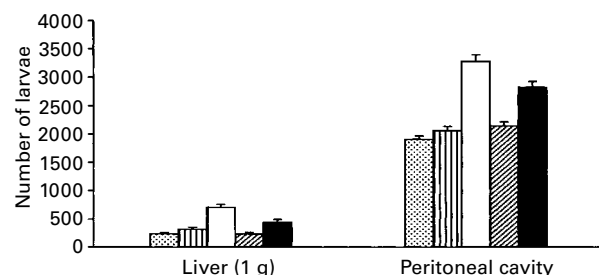


Fig. 1. Effect of nine doses of glucan (▨), liposomized glucan (▩), liposomized glucan in combination with vitamin C (▧) and empty liposomes (□) on the number of *Mesocostoides corti* larvae in the liver (expressed as per 1 g tissue) and peritoneal cavity on day 65 p.i., compared to the control group (■). The administration of the drug started on day 7 p.i., and all preparations were administered every third day up to day 31 p.i.

Table 1. Hydroxyproline content ($\mu\text{g g}^{-1}$ of liver tissue) in the liver of mice infected with *Mesocestoides corti*, untreated and treated with liposomized glucan in combination with vitamin C, liposomized glucan, empty liposomes or glucan.

Days post-infection	Lip. glucan+vit. C mean \pm SD	Liposomized glucan mean \pm SD	Empty liposomes mean \pm SD	Glucan mean \pm SD	Control mean \pm SD
0	0.08 \pm 0.0015	0.08 \pm 0.0015	0.08 \pm 0.0015	0.08 \pm 0.0015	0.08 \pm 0.0015
11	0.18 \pm 0.0005*	0.14 \pm 0.0005	0.12 \pm 0.0010	0.17 \pm 0.0015*	0.11 \pm 0.0010
15	0.21 \pm 0.0010*	0.15 \pm 0.0010*	0.13 \pm 0.0005	0.16 \pm 0.0010*	0.11 \pm 0.0010
21	0.20 \pm 0.0010*	0.15 \pm 0.0010	0.15 \pm 0.0015	0.19 \pm 0.0005*	0.13 \pm 0.0005
28	0.26 \pm 0.0005*	0.22 \pm 0.0010*	0.22 \pm 0.0010*	0.20 \pm 0.0010	0.18 \pm 0.0005
32	0.39 \pm 0.0010*	0.33 \pm 0.0015	0.30 \pm 0.0005	0.32 \pm 0.0010	0.30 \pm 0.0015
42	0.45 \pm 0.0015*	0.43 \pm 0.0015*	0.40 \pm 0.0005	0.43 \pm 0.0005*	0.35 \pm 0.0025
50	0.57 \pm 0.0025*	0.48 \pm 0.0015*	0.42 \pm 0.0025	0.57 \pm 0.0020*	0.43 \pm 0.0015
65	0.61 \pm 0.0020*	0.57 \pm 0.0020*	0.51 \pm 0.0015*	0.55 \pm 0.0020*	0.43 \pm 0.0010

*Significantly higher than controls ($P < 0.001$).

gradual increase of AST serum levels in control mice (table 2). The administration of glucan increased AST activities only slightly during the entire examination period. However, when glucan incorporated in liposomes was administered, a further elevation of AST levels was recorded. The co-administration of vitamin C with this liposomized glucan resulted in even higher serum levels within the first month p.i. Maximum AST activities were recorded in groups treated with liposomal formulations between days 28 and 32 p.i., corresponding to the end of administration of all formulations. During the following period, up to the end of examination, activities of AST in serum of mice from these groups dropped significantly ($P < 0.001$). This drop was most pronounced in the liposomized glucan and vitamin C-treated group.

Serum activities of ALT

Alanine aminotransferase is confined to the cytoplasm of parenchymal cells. Therefore, total serum activities are lower than those recorded for AST. Levels of this enzyme increased gradually in control mice (table 3). Though the glucan-treated group was similar to control, groups treated with all three liposomal formulations showed a significant elevation ($P < 0.001$) of ALT activities in the serum. Again this was most pronounced after the liposomized glucan administration with vitamin C. The administration of empty liposomes resulted in an elevation of activity up to day 42 p.i. ($P < 0.001$). In all

treated groups the activity of this enzyme reached maximal values between days 28 and 32 p.i., then declined, indicating a progressive reparation of cell membranes in the chronic stage of infection.

Cholesterol levels in serum

Parenchymal cells are involved in the metabolism of lipids and this activity is limited after tissue injury. In contrast to the increasing enzyme activities in control mice with ongoing infection, cholesterol levels declined in this group (table 4). The most pronounced elevation of cholesterol was found in mice given empty liposomes ($P < 0.001$). While treatment with glucan had no significant impact on this parameter, liposomal formulations of glucan resulted in a significant increase ($P < 0.001$) in serum cholesterol levels. Peak levels in all groups were observed between days 28 and 32 p.i., concurrent with termination of therapy. After this, levels of cholesterol gradually decreased in all groups.

Discussion

Fibrosis and formation of collagenous capsules around parasites in the liver is one of the most severe consequences of infection by *M. corti*. This process also reduces the amount of drug to which larvae are exposed (Hrčková *et al.*, 1997). We demonstrated that soluble glucan, administered consecutively in low

Table 2. Aspartate aminotransferase (AST) activities ($\mu\text{kat l}^{-1}$) in the serum of mice infected with *Mesocestoides corti*, untreated and treated with liposomized glucan in combination with vitamin C, liposomized glucan, empty liposomes or glucan.

Days post-infection	Lip. glucan+vit. C mean \pm SD	Liposomized glucan mean \pm SD	Empty liposomes mean \pm SD	Glucan mean \pm SD	Control mean \pm SD
0	0.642 \pm 0.0150	0.642 \pm 0.0150	0.642 \pm 0.0150	0.642 \pm 0.0150	0.642 \pm 0.0150
11	1.251 \pm 0.0280*	1.150 \pm 0.0820	1.198 \pm 0.0040	1.120 \pm 0.0200	1.141 \pm 0.0450
15	1.255 \pm 0.0285*	1.230 \pm 0.0285*	1.194 \pm 0.0285*	1.141 \pm 0.0085	1.100 \pm 0.0700
21	1.257 \pm 0.0025*	1.204 \pm 0.0260*	1.238 \pm 0.0205*	1.075 \pm 0.0245	1.059 \pm 0.0245
28	1.639 \pm 0.0775*	1.467 \pm 0.0085*	1.500 \pm 0.0205*	1.418 \pm 0.0165	1.369 \pm 0.0040
32	1.553 \pm 0.0000*	1.492 \pm 0.0490*	1.369 \pm 0.0165	1.476 \pm 0.0225*	1.394 \pm 0.0045
42	1.141 \pm 0.0245**	1.259 \pm 0.0155**	1.255 \pm 0.0165**	1.486 \pm 0.0285	1.508 \pm 0.0025
50	1.253 \pm 0.0130**	1.320 \pm 0.0165**	1.361 \pm 0.0000**	1.447 \pm 0.0020	1.480 \pm 0.0095
65	1.190 \pm 0.0240**	1.279 \pm 0.0205**	1.320 \pm 0.0125**	1.431 \pm 0.0085	1.461 \pm 0.0029

*Significantly higher than controls ($P < 0.001$); **significantly lower than controls ($P < 0.001$).

Table 3. Alanine aminotransferase (ALT) activities ($\mu\text{kat l}^{-1}$) in the serum of mice infected with *Mesocostoides corti*, untreated and treated with liposomized glucan in combination with vitamin C, liposomized glucan, empty liposomes or glucan.

Days post-infection	Lip.glucan+vit. C mean \pm SD	Liposomized glucan mean \pm SD	Empty liposomes mean \pm SD	Glucan mean \pm SD	Control mean \pm SD
0	0.697 \pm 0.0088	0.697 \pm 0.0088	0.697 \pm 0.0088	0.697 \pm 0.0088	0.697 \pm 0.0088
11	0.950 \pm 0.0140*	0.944 \pm 0.0120*	0.820 \pm 0.0260*	0.724 \pm 0.0120	0.736 \pm 0.0080
15	0.889 \pm 0.0190*	0.850 \pm 0.0120*	0.880 \pm 0.0600*	0.799 \pm 0.0230	0.710 \pm 0.0100
21	0.882 \pm 0.0110*	0.839 \pm 0.0100*	0.822 \pm 0.0280*	0.780 \pm 0.0130	0.770 \pm 0.0100
28	1.254 \pm 0.0160*	1.152 \pm 0.0080*	1.230 \pm 0.0050*	0.810 \pm 0.0050	0.791 \pm 0.0080
32	1.125 \pm 0.0040*	1.035 \pm 0.0070*	1.042 \pm 0.0110*	1.015 \pm 0.0190	0.975 \pm 0.0200
42	0.846 \pm 0.0390*	0.838 \pm 0.0120*	0.831 \pm 0.0030*	0.832 \pm 0.0480*	0.780 \pm 0.0270
50	0.869 \pm 0.0080*	0.866 \pm 0.0110	0.850 \pm 0.0040	0.785 \pm 0.0060**	0.848 \pm 0.0080
65	0.814 \pm 0.0070*	0.810 \pm 0.0270*	0.775 \pm 0.0150	0.767 \pm 0.0130	0.771 \pm 0.0180

*Significant higher than controls ($P < 0.001$); **significantly lower than controls ($P < 0.001$).

doses within the acute phase of infection, significantly reduced larval burdens in *M. corti* infections. This is accompanied by an increase in the collagen content in the liver. The larvicidal effect of glucan, either alone or in liposomized formulation, was higher in the peritoneal cavity of mice than in the liver. These data are consistent with the findings of White *et al.* (1988), who examined the effects of another polysaccharide, lentinan, on *M. corti* (*vogae*) infections, where multiple doses of lentinan resulted in a marked reduction of parasites in the peritoneal cavity. Glucan also reduced the number of migrating ascarid larvae in the lungs of pigs (Benková *et al.*, 1992) and in brain and muscles of mice with toxocarasis (Soltys *et al.*, 1996).

Glucans bind to the lectin domain within the complement receptor type 3 (CR3) on leukocytes (Thornton *et al.*, 1996) triggering a signal transduction pathway and resulting in the activation of a respiratory burst and an increased production of reactive oxygen radicals (Gallin *et al.*, 1992) and the stimulation of phagocytosis (Topley *et al.*, 1987; Steadman *et al.*, 1990). In the present study, we did not examine macrophage functions, but on the basis of the above findings, we hypothesize that the larvicidal activity of peritoneal macrophages was restored upon stimulation by glucan.

When incorporated into liposomes, glucan is cleared from the circulation by macrophages via endocytosis (Straubinger *et al.*, 1983) or by phagocytosis when liposomes are opsonized (Wassef & Alving, 1987).

However, it is not clear how glucan in liposomes, taken up by cells, can stimulate macrophage functions when binding to a CR3 receptor is circumvented. It is possible that in cells glucan liberated from liposomes directly stimulates effector macrophage functions. For example, the activation of Fc receptor-mediated phagocytosis by macrophages following the intraperitoneal administration of liposomes was described by Aramaki *et al.* (1994). In the present study, treatment with empty liposomes led to an increase in the number of peritoneal larvae on day 65 p.i. ($P < 0.01$). The increase in parasite burden in the liver was less prominent. One possible explanation for this result is that phospholipids released from liposomes in the peritoneum were utilized by macrophages and the parasites.

In metacestode infections that invade the liver, such as *E. multilocularis* (Guerret *et al.*, 1998) or *M. corti* (Specht & Widmer, 1972) fibrosis is an important aspect of the host immune reaction against parasite development. This process is more pronounced in resistant mice leading to lower parasite burdens (Pollacco *et al.*, 1978). It was postulated that the cytokines IL-1, IL-6, TNF- α , IL-12 and IFN- γ are involved in the regulation of fibrosis (Hoffmann *et al.*, 1998). For example, in alveolar echinococcosis, pro-inflammatory cytokines IL-1 β , IL-6 and TNF- α were consistently produced by macrophages at the periphery of periparasitic granulomas and served as mediators of fibrosis (Bresson-Hadni *et al.*, 1994). In *M. corti* (*vogae*) infection, Jenkins *et al.* (1992) showed that TNF- α activity

Table 4. Cholesterol levels (mmol l^{-1}) in the serum of mice infected with *Mesocostoides corti*, untreated and treated with liposomized glucan in combination with vitamin C, liposomized glucan, empty liposomes or glucan.

Days post-infection	Lip. glucan+vit. C mean \pm SD	Liposomized glucan mean \pm SD	Empty liposomes mean \pm SD	Glucan mean \pm SD	Control mean \pm SD
0	2.141 \pm 0.0214	2.141 \pm 0.0214	2.141 \pm 0.0214	2.141 \pm 0.0214	2.141 \pm 0.0214
11	2.374 \pm 0.0000*	2.188 \pm 0.0295	2.470 \pm 0.0275*	2.206 \pm 0.0165*	2.033 \pm 0.0705
15	2.126 \pm 0.0080*	2.077 \pm 0.0005*	2.290 \pm 0.0210*	2.041 \pm 0.0535*	1.978 \pm 0.0165
21	2.243 \pm 0.0080**	2.318 \pm 0.0280	2.639 \pm 0.0675*	2.308 \pm 0.0045	2.333 \pm 0.0055
28	2.371 \pm 0.0175*	2.460 \pm 0.0100*	2.480 \pm 0.0250*	2.193 \pm 0.0295	2.017 \pm 0.0440
32	1.892 \pm 0.0490*	1.962 \pm 0.0265*	2.263 \pm 0.0005*	1.758 \pm 0.0160	1.808 \pm 0.0125
42	1.962 \pm 0.0550*	2.051 \pm 0.0595*	2.005 \pm 0.0595*	1.562 \pm 0.0640	1.492 \pm 0.0080
50	2.060 \pm 0.0210*	2.013 \pm 0.0105*	2.004 \pm 0.0025*	1.833 \pm 0.0285	1.754 \pm 0.0130
65	1.818 \pm 0.0215	1.797 \pm 0.0500	1.969 \pm 0.0200*	1.804 \pm 0.0140*	1.750 \pm 0.0130

*Significantly higher than controls ($P < 0.001$); **significantly lower than controls ($P < 0.001$).

was markedly elevated on day 14 p.i., but IFN- γ production by T-cells was down-regulated during infection (Jenkins *et al.*, 1990). IFN- γ is a very strong immunomodulatory cytokine and was found to decrease collagen synthesis in cultured myofibroblasts (Mallat *et al.*, 1995). However, in *M. corti* infections, detailed studies on the role of cytokines in liver fibrosis are lacking.

In the present study, the progression of fibrosis was investigated by measuring the hydroxyproline content in the liver during the administration of nine doses of the glucan and liposomized glucan preparations alone or co-administered with vitamin C, and empty liposomes up to day 65 p.i. The collagen content in the liver increased gradually in all groups. On histological examination, fibrosis was more intense with progressing infection. In *E. multilocularis* infection Guerret *et al.* (1998) found that the concentration of lysyl oxidase, an enzyme that correlates with cross-linking activity of mature collagen (type I), increased with progressing fibrosis and growth of parasite cysts. Histologically, a similar increase in granulomatous response, collagen deposition and fibrosis was observed after the administration of multiple doses of lentinan to *M. corti* infected mice (White *et al.*, 1988). In the latter and the present studies many larvae were damaged or appeared dead. We recorded only minor differences in collagen content between glucan alone versus liposomized glucan-treated groups. We found the most intense fibrosis after the administration of liposomized glucan in combination with vitamin C. The role of vitamin C in the organism is well defined, and its presence for the synthesis of collagen is essential (Murad *et al.*, 1981). Thus we hypothesize that the addition of vitamin C contributed to the increased collagenesis in addition to the stimulation of this process by glucan.

The stimulation of liver fibrosis is an extremely complex event in which hepatic stellate cells (HSC) are responsible for the synthesis of collagenous and non-collagenous extracellular matrix components and other biologically active molecules (Friedman, 1993). *In vivo*, activated HSC are present in chronically diseased liver, especially during liver fibrosis. In acute injuries, the factor responsible for transformation of perisinusoidal cells to HSC is hepatocyte necrosis followed by activation of Kupffer cells (Burt, 1993). There are several factors that induce this transformation including growth factors, cytokines and chemokines. These are products of oxidative stress released mainly from Kupffer cells (Tsukamoto, 1999). Thus, we hypothesize that in glucan-enhanced fibrosis, recruited macrophages (including Kupffer cells) probably produced a stronger activating signal for HSC. In the case of treatment with liposomized formulations of glucan, TNF- α was probably not the main factor responsible for activation of HSC. This is based on the findings of Tonks *et al.* (2001), who showed that dipalmitoyl phosphatidylcholine (DPPC), the major component of liposomes used in the present study, can significantly inhibit the release of TNF- α from monocytes. In agreement with this hypothesis was our finding that empty liposomes did not modify collagen content except for on the last day of the experiment.

Damage of parenchymal cells causes release of their intracellular constituents into the blood. Sensitive indicators of such damage include plasma levels of the

enzymes AST and ALT (Zilva & Pannall, 1984). Following *M. corti* infection, the activity of both enzymes gradually increased, peaking between days 32 and 42 p.i., then declining over the next month. Levels of ALT, which is restricted to the cytoplasm, were lower in comparison to AST, which is localized in the mitochondria and cytoplasm (Zilva & Pannall, 1984). The same pattern in both enzyme activities during *M. corti* infection was observed by White *et al.* (1988) and Velebný & Hřčková (1995). Glucan administration did not result in a marked elevation of both enzymes activities. Liposomized glucan and empty liposomes significantly elevated ALT levels, most prominently when co-administered with vitamin C. This finding correlates with increased collagen deposition. Under conditions in which cell membrane damage occurs, more ALT than AST leaks into the blood (Zilva & Pannall, 1984). It is possible that redistribution of liposomal phospholipids from Kupffer cells to hepatocytes influenced the permeability of their membranes (Scherphof *et al.*, 1983), thus some leakage of ALT might occur. On the other hand, AST activities decreased significantly from day 32 p.i. in groups with liposomal formulations. As this enzyme activity is indicative of whole cell damage (Zilva & Pannall, 1984), the administration of glucan in a liposomal carrier seems to stimulate regeneration of liver tissue. Liver regeneration is a fundamental response to injury of hepatic tissue, due to the capacity of hepatocytes to proliferate rapidly. It was postulated by Takeishi *et al.* (1999) that in this process, Kupffer cells play a key role by stimulating the expression of hepatocyte growth factor (HGF) in HSC. HGF is the most potent mitogen for hepatocytes and its specific binding to liver collagens was described by Schuppan *et al.* (1998). The more intense collagenesis and repair of liver parenchyma in liposomized glucan- and vitamin C-treated mice could be a consequence of HGF activity.

Hepatocytes play an important role in the metabolism of exogenous and endogenous lipids, and liver injury often reduces metabolic and biosynthetic activities of these cells. Indeed, all chronic liver diseases lead to a depression of total serum cholesterol levels (George & Chandrakasan, 2000). Therefore we monitored serum cholesterol levels during and after therapy. It is important to note that serum cholesterol levels reflect a dynamic ratio between cholesterol excretion from other cells, its uptake by the parenchymal cells and synthesis (Musil, 1990). Higher levels of cholesterol in the control group during the acute phase of infection by *M. corti* might reflect liver damage leading to the release of more cholesterol into the blood. As liver repair began on days 28–32 p.i. (Specht & Widmer, 1972; personal observations), cholesterol levels declined but did not reach the physiological levels, probably because fibrous tissues replaced a substantial portion of the liver parenchyma. Decreased cholesterol synthesis in hepatocytes from a cirrhotic liver was described by Zimmermann *et al.* (1992). Glucan administration did not modify significantly cholesterol levels, ALT and AST activities, although the collagen content increased significantly. We might conclude that although glucan treatment activates the larvicidal capacity of phagocytic cells, one result of which is increased fibrosis, it did not stimulate regenerative processes in livers infected with larval stages of *M. corti*.

The administration of a liposomal formulation of glucan or empty liposomes lead to a significant elevation of serum cholesterol levels up to days 28–32 p.i. Following that, as in the control group, cholesterol values decreased. This might suggest that the synthetic capacity of liver cells for cholesterol was improved due to the utilization of exogenous lipids correlating with lowered serum AST activities, a marker for hepatocyte necrosis. Later, when re-utilization of liposomal lipids was probably completed, cholesterol levels in the serum levelled down in all groups, but remained significantly higher than in untreated controls.

In summary, the present study has shown that intense fibrosis in the liver of mice treated with liposomized glucan and vitamin C did not result in extensive parenchymal cell injury but, rather, in a higher efficacy of treatment. Moreover, liposomal lipids may be utilized by liver cells in the process of regeneration. Targeting of liposomized immunomodulator glucan directly to the phagocytic cells may overcome problems associated with their suppressed accessory and effector functions.

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