Efficacy of praziquantel and liposome entrapped glucan on larval *Mesocestoides vogae* infection in mice, the type I and III collagen distribution and collagen content in the liver

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Summary

The larvicidal effect of praziquantel (PZQ), liposomized glucan (LG) alone or co-administered with PZQ and impact of these drugs on collagen content and distribution in the liver of mice infected with *Mesocestoides vogae* tetrathyridia was investigated on days 1, 7 and 14 after treatment (i.e. on days 16, 22 and 29 post infection, p.i.). Drugs were administered in acute phase of infection from day 13 to day 15 p.i. The highest larvicidal effect in the liver and peritoneal cavity of mice was found during the first week after co-administration of PZQ and LG and was inversely related to collagenesis in the liver. Co-administration of these drugs did not increase collagen content in comparison with control. However, in spite of the increased total collagen content in the liver of PZQ- and LG-treated mice, biodegradable type III collagen was observed in the greater measure than the cross-linked type I collagen in these groups in comparison with control mice. We suppose that stimulation of various hepatic cell types with glucan targeted to the liver by means of liposomes and reversibility of hepatic fibrogenesis after treatment could enhance the defence processes of mice infected with *M. vogae* tetrathyridia.

Key words: *Mesocestoides vogae*, mouse; hepatic fibrogenesis; collagen; praziquantel; β-glucan; liposomes

Introduction

Among parasitic diseases manifested by hepatic fibrosis schistosomosis and echinococcosis is of medicinal importance. Platyhelminth *Schistosoma mansoni* (Trematoda) induces this hepatic disease by ova (Wyler et al., 1978) and tape worm *Echinococcus multilocularis* (Cestoda) by cysts (Mehlhorn et al., 1983). In pharmacological experiments *Mesocestoides vogae* (syn. *M. corti*, Cestoda) is used as a model for slower developing metacestodes *E. multilocularis* (Mitchell et al., 1977) and *E. granulosus* (WHO, 1984). Tissue damage, granulomatous inflammation and hepatic fibrosis are the relevant harmful effects of parasitation of these parasites. Hepatic fibrosis results from an imbalance between synthesis and degradation of extracellular matrix (ECM) molecules (Friedman, 1993, 2000). Hepatic stellate cells (HSC) are responsible for the excess production of ECM components. The activation of HSC is mediated by various cytokines and reactive oxygen species released from the damaged hepatocytes and activated Kupffer cells (reviewed by Wu & Zern, 2000). Quantitative and qualitative changes induced in the hepatic ECM by alveolar echinococcosis (Vuitton et al., 1986) or schistosomosis (Badawy et al., 1996) were often investigated, however, little is known about collagen types and their distribution in the liver of mice during *M. vogae* infection. Treatment of hepatic fibrosis induced by parasite requires drugs with larvicidal and antifibrotic effect. Knowledge of the nature of hepatic fibrogenesis leads to the inhibition of HSC activation which is crucial goal for intervention in this process (Wu & Zern, 2000). Praziquantel (PZQ) is a broad-spectrum anthelmintic highly effective against trematode and cestode infections in humans and animals (Thomas & Gönner, 1978). However, PZQ did not kill all asexually multiplying *M. vogae* tetrathyridia in mice even at high doses (Novak, 1977; Richards et al., 1988). Stimulation of humoral and cellular components of the host’s immunity, suppressed by helminth infections (Jenkins et al., 1990), might contribute to the enhanced larvicidal effect of this drug. In our previous experiments with fungal polysaccharide β-glucan (alone and entrapped in liposomes) on mice infected with *M. vogae* (Ditteová et al., 2003a,b) significant reduction of larval counts has been demonstra-
ted, which was higher after liposomized glucan than after administration of free glucan. Larvicidal effect of β-glucan alone has also been documented in the treatment of ascara-
iosis (Benková et al., 1992; Šoltýs et al., 1996), however, it has not been examined yet after co-administration of PZQ with liposome entrapped β-glucan. Glucans have been shown to activate the innate immune system (reviewed by Bohn & BeMiller, 1995). Liposomes served as the drug delivery system (Fielding, 1991).

As to the antifibrotic effect it would be desirable if the in-
crease of PZQ efficacy against M. vogae tetrathyridia si-
multaneously contribute to collagen degradation and re-
sorption. This possibility could result from the reversibility of hepatic fibrogenesis (Burt & Oakley, 1993), i.e. from changing of the ratio between content of the cross-linked type I and the biodegradable type III collagen in favour of type III. Effect of PZQ alone or co-administered with lipo-
some entrapped β-glucan on fibrogenesis during larval ces-
tode infection has not been reported till now.

In this study the larvicidal effect of praziquantel co-admi-
istered with liposome entrapped β-glucan as well as im-
 pact of these drugs on the total collagen content as well as on the localization of type III and type I collagen in the li-
ver of mice during M. vogae infection was examined.

**Material and Methods**

**Chemicals and preparation of drug formulations**

Praziquantel (SIGMA) was suspended in 0.4 % cremophor oil (SIGMA) in distilled water giving rise to the suspension with concentration of the drug 7 mg.ml⁻¹.

β-glucan, isolated from the fungi Pleurotus ostreatus and carboxymethylated (CM-glucan) (MEVAK, Slovak Republic), was entrapped into liposomes with a negative sur-
face charge according to the method of Velebný et al., (2000). Briefly, a chloroform - methanol (4:1 v/v) solution of the mixture consisting of 0.031 mmol phospholipon 100 H (PHOSPHOLIPID, Germany), 0.009 mmol cholesterol and 0.004 mmol dicetylphosphate (both compounds were purchased from SERVA) was dried under the reduced pres-
sure on a rotary vacuum evaporator to form a film which was hydrated by 20 ml of 0.1 % solution of CM-glucan in saline. Prepared liposomal suspension contains CM-glucan and lipids in concentration 1 mg.ml⁻¹ and 1.5 mg.ml⁻², respectively.

**Animals and infection**

All experiments were carried out on 8-week-old outbred ICR male mice. Animals were divided in four groups com-
prising ten individuals in each group. They were kept on a standard diet (commercial pellets) with the access to water ad libitum.

Tetrathyridia of M. vogae were maintained by intraperito-
neal passage through outbred ICR mice. Animals were in-
fected orally with 55 – 60 tetrathyridia in warm (32 – 34°C) Hanks Balanced Salt Solution (HBSS) (SIGMA).

**Experimental design**

Administration of drug formulations started on day 13 post infection (p.i.). PZQ was given orally at a dose rate 35 mg.kg⁻¹ body weight twice a day for the three consecutive days (Group 1). Group 2 received the same dosage of PZQ together with liposomized CM-glucan, which was co-
administered intramuscularly at a dose rate 5 mg.kg⁻¹ body weight twice on day 13 p.i. (one dose into the muscles of each hind leg of animals). Liposomized CM-glucan alone was given to animals (Group 3) at the same dosage and route as in the previous group. The average dose of lipids in liposomes was 7.5 mg.kg⁻¹ body weight. Last group of infected and untreated mice served as the control.

**Isolation of Mesocestoides vogae tetrathyridia and drug efficacy**

Tetrathyridia were recovered from peritoneal cavity of mice by rinsing with saline. Isolation of tetrathyridia from the livers of the same mice was carried out by digesting in 0.25 % trypsin solution on days 1, 7 and 14 post treatment (p.t.), i.e. on day 16, 22 and 29 p.i. Parasites recovered from all groups of mice were re-sus-
pended in 0.1 % agar solution and counted. The total num-
ber of tetrathyridia isolated from peritoneal cavity of mice and number per 1 g of the liver was calculated from the three counts per sample and expressed as a mean ± standard deviation (SD). Larvicidal effect of the drug was cal-
culated by means of reduced larval counts in treated mice in comparison with untreated ones and expressed in per cent (Hrčková & Velebný, 1995).

**Determination of collagen content in the liver**

Hydroxyproline as the most abundant amino acid of col-
gen was used for indication of the content of this protein in the liver. Samples of the homogenized liver from three animals in control group were collected on days 7 and 13 p.i., in all groups on days 16, 22 and 29 p.i. Hydroxypro-
line content in liver homogenates was determined by a colorimetric method (Woessner, 1961). Briefly, samples of the homogenized liver were hydrolyzed by 6N HCl at 110°C for 18 hrs. Liver hydrolysates were transferred in the 96-well plate and then evaporated to dryness at room temperature. Dried samples were incubated with consecu-
tively added solutions of chloramine T and perchloric acid at room temperature and then with p-dimethylamino ben-
zaldehyde at 60°C. Absorbance of the coloured solutions in the wells was measured at 570 nm. Concentration of hy-
droxyproline was read out from the standard curve and ex-
pressed in µg.g⁻¹ of the liver (wet weight).

**Immunohistochemistry of the liver**

The liver specimens were fixed in 10 % neutral formalin and embedded in paraffin. Liver sections (6 µm thick) were deparaffinized, rehydrated in a graded series of ethanol and quenched for endogenous peroxidase activity by soaking in methanol containing 0.3 % hydrogen peroxide for 30 mi-

utes. Sections were pretreated with diluted (1:2000, v/v)
Table 1. Number of *Mesocestoides vogae* tetrathyridia in the liver of mice treated by praziquantel, liposomized glucan and by their co-administration

<table>
<thead>
<tr>
<th>Group of mice</th>
<th>Days p.i./Days p.t.</th>
<th>Larval count (μg/g)</th>
<th>Larval count (μg/g)</th>
<th>Larval count (μg/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>16/1</td>
<td>22/7</td>
<td>29/14</td>
<td></td>
</tr>
<tr>
<td>PZQ</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>162 ± 25</td>
<td>339 ± 46</td>
<td>602 ± 45</td>
<td></td>
</tr>
<tr>
<td>PZQ + LG</td>
<td>201 ± 31</td>
<td>160 ± 12</td>
<td>562 ± 35</td>
<td></td>
</tr>
<tr>
<td>LG</td>
<td>225 ± 36</td>
<td>548 ± 52</td>
<td>648 ± 43</td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>437 ± 47</td>
<td>705 ± 75</td>
<td>793 ± 82</td>
<td></td>
</tr>
</tbody>
</table>

Each value represents mean ± SD of six counts; R – reduction (%) of larval count in comparison with control; PZQ – praziquantel; p.i. – post infection; LG – liposomized glucan; p.t. – post treatment. Significance of difference from control on certain day: * – P < 0.001; ** – P < 0.01; *** – P < 0.05

Table 2. Number of *Mesocestoides vogae* tetrathyridia in peritoneal cavity of mice treated by praziquantel, liposomized glucan and by their co-administration

<table>
<thead>
<tr>
<th>Group of mice</th>
<th>Days p.i./Days p.t.</th>
<th>Larval count (μg/g)</th>
<th>Larval count (μg/g)</th>
<th>Larval count (μg/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>16/1</td>
<td>22/7</td>
<td>29/14</td>
<td></td>
</tr>
<tr>
<td>PZQ</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>87.5 ± 23</td>
<td>117.4 ± 41</td>
<td>205.0 ± 21</td>
<td></td>
</tr>
<tr>
<td>PZQ + LG</td>
<td>34.4 ± 8</td>
<td>82.6 ± 29</td>
<td>172.0 ± 40</td>
<td></td>
</tr>
<tr>
<td>LG</td>
<td>58.2 ± 15</td>
<td>177.0 ± 43</td>
<td>260.0 ± 32</td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>124.2 ± 32</td>
<td>262.2 ± 48</td>
<td>337.5 ± 52</td>
<td></td>
</tr>
</tbody>
</table>

Legend: See Table 1

Fig. 1. Hydroxyproline (HP) content in the liver of mice infected with *Mesocestoides vogae* tetrathyridia and treated by praziquantel (PZQ), liposomized glucan (LG) and by their co-administration

Footnote: Each value represents mean ± SD of six measurements.

PZQ = praziquantel; LG = liposomized glucan; p.i. = post infection. Significance of the difference from the control on certain day: * – P < 0.001, ** – P < 0.01

Statistical analysis
Numerical results were expressed as a mean ± SD. Statistical evaluation of data used one-way Kruskal-Wallis ANOVA (P < 0.001). For the multiple comparison procedure the Schaffé post-hoc ANOVA test (P level is indicated in text) (Statistica 6.0, Stat Soft Inc., Tulsa, USA) was used.

Results

Larvicidal effect of drugs
The larval counts in the liver of control mice gradually increased on selected days p.i. Treatment of mice resulted in significantly (P < 0.001) lower larval counts in the liver comparing to control mice (Table 1). Number of larvae was the most reduced after simultaneous treatment with PZQ and LG on day 7 (160 ±12) and after treatment with PZQ on day 1 post therapy (162 ± 25). Moreover, LG alone also significantly (P < 0.01) reduced larval counts in the liver. Worm burden in peritoneal cavity of all treated mice (Table 2) was significantly lower than that in control group (P value is indicated in Table 2). The lowest worm burden was recorded after combined treatment with PZQ and LG during the whole period examined (P < 0.001).
Collagen content in the liver

Parasitation of *M. vogae* tetrathyridia in the liver of mice resulted in gradual increasing of collagen content (indicated by hydroxyproline) that significantly (P < 0.01) differed from intact mice (78.64 ± 5.12) from the day 13 p.i. (145.92 ± 9.51) (Fig. 1). Treatment of mice with PZQ or LG significantly (P < 0.01 and P < 0.001, respectively) increased hepatic collagen content in comparison with control (174.76 ± 8.62) on day 1 after the last dose of these drugs. However, simultaneous administration of PZQ and

Fig. 2. Immunohistochemical staining with the monoclonal antibody directed against type I and type III collagen of the hepatic sections from mice with *Mesocestoides vogae* infection on day 29 p.i.: a – Granulomatous infiltration with type III collagen localized between inflammatory cells (in the control and treated groups); b – Type I collagen in the fibrotic diffuse lesions and in fibroblasts (control group); c – Type III collagen immunoreactivity in the granulomatous infiltration of cells (PZQ + LG-treated group); d – Fibres of type III collagen scattered in diffuse fibrotic/inflammatory lesions (PZQ-treated group); e – Type I collagen in the capsule around tetrathyridia (PZQ + LG-treated group); f – Type III collagen in the form of fibres formed the capsule around tetrathyridia (LG-treated group)

Immunoreactivity to the collagen types is presented as brown colour.
LG was manifested by significant (P < 0.01) decrease of hepatic collagen content in comparison with control on day 14 after treatment. Effect of two doses of LG (5 mg.kg⁻¹) on the hepatic collagen content was also monitored in healthy mice. It was found that collagen content ranged between 71.56 ± 8.61 and 83.44 ± 5.62 μg.g⁻¹ within two weeks after administration of LG and did not differ significantly (P > 0.01) from value 78.64 ± 5.12 μg.g⁻¹ determined before LG administration.

**Immunohistochemical localization of type I and III collagen on the liver sections**

Immunoreactivity (IR) to the type I and III collagen was examined on days 22 and 29 p.i and was found on liver sections in all examined groups of mice. Both collagen types were present in the form of fibrils and IR was seen in flattened cells (fibroblasts) and also in other cell type (probably hepatic stellate cells). Regarding distribution of both collagen types in the liver, IR was localized in granulomatous infiltrations composed of inflammatory cells (Fig. 2a), in the diffuse fibrotic lesions, it lined the migratory tracks of larvae and formed the capsules surrounding most of the larvae. At the end of experiment (day 29 p.i.), in control group more intense and abundant overall staining of type I collagen than of type III collagen was observed (Fig. 2b). On the contrary, in all treated groups IR to type III collagen prevailed over IR to type I, predominantly in granulomas (Fig. 2c), and in diffuse fibrotic lesions (Fig. 2d). The type I collagen IR in treated groups was localized mainly in fibroblasts and fibrils surrounding larvae (Fig. 2e), however, IR to type III collagen was also observed here (Fig. 2f).

**Discussion**

*M. corti* tetrahyridia, which proliferate rapidly and are infective, serve as a model for the slower developing metacestodes *Echinococcus granulosus* and *E. multilocularis* (WHO, 1984). For the treatment of cystic or alveolar echinococcosis are currently used benzimidazoles (albendazole, mebendazole) given alone or in combination with praziquantel (El-On, 2003; Wen et al., 1994). Praziquantel was used for the treatment of *M. vogae* infection in our previous experiments (Ditteová et al., 2003a,b; Hrčková & Velebný, 1995). Larvicidal effect of PZQ is limited due to the pharmacokinetic properties of the drug (Steiner et al., 1976; Andrews, 1983). Suppressed effector macrophage functions due to helminth infections (Jenkins et al., 1990) contribute to the limited immunological defence against these infections. In order to activate larvicidal function of macrophages, polysaccharide β-glucan entrapped in liposomes was used in our study. Activation of monocyte effector functions, e.g. phagocytic ability, production of reactive oxygen forms (Wakshull et al., 1999), cytokines and inflammatory mediators (Williams et al., 1996) was documented by β-glucans (reviewed by Kogan, 2000). Co-administration of LG and PZQ resulted in a significantly higher reduction of worm burden in comparison with anthelmintic alone in the peritoneal cavity within the whole examined period and in the liver on day 7 p.i. Activation of cytotoxic functions of effector immune cells might be indicated by observed reduction of larval counts after termination of LG treatment. After injection of the soluble immunomodulator only a small part of the dose can reach macrophages in order to be activated. This disadvantage has been overcome by administration of β-glucan entrapped in conventional liposomes that are suitable carriers for site-specific delivery of drugs to macrophages (Gregoriadis, 1988; Fielding, 1991). This monocytes are able to internalise liposome particles either via complement receptors or by phagocytosis or endocytosis what led to their activation (Szebeni, 1998). We assume that in our studies glucan liberated from taken up liposomes affected activity of mononuclear phagocytic cells directly, i.e. without binding to the glucan receptors on the cells (Brown & Gordon, 2003).

Some investigators evaluated impact of PZQ on fibrogenesis induced by some liver dwelling parasites. Badawy et al. (1996) found the stage dependent effect of this drug on hepatic collagen content in murine schistosomosis. Treatment of hamsters with opisthorchiosis by PZQ resulted in decreased hepatic collagen content (Hutadilok et al., 1983). We assume, that hepatic fibrosis after PZQ-treatment of *M. vogae* infected mice was stimulated by antigens released from the damaged larvae which triggered a cascade of profibrotic reactions (Burt & Oakly, 1993; Tsukamoto, 1999). The greatest stimulation of fibrogenesis was determined after PZQ- or LG-treatment alone. It is known that the reactive oxygen forms play important role in hepatic fibrosis (Britton & Bacon, 1994) and this result is in agreement with the weak antioxidant activity of glucan reported by Tsiapali et al. (2001). Increase of the collagen content in the liver of mice accompanied by reduction of *M. corti* larval counts after β-glucan administration was also observed in our previous studies (Ditteová et al., 2003a,b). White et al. (1988) observed similar effects after administration of T-cell dependent immunomodulator lentinan. It was believed that glucan stimulates collagen deposition in the wounds indirectly via release of macrophage profibrotic factors. However, in vitro studies revealed that glucan can directly activate collagen synthesis in human skin fibroblasts (Kougias et al., 2001). Therefore, it is possible that higher hepatic hydroxyproline content after LG-treatment.
could partially be result of a direct stimulation of hepatic fibroblasts. This assumption is based on the results of our investigation concerning the role of mast cells in regulation of collagen synthesis in the liver (Hrčková et al., in press). This study showed, that elevation of the hepatic mast cells numbers preceded an increase of fibrosis. However, hepatic injury due to parasitisation and larval somatic antigens seems to be the initiating factors of fibrogenesis in this organ.

Response of mouse liver to infection with tetrathyridia of Mesocestoides was described by some investigators (Specht & Widmer, 1972; White et al., 1982). The type III collagen is synthesised after acute hepatic injury, production of the type I collagen increased later (Burt et al., 1990). In chronic phase of hepatic fibrogenesis collagen molecules are cross-linked by pyridinoline (Burt & Oakley, 1993) and they are more resistant to degradation by specific proteolytic enzymes. Our immunohistochemical observations showed increased amount of collagen fibres in the liver which formed capsules around M. vogae tetrathyridia from day 14 p.i. (Fig. 2e). This is in correlation with enhanced collagen content found in the liver from the control and treated groups. However, total collagen content does not indicate reversibility of hepatic fibrogenesis which resides in changing of the ratio between type III and type I collagen and which is important for the pathology of infection. Our preliminary immunohistochemical study revealed, that in spite of the increased total collagen content in the liver of PZQ- and LG-treated mice, biodegradable type III collagen seems to be present in a greater measure than the cross-linked type I collagen in comparison with the control. It might indicate that these drugs triggered a cascade of events leading to remodelling of hepatic ECM associated with the degradation of a number of highly cross-linked type I collagen molecules. Similar effect was described after treatment of skin alveolar echinococcosis with albendazole (Ribard-Blum et al., 1998). Nevertheless, the collagen deposition around M. vogae tetrathyridia is one of the most important defence mechanisms of the host against their migration and multiplication (Pollacco et al., 1978) and both collagen types were detected around tetrathyridia in our study.

In this study we have shown that anthelmintic activity of PZQ against metacestode M. vogae has been potentiated by stimulation of suppressed immune effector cells when co-administered with non-specific immunomodulator β-glucan entrapped in liposomal carrier. Co-administration of these drugs did not change significantly hepatic collagen content in comparison with untreated infected mice while liposomized glucan and praziquantel alone showed moderate profibrotic effect. In spite of the increased total hepatic collagen content in PZQ- and LG-treated group more biodegradable type III collagen than the cross-linked type I collagen was observed in comparison with control. This finding could indicate the reversibility of hepatic fibrogenesis which is important for the effective treatment of this infection.

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