Evaluation of local immune response to *Fasciola hepatica* experimental infection in the liver and hepatic lymph nodes of goats immunized with Sm14 vaccine antigen

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Protection against *Fasciola hepatica* in goats immunized with a synthetic recombinant antigen from *Schistosoma mansoni* fatty acid-binding protein 14 (rSm14) was investigated by assessing worm burdens, serum levels of hepatic enzymes, faecal egg count and hepatic damage, which was evaluated using gross and microscopic morphometric observation. The nature of the local immune response was assessed by examining the distribution of CD2⁺, CD4⁺, CD8⁺ and γδ T lymphocytes along with IgG⁺, IL-4⁺ and IFN-γ⁺ cells in the liver and hepatic lymph nodes (HLN). The goats used consisted of group 1 (unimmunized and uninfected), group 2 [infected control - immunized with Quillaia A (Quil A) and group 3 (immunized with rSm14 in Quil A and infected), each containing seven animals. Immunization with rSm14 in Quil A adjuvant induced a reduction in gross hepatic lesions of 56.6% (p < 0.001) and reduced hepatic and HLN infiltration of CD2⁺, CD4⁺, CD8⁺ and γδ T lymphocytes as well as IL-4⁺ and IFN-γ⁺ cells (p < 0.05). This is the first report of caprine immunization against *F. hepatica* using a complete rSm14 molecule derived from *S. mansoni*. Immunization reduced hepatic damage and local inflammatory infiltration into the liver and HLN. However, considering that Quil A is not the preferential/first choice adjuvant for Sm14 immunization, further studies will be undertaken using the monophosphoryl lipid A-based family of adjuvants during clinical trials to facilitate anti-*Fasciola* vaccine development.

Key words: Sm14 - *Fasciola hepatica* - vaccines - goat - liver - parasite infection

Fasciolosis, caused by *Fasciola hepatica*, is the most important parasitic livestock disease and an economically important disease in ruminants in Europe, the Americas, Australia and New Zealand. Fasciolosis represents a recognized, unsolved agricultural problem responsible for economic losses estimated at around US$ 3 billion per year (Spithill et al. 1999). Recent reports indicate that it is also a major human pathogen and is increasingly being recognized as a cause of significant human diseases (Mas-Coma et al. 2005).

Liver fluke infections can be highly pathogenic and can lead to severe morbidity and even death of the host. The flukicide triclabendazole is still the drug of choice used in *Fasciola* control programs (Overend & Bowen 1995); however, the high cost of treatment prevents widespread use by rural producers in developing countries. Moreover, resistance to triclabendazole has been reported in sheep infected with *F. hepatica* (Overend & Bowen 1995, Moll et al. 2000), suggesting that selection of resistant parasites may eventually compromise the use of this drug. Furthermore, although chemotherapy has been used for more than two decades with some efficacy in reducing morbidity rates, it represents only a palliative measure, leaving transmission rates unaltered due to continuous re-infection in endemic areas (Spithill et al. 1999, Dalton et al. 2003, Meeusen & Piedrafita 2003). Vaccines represent the most attractive long-term alternative to solve this dilemma, as they represent an environmentally-friendly method for the control of liver fluke disease in livestock.

Indeed, the prevalence of fasciolosis in livestock has increased dramatically in the last few years. For example, up to 12-fold increases in infection rates have been observed in several European Union member states. In the United Kingdom, the prevalence of infection in cattle ranges from 45-84% and in Ireland alone, annual losses have been estimated at €60 million (Tendler & Simpson 2008).

Focusing on molecular characterization of secretor-excretory (SE) components with the goal of identifying immunoprotective components contained therein, the recombinant antigen from *Schistosoma mansoni* fatty acid-binding protein 14 (rSm14) molecule was selected from a mixture of adult schistosome components obtained from living worms that have been previously shown to protect mice against infection. This molecule was identified by a long-term investigation focusing on vaccination experiments in populations of out-bred animals and further characterized by gene cloning, producing a 14 kDa molecule named Sm14, belonging to a
family of fatty-acid binding proteins (Moser et al. 1991, Tendler et al. 1996). It has been previously demonstrated that Sm14 can induce high levels of SE antigens against S. mansoni in experimental infections and is also protective against F. hepatica (Tendler et al. 1995, 1996).

rSm14 is now being actively developed as a bivalent anti-helminth vaccine for use in both livestock disease caused by F. hepatica and human endemic schistosomiasis (Tendler & Simpson 2008).

The aim of this study was to evaluate protection against F. hepatica in goats immunized with rSm14 by assessing worm burdens, serum levels of hepatic enzymes, faecal eggs counts, gross and microscopic hepatic lesions and the local immune response.

MATERIALS AND METHODS

Experimental design - This study consisted of a 10 week period of immunization followed by an experimental infection (17 weeks) and subsequent sacrifice of all animals, 27 weeks in all. Animals were first acclimatized for 30 days to the experimental conditions.

The sample included 21 Florida Sevillana goats. Animals were four months of age and free of parasitic and infectious diseases, as indicated by faecal analysis and absence of clinical signs. Animals were randomly allocated into three groups of seven each. Group 1 was used as an uninfected control. The other two groups were immunized twice, on the 1st and 4th week. The 2nd group was used as an infection control and injected with 1 mL of phosphate buffered saline (PBS) in 1 mL of adjuvant QuillaiA (Quil A). The 3rd group was immunized with 100 μg of rSm14 in 1 mL of Quil A with quantum satis of 2 mL of PBS. rSm14 was kindly provided by Prof. Miriam Tendler from the Oswaldo Cruz Foundation, Brazil.

In the 10th week, groups 2 and 3 were infected with 200 F. hepatica metacercariae of ovine origin (Bristol University, Bristol, UK). The doses were administered orally in gelatine capsules. Infected and control goats were killed by intravenous injection of thiobarbital 17 weeks after infection and subjected to necropsy. The experiment was approved by the Bioethical Committee of the University of Cordoba (7119) and was carried out taking into account the following criteria: (i) the area of portal spaces and microscopic hepatic damage (chronic tracts, cholangitis with eosinophils, lymphocytes and plasma cell infiltration, fibrosis and granulomas) and (ii) the area of bile ducts. Ten low-power microphotographs per animal were taken, each measuring 8.5 mm². These microphotographs were randomly taken of damaged hepatic areas. First, hepatic damaged areas and portal spaces were delineated and the area was obtained. Second, the outer perimeter of bile ducts was delineated and the area was obtained. The results are expressed as percentage [mean ± standard deviation (SD)] per group.

Microscopic liver morphology was examined to evaluate: (i) the area of portal spaces and microscopic hepatic damage (chronic tracts, cholangitis with eosinophils, lymphocytes and plasma cell infiltration, fibrosis and granulomas) and (ii) the area of bile ducts. Ten low-power microphotographs per animal were taken, each measuring 8.5 mm². These microphotographs were randomly taken of damaged hepatic areas. First, hepatic damaged areas and portal spaces were delineated and the area was obtained. Second, the outer perimeter of bile ducts was delineated and the area was obtained. The results are expressed as the percentage of cortex occupied by lymphoid follicles (mean ± SD) group.

Immunohistochemistry - Tissue samples from the left and right hepatic lobes and HLN were snap-frozen in optimal cutting temperature (Miles, Elkhart, IN, USA) and immersed in liquid nitrogen-cooled 2-methylbutane (Merck, Darmstadt, Germany). Frozen samples were stored at -80°C until they were cut in serial tissue sections of 5 μm thickness using a cryostat at -25°C and stored again at -80°C until used.

The avidin-biotin-peroxidase (ABC) method was used for immunohistochemistry on snap-frozen tissue samples (Pérez et al. 1998). Endogenous peroxidase activity was blocked by incubation with 0.05% phenyl-hydrazine (Sigma®, St Louis, MO, USA) in PBS at pH 7.6 for 30 min. Tissue sections were incubated with 5% normal goat serum (Vector Laboratories, Burlingame, CA, USA) for 30 min at room temperature. Then primary antibodies were applied overnight (18 h) at 4°C.

Details of primary antibodies are shown in Table I. Monoclonal antibodies (mAb) used for detecting lymphocyte subsets (mouse anti-bovine CD2⁺, CD4⁺, CD8⁺, TCR⁺ and IgG⁺) have shown cross-reactivity with caprine antigens (Navarro et al. 1996, Pérez et al. 1998). Mouse anti-bovine IL-4 and IFN-γ mAbs cross react with caprine antigens according to the supplier’s information. After three 10 min rinses in PBS, biotinylated goat anti-mouse immunoglobulin serum (Dako, Denmark) diluted 1:50 was applied to slides incubated with primary mAbs for 30 min and a goat anti-rabbit immunoglobulin serum (Dako, Denmark) diluted 1:200 was applied to slides incubated with the primary rabbit anti-goat IgG polyclonal antibody for 30 min.
TABLE I

Details of primary antibodies used, dilution and source

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Specificity</th>
<th>Origin</th>
<th>Dilution</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>BAQ95A</td>
<td>CD2</td>
<td>Mouse</td>
<td>1:1000</td>
<td>VMRD Inc</td>
</tr>
<tr>
<td>GC50A1</td>
<td>CD4</td>
<td>Mouse</td>
<td>1:50</td>
<td>VMRD Inc</td>
</tr>
<tr>
<td>CACT80C</td>
<td>CD8</td>
<td>Mouse</td>
<td>1:500</td>
<td>VMRD Inc</td>
</tr>
<tr>
<td>CACTB6A</td>
<td>γ/δ</td>
<td>Mouse</td>
<td>1:200</td>
<td>VMRD Inc</td>
</tr>
<tr>
<td>AI-5000</td>
<td>IgG</td>
<td>Rabbit</td>
<td>1:1000</td>
<td>Vector</td>
</tr>
<tr>
<td>MCA1820</td>
<td>IL-4</td>
<td>Mouse</td>
<td>1:50</td>
<td>Serotec</td>
</tr>
<tr>
<td>MCA1783</td>
<td>IFN-γ</td>
<td>Mouse</td>
<td>1:50</td>
<td>Serotec</td>
</tr>
</tbody>
</table>

VMRD Inc, Pullman, WA, USA; Serotec, Oxford, UK; Vector Laboratories, Inc, Burlingame, CA, USA.

After two 10 min rinses in PBS, an ABC complex (Vector, Burlingame, USA) diluted 1:50 was applied for 1 h as a 3rd reagent. Then tissue sections were incubated with 3,3'-diaminobenzidine tetrahydrochloride (Sigma) diluted to 0.035% in Tris-buffered saline (pH 7.2) with 0.1% hydrogen peroxide for 1 min, rinsed in distilled water, lightly counterstained with Mayer’s haematoxylin and mounted with Immumount® (Shandon, Pittsburgh, PA, USA). Tissue sections in which the specific primary antibodies were substituted with PBS or non-specific isotopic serum were used as negative controls. Caprine lymph node tissue sections were used as positive controls for all of the primary antibodies.

Immunoreactive cells were counted using 10 photomicrographs per animal at 200X magnification (0.2 mm²/photomicrograph). Photomicrographs were used to count positively-staining cells. In liver tissue sections, areas were randomly selected, including portal spaces. In HLN, positive cells were counted using the same methodology in both medullar and follicular areas. The results are presented as means ± SD. Image Pro-plus 4.5 biomedical software (Media Cybernetics, Silver Spring, USA) was used for cell counting. Macros were calibrated for staining intensity and cell size to include all immunostained cells.

Statistical analyses - Statistical analysis was carried out using SPSS 14.0 software for Windows (SPSS Inc, Chicago, USA). The Kolmogorov-Smirnov test was applied to decide whether distributions were parametric. The results between groups were compared using the Student t test when distributions were parametric and the Mann-Whitney U test when distributions were non-parametric. Correlation studies were carried out using the Spearman correlation test for non-parametric distributions; p < 0.05 was considered significant.

RESULTS

Faecal egg counts - F. hepatica eggs began to appear in faeces nine weeks after infection. There was a good deal of individual variation in egg counts between different animals. However, egg counts rose gradually from week 9 post-infection until the end of the experiment, showing no statistical differences between the immunized and infected control groups.

Fluke burdens - Results for fluke burdens in group 2 and 3 are shown in Table II. The mean number of flukes recovered was 92.0 ± 53.1 and 101.8 ± 61.9 in groups 2 and 3, respectively. No significant differences were found between groups 2 and 3. The fluke implantation rate was 46% and 50.9% in the infected control and immunized groups, respectively. It was remarkable that two goats in group 3 presented only 65 and 50 flukes, respectively, given the mean burden of this group.

Plasma levels of hepatic enzymes - Plasma levels of GLDH and GGT were within the normal range for the three groups prior to challenge (week 10). Three weeks after infection (week 13), GLDH levels were elevated in both infected groups until week 18, when a maximum level was reached (108.0 UI/L in group 2 and 108.5 UI/L in group 3), decreasing gradually until week 27. GGT increased in week 16, reached maximum levels in week 20 (213.7 UI/L in group 2 and 116.1 UI/L in group 3) and decreased quickly to baseline levels. No significant difference was noted between groups during the study.

Histopathology and morphometric study of the liver - Livers from infected animals showed superficial scars and whitish tortuous tracts, mainly involving the left hepatic lobe, whereas gross changes in the right and quadrate lobes were variable and generally less severe. Animals in groups 2 and 3 showed moderate to severe gross hepatic changes. Gall bladders and large superficial bile ducts were whitish in color and enlarged. The two animals from group 3 that died at four days post-infection (dpi) showed a few tiny whitish spots over the left hepatic lobe.

Hepatic histopathological changes consisted of fibrous perihepatitis, chronic tracts composed of an abundant infiltrate of macrophages containing brown pigment, fewer lymphocytes and plasma cells and fibrosis. In portal spaces, marked bile duct hyperplasia accompanied by fibrosis and inflammatory infiltration of eosinophils, lymphocytes and plasma cells were often found. Some animals also showed infiltration of globule

TABLE II

Results (mean ± standard deviation) of the fluke burdens, macroscopic and microscopic liver morphometric analyses in goats from group 2 (Quillaia A) and group 3 (Schistosoma mansoni fatty acid-binding protein 14)

<table>
<thead>
<tr>
<th>Group</th>
<th>Fluke burden</th>
<th>Gross damage</th>
<th>Microscopic damage</th>
<th>Bile duct</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>-</td>
<td>-</td>
<td>5.6 ± 0.9</td>
<td>0.84 ± 0.43</td>
</tr>
<tr>
<td>2</td>
<td>92.0 ± 53.0</td>
<td>50.02 ± 17.50</td>
<td>44.48 ± 26.82</td>
<td>5.76 ± 3.58</td>
</tr>
<tr>
<td>3</td>
<td>101.8 ± 61.9</td>
<td>21.70 ± 8.33</td>
<td>34.46 ± 10.18</td>
<td>4.33 ± 1.58</td>
</tr>
</tbody>
</table>

a: area occupied by portal spaces in group 1 and by portal spaces, inflammatory infiltrates, fibrosis and granulomas in groups 2 and 3; b: p < 0.001 comparing group 3 with 2.
leukocytes in the bile ducts. Lymphoid follicles with germinal centres were also observed in some portal spaces. Granulomas, containing an eosinophilic necrotic centre surrounded by macrophages, multinucleated giant cells, lymphocytes, plasma cells and fibrosis, were found in some animals. High individual microscopic variability was found in both infected groups. The two animals from group 3 dying at four dpi showed subcapsular granulomas containing numerous macrophages, some multinucleated giant cells, eosinophils and lymphocytes in the centre; refractile material similar to larval cuticula could be identified (Fig. 1).

Results of gross morphometric examination of animals in groups 2 and 3 are shown in Table II. Gross morphometric observation revealed that 50% ± 14.9 (group 2) and 21.7% ± 8.3 (group 3) of the liver surface was damaged. This difference was significant (p < 0.001). Gross liver damage showed a significant correlation (p = 0.01, R = -0.691) with experimental grouping. However, correlation between gross damage area and fluke burden was low (R = -0.06) and non-significant (p = 0.79).

Results of microscopic liver analysis are shown in Table II. The percentage of area occupied by portal spaces and damaged areas of hepatic parenchyma (chronic tracts, cholangitis with inflammatory infiltration, fibrosis and granulomas) revealed a marked increase in the two infected groups with respect to the area of portal spaces in the uninfected control group. The percentage of damaged area in groups 2 (44.48% ± 26.82) and group 3 (34.46% ± 10.18) showed no significant differences. The percentage of area occupied by bile ducts showed a very significant increase (p < 0.001) in the two infected groups with respect to the uninfected control group; this difference was slightly higher in group 2 (5.76% ± 3.58) compared to group 3 (4.33% ± 1.58), although the difference was not significant.

Statistical correlations were found between macroscopic and microscopic liver morphology (R = 0.477, p = 0.033) and between microscopic damage and bile duct hyperplasia (R = 0.623, p = 0.003).

Histopathology and morphometric study of the HLN - The mean weight (grams) of the HLN was 18 ± 2.79, 12.57 ± 4.68 and 8.5 ± 2.08 in the negative control, infected control and immunized group, respectively. A statistically significant increase in weight was found in the two infected groups compared to the negative control group (p < 0.05). However, no significant differences were found between infected controls and the immunized group (p = 0.80). Grossly, HLNs from all infected goats showed severe cortex and medullar hyperplasia.

Histopathological examination of the HLN revealed marked hyperplasia of lymphoid follicles and medullary cords in infected goats. Infiltration of eosinophils was variable in paracortical areas and the medulla of infected animals, demonstrated by haemosiderin pigment in the cytoplasm of variable numbers of medullary macrophages.

The percentage of cortex occupied by lymphoid follicles was 14.85 ± 2.79, 26.36 ± 9.36 and 27.44 ± 8.40 in groups 1, 2 and 3, respectively. Statistical analysis revealed a significant increase in the area occupied by lymphoid follicles in the two infected groups with respect to uninfected controls (p < 0.05), while no differences between the infected control and immunized group were observed (p > 0.05).

Immunohistochemical study of the liver - Results of immunohistochemical analysis of hepatic lesions are shown in Table III. Negative controls showed occasional positive cells.

All goats from the infected groups showed severe infiltration of CD2+, CD4+ and CD8+ cells, located primarily within portal spaces (Fig. 2). The ratio of CD4+/CD8+ cells was 1.34, 1.56 and 0.98 in groups 1, 2 and 3, respectively. Interestingly, there were occasional cells expressing IL-4+ and TCR+ in hepatic lesions from both infected groups. All infected animals showed a significant increase in all cell populations examined in comparison with uninfected controls (p < 0.001). In the rSm14-immunized group (group 3), infiltration of CD8+ and TCR+ (p < 0.05) as well as CD2+ and CD4+ (p < 0.001) cells was markedly reduced in comparison with infected controls (group 2). No significant difference was noted in IgG+ populations.

Immunohistochemical study of the HLN - The distribution of different cell subsets in HLN is summarized in Table III. Both infected groups showed a significant increase in all cell populations studied in comparison with uninfected controls (p < 0.001). In the follicular areas, the number of CD2+, CD4+, CD8+, IL-4+, IFN-γ+ and TCR+ cells were markedly lower (p < 0.001) in group 2 (infected control) than in group 3 (immunized). Similar results were observed in the medullary area for CD4+ and IgG+ cells (p < 0.05) as well as for CD2+, CD8+, IL-4+ and IFN-γ+ cells (p < 0.001). In the two infected groups, the number of IL-4+ cells was higher than the number of IFN-γ+ cells (Fig. 3).
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**Discussion**

Goats immunized with rSm14 in Quil A did not show reduction in fluke burdens with respect to the infected control group. In a previous study using a synthetic peptide derived from the Sm14 antigen adjuvanted with Ribi and Alum, the number of flukes was 45.9% lower than the infected control, although a high degree of individual variability was recorded (Zafra et al. 2008). Additionally, Sm14 in Ribi adjuvant with Alum reduced fluke burdens by between 51.7% and 100% in mice (Tendler et al. 1996, Ramos et al. 2001, Vilar et al. 2003) as well as in sheep (Almeida et al. 2003). In all of these experiments, significant hepatic damage was also reported in vaccinated mice, sheep and goats. Therefore, the results of the present study suggest that Quil A is not a suitable adjuvant for formulating vaccines against *F. hepatica* using the Sm14 antigen. Because the best results with this antigen were obtained using Ribi, which is based on Lipid A, as an adjuvant, Sm14 is being developed as an anti-*Fasciola/schistosome* vaccine in formulations that include Lipid A-derived adjuvants.

In the present paper, implantation rates (46% and 50.9%) in the infected control and immunized group, respectively, were higher than reported in previous studies in goats (Martínez-Moreno et al. 1997, 1999, Zafra et al. 2008). However, in some experimental infections, implantation rates varying between 42-51% have been reported in sheep (Almazán et al. 2001, Ramajo et al. 2001).

Sinclair (1967) reported that after 90 h of infection, the first larvae reached the liver. Additionally, Presidente et al. (1974) reported that on the 7th dpi, many pale gray,

### Table III

Mean ± standard deviation of T cell subsets per field (0.2 mm²) in the liver and hepatic lymph nodes of goats from group 1 (negative control), 2 (Quillaia A) and 3 (*Schistosoma mansoni* fatty acid-binding protein 14)

<table>
<thead>
<tr>
<th>Group</th>
<th>Area</th>
<th>CD2</th>
<th>CD4</th>
<th>CD8</th>
<th>TCR</th>
<th>IgG</th>
<th>IFN-γ</th>
<th>IL-4</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>L</td>
<td>8.86 ± 1.8</td>
<td>6.2 ± 1.8</td>
<td>4.63 ± 2.41</td>
<td>0.3 ± 0.1</td>
<td>2.1 ± 0.9</td>
<td>0.06 ± 0.01</td>
<td>0.1 ± 0.03</td>
</tr>
<tr>
<td>C</td>
<td>38.7 ± 8.2</td>
<td>32.9 ± 6.7</td>
<td>14.7 ± 3.1</td>
<td>2.1 ± 0.4</td>
<td>2.3 ± 0.3</td>
<td>0.2 ± 0.01</td>
<td>0.02 ± 0.01</td>
<td></td>
</tr>
<tr>
<td>M</td>
<td>117.5 ± 33.2</td>
<td>69.5 ± 11.4</td>
<td>30.5 ± 7.6</td>
<td>12.4 ± 3.3</td>
<td>5.6 ± 1.2</td>
<td>0.01 ± 0.01</td>
<td>0.03 ± 0.04</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>L</td>
<td>525.7 ± 29.0</td>
<td>246.7 ± 55.3</td>
<td>157.7 ± 26.5</td>
<td>16.9 ± 6.6</td>
<td>33.1 ± 22.5</td>
<td>0.4 ± 0.5</td>
<td>2.8 ± 1.9</td>
</tr>
<tr>
<td>C</td>
<td>1359.1 ± 141.6</td>
<td>699.1 ± 124.8</td>
<td>502.0 ± 90.1</td>
<td>565.3 ± 199.8</td>
<td>39.1 ± 9.2</td>
<td>5.5 ± 3.3</td>
<td>24.2 ± 8.6</td>
<td></td>
</tr>
<tr>
<td>M</td>
<td>782.7 ± 43.2</td>
<td>297.9 ± 50.1</td>
<td>370.0 ± 41.6</td>
<td>224.7 ± 95.5</td>
<td>383.4 ± 42.6</td>
<td>4.9 ± 4.3</td>
<td>19.6 ± 2.7</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>L</td>
<td>301.2 ± 104.9</td>
<td>100.6 ± 63.0</td>
<td>56.8 ± 35.6</td>
<td>5.6 ± 2.4</td>
<td>14.2 ± 10.9</td>
<td>0.2 ± 0.4</td>
<td>1.8 ± 1.8</td>
</tr>
<tr>
<td>C</td>
<td>829.4 ± 50.5</td>
<td>368.2 ± 47.2</td>
<td>251.4 ± 54.6</td>
<td>15.8 ± 12.6</td>
<td>52.8 ± 16.0</td>
<td>0.6 ± 1.1</td>
<td>2.6 ± 1.8</td>
<td></td>
</tr>
<tr>
<td>M</td>
<td>510.0 ± 68.3</td>
<td>198.3 ± 50.1</td>
<td>150.6 ± 38.8</td>
<td>14.0 ± 3.4</td>
<td>602.9 ± 177.1</td>
<td>0.50 ± 0.84</td>
<td>1.0 ± 1.7</td>
<td></td>
</tr>
</tbody>
</table>

*a*: p < 0.05 comparing group 3 with 2; *b*: p < 0.01 comparing group 3 with 2; C: cortical areas of hepatic lymph nodes (HLN); L: liver; M: medullar areas of HLN; statistical analyses revealed significant differences (p < 0.001) in group 2 and 3 compared with group 1, for all tested antibodies.

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Fig. 2: liver. Portal space showing bile duct surrounded by abundant inflammatory infiltration composed mainly of CD2⁺ (A, B), CD4⁺ (C, D) and CD8⁺ (E, F) T lymphocytes (arrow); group 2 (B, D, F) and group 3 (A, C, E). Avidin-biotin-peroxidase method, haematoxylin counterstain. Bar = 50 μm.
irregularly shaped areas, 4-12 mm in size, were found on the ventral half of the left and middle lobes. In this study, the two goats from group 3 dying at four dpi showed a few scars consistent with larval migratory lesions in the liver parenchyma. Moreover, granulomas containing cuticular debris surrounded by macrophages, eosinophils and lymphocytes indicate that the host response may have resulted in the death of some of the larvae reaching the liver parenchyma. However, results of fluke burdens indicated that only a small number of the larvae reaching the liver had died during the migratory stage, showing no significant differences with respect to the control group. It is of interest to investigate the local host immune response during early post-infection stages to determine the pattern of the host response in immunized and non-immunized animals due to the fact that protective response occurs during early post-infection stages in laboratory animals (Kesik et al. 2007).

Evaluation of hepatic damage in experimental fasciolosis has traditionally been assessed by visual inspection, conventional histopathology, scoring results by crosses or numbers, or even by serum levels of hepatic enzymes (Meeusen et al. 1995, Dalton et al. 1996, Martínez-Moreno et al. 1999, Mulcahy & Dalton 2001, Raadsma et al. 2008). These methods are subjective and do not allow correlation with other factors. Recently, a morphometric study was conducted to evaluate hepatic damage in goats infected with *F. hepatica* (Zafra et al. 2008).

Gross morphometric examination of the liver showed that lesions were significantly reduced in the immunized group with respect to the infected control, despite the fact that the number of flukes in both groups was quite similar. This result could be due to a lower capacity of larvae to induce hepatic damage in the immunized goats or to the random migration of larvae. In some groups, large numbers of flukes may have randomly migrated thorough the left hepatic lobe leaving the rest of the liver virtually unaffected, while other animals with lower number of flukes presented numerous scars in both hepatic lobes. This finding may explain the lack of correlation between gross hepatic damage and fluke burdens, which was also reported in a previous trial in goats (Zafra et al. 2008, Pérez-Écija et al. 2009).

In the present study, a total of 85 mm$^2$ of liver per animal were evaluated using 10 randomly selected photomicrographs from four tissue samples, an area higher than that evaluated by Coutinho et al. (2007) and Vizzotto et al. (2002) who used 4.9 mm$^2$ and 1.3 mm$^2$, respectively. The statistical correlation between the macroscopic and microscopic morphometric liver analyses and between microscopic damage and bile duct hyperplasia corroborate the idea that the sampling method used in the microscopic study was representative.

The marked enlargement of the HLN in the two infected groups with respect to uninfected controls suggests a strong immune response to *F. hepatica* infection. Histopathological and morphometric evaluation revealed that HLN hyperplasia was due mainly to hyperplasia of lymphoid follicles and medullary cords, suggesting evident robust humoral immune response, which agrees with experiments performed in sheep (Meeusen et al. 1995, Pérez et al. 2002) and goats (Martínez-Moreno et al. 1999, Zafra et al. 2009). The high infiltration of CD2$, CD4$ and CD8$^+$ cells into the liver and HLN in this study suggests a strong local cellular immune response against *F. hepatica*. These results agree with those previously reported in sheep (Meeusen et al. 1995, Chauvin & Boulard 1996, Pérez et al. 2002, 2005) and goats (Martínez-Moreno et al. 1999, Pérez et al. 1999, Zafra et al. 2008) chronically infected with *F. hepatica*. The increase in IgG$^+$ plasma cells in the liver and HLN of the two infected groups agrees with the increase in serum levels of IgG anti-rSm14 found in group 3. However, as in previous infections in sheep and goats, the host immune response in the two infected groups was non-protective as indicated by similar fluke burdens in both groups.

Reduced hepatic infiltration of CD2$, CD4$ and CD8$^+$ T lymphocytes observed in group 3 (rSm14) compared to group 2 (Quil A) could be related to the lower hepatic damage provoked rather than the number of flukes. Similar results were recently found in goats immunized with a synthetic peptide derived from the Sm14 antigen (Zafra et al. 2009), suggesting that evaluation of the local response during early post-infection stages is important to discriminate between the host response against tissue damage caused by migrating larvae and host response against migratory larvae.

Bovine anti-IL4 and anti-IFN-γ positively stained cells in the hepatic infiltrate and lymphocytes in HLN in the present study, confirming that both antibodies cross-reacted with caprine tissues as the supplier reported. To date, expression of these antigens has only been successfully used for immunohistochemistry in fasciolosis in HLN (Zafra et al. 2009), although tissue expression...
of cytokines has been used to evaluate tissue response profiles in cattle using the antibodies used in the present study (Johnson et al. 2006). The low expression of IFN-γ and higher expression of IL-4, particularly in the HLN, suggest a Th2-polarised response, a finding also reported in chronic F. hepatica infections in goats (Zafra et al. 2009), cattle (Waldvogel et al. 2004) and sheep (Zhang et al. 2005).

Despite the similarity of fluke burdens, we observed a significant reduction in both gross hepatic damage and local inflammatory infiltrates in animals immunized with Sm14 in Quil A. We speculate that a reduced immune response induced in immunized animals was responsible for this action. This effect could be due to inhibited function of Sm14 in the parasite, compromising the uptake, transport and compartmentalization of host-derived fatty acids due to the organized immune response against this protein. This response was not able to kill the parasite per se, however it produced a desirable result in an immunization-based control program, which could be improved with further studies.

In conclusion, goats immunized with rSm14 in Quil A and subsequently infected with F. hepatica did not show any reduction in fluke burdens and faecal egg counts, but gross hepatic lesions were significantly reduced in the immunized group with respect to the infected control group. A strong local immune response, represented by infiltration of Th2-polarised CD2+, CD4+ and CD8+ T lymphocytes, was recorded in the liver and HLN of both infected groups and was more severe in the infected control group than in the immunized group. However, in both groups, the host response was non-protective. Further studies are needed to investigate the nature of the local immune response in the context of new vaccine formulations in a Lipid A-based adjuvant to evaluate the potential usefulness of Sm14 antigen as a vaccine for fascioliasis in ruminants.

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