LOCAL IMMUNE RESPONSE TO EXPERIMENTAL *FASCIOLA HEPATICA* INFECTION IN SHEEP

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Summary :

Macrophages, eosinophils, neutrophils and lymphocyte subpopulations (OvCD5+, OvCD4+, OvCD8+, OvWC1+ and Ig+) were identified in sections of hepatic tissue and hepatic lymph nodes from sheep experimentaly infected with *F. hepatica* and necropsied 14, 28, 42 or 56 days post infection. The migratory tunnels produced by juvenile flukes appeared as focal areas of necrosis surrounded by infiltrating inflammatory cells, particularly numerous macrophages, eosinophils and OvCD4+ lymphocytes. In addition, B lymphocyte proliferation was observed in hepatic lymph nodes and in hepatic portal tracts. Only three juvenile flukes were identified in the sections. They where partially in contact with healthy tissue and partially with neutrophils, macrophages and eosinophils; they were covered by IgM. Host-parasite interactions resulting from immune response regulation by helper T lymphocytes and from immune evasion by the parasite are discussed.

KEY WORDS : *Fasciola hepatica*, sheep, lymphocytes subpopulations, immunohistochemistry.

Résumé : RÉPONSE IMMUNITAIRE LOCALE CHEZ LE MOUTON INFESTÉ EXPÉRIMENTALEMENT PAR *FASCIOLA HEPATICA*

Les macrophages, les éosinophiles, les neutrophiles et les sous-populations lymphocytaires (OvCD5+, OvCD4+, OvCD8+, OvWC1+ et Ig+) ont été identifiés dans des coupes de tissu hépatique et de nœud lymphatique hépatique (NLH) de moutons infestés expérimentalement par F. hepatica *et autopsiés 14, 28, 42 et 56 jours après l'infestation. Les lésions provoquées par les parasites en migration sont constituées d'un centre nécrotique entouré essentiellement de macrophages, d'éosinophiles et de lymphocytes OvCD4+. Par ailleurs, une forte prolifération de lymphocytes B a été observée dans le NLH et les espaces portes hépatiques. Seules, trois douves immatures ont pu être mises en évidence ; elles étaient en partie au contact de tissu sain et en partie au contact de neutrophiles, de macrophages et d'éosinophiles. De plus, elles étaient couvertes d'IgM. Les possibilités d'interactions hôte-parasite résultant d'une régulation de la réponse anti-*F. hepatica *par des lymphocytes T helper et des mécanismes d'échappement du parasite sont discutées.*

MOTS CLÉS : Fasciola hepatica, *mouton, sous-populations lymphocytaires, immunohistochimie.*

INTRODUCTION

After primary infection of sheep or cattle, *Fasciola hepatica* migrates in the hepatic parenchyma from the first week post-infection (WPI 1) to WPI 8. During this migration period, both natural (cattle, sheep) and experimental (rat) hosts develop a cellular response against the parasite. In particular, *in vitro* lymphocyte proliferation responses against *F. hepatica* antigens occur between WPI 2 to 5 (Oldham, 1985; Oldham & Williams, 1985; Poitou *et al.*, 1992; Chauvin *et al.*, 1995) and then decrease. In addition, in sheep, a dramatic systemic eosinophilia occurs with peaks in WPI 4-5 and WPI 9-11 (Chauvin *et al.*, 1995). To be effective during this early migration, the host defense mechanisms should occur in the hepatic parenchyma around the young flukes. Various studies in sheep (Rushton, 1977; Rushton & Murray, 1977; Meeusen *et al.*, 1995), cattle (Dow *et al.*, 1967; Doy & Hughes, 1984) or mice (Masake *et al.*, 1978) have showed that juvenile flukes induce granulomatous lesions in hepatic parenchyma with numerous macrophages, lymphocytes and eosinophils. Furthermore, Meeusen *et al.* (1995) observed that 10 days after infection, the lymphocytes are primarily OvCD4+ cells, but that four months post infection OvCD8+ cells became more numerous. Similarly, Poitou *et al.* (1993) observed a marked decrease in $CD8^+$ cells in rat spleens from WPI 2 to WPI 6 followed by a return to control levels in WPI 7, 8 and 9, when the flukes reached the bile ducts.

The purpose of the present study was to describe the kinetics of cellular responses (macrophages, eosinophils, lymphocytes subpopulations) between WPI 2 and WPI 8 in hepatic parenchyma around juvenile flukes and in the migratory tunnels and to investigate the immune response in hepatic lymph nodes (HLN).

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MATERIAL AND METHOD

Experimental design

Twenty Vendéen castrated male sheep, approximately six months old, were randomized in five groups: C1, C2, C3, C4 and C5. Group C1 were control animals. Sheep from Groups C2, C3, C4 and C5 were orally infected with 200 metacercariae per animal. Animals of Group C2 were necropsied 14 days post-infection (DPI 14), those of Group C3 at DPI 28, those of Group C4 at DPI 42, and those of Group C5 at DPI 56. One animal from Control Group was also necropsied at DPI 14, DPI 28, DPI 42 and DPI 56.

Necropsy and histological preparation

Sheep were killed by intravenous sodium pentobarbital. The livers and the hepatic lymph nodes were immediatly removed. Several 1 cm liver cubed samples of liver tissue were dissected from the macroscopically visible lesions in each sheep, embedded in OCT compound (Tissue-tek; Miles, USA) and rapidly immersed in isopentane cooled by liquid nitrogen. The hepatic lymph nodes were cut into small pieces and similarly embedded and frozen. Healthy hepatic tissues and HLN samples were recovered from control animals and processed in the same way. All samples were stored at – 80 °C until further processing. For each animal, 7-8 µm thick sections of two to four liver samples and of one hepatic lymph node sample were serially sectioned using a cryostat (Rua, France), placed on clean slides and air dried overnight.

Staining

Eosinophils and macrophages were identified on hemalum-eosin (HE) or May-Grünwald Giemsa (MGG) stained sections. Lymphocyte subpopulations were characterized by immunohistochemistry with monoclonal antibodies (MAb) against Ovine Cluster of Differentiation (OvCD, Naessens, 1993).

T lymphocytes were identified using MAbs purchased from Professor M.R. Brandon, Melbourne University, Australia: an anti-OvCD5 (MAb 25-91, Keech & Brandon, 1991 *a*); an anti-OvCD4 (MAb 44-38 and 44-97, Hopkins, 1991, Hopkins *et al.*, 1993) and an anti-OvCD8 (MAb 36-65, Keech & Brandon 1991 *b*). A MAb against Workshop Cluster 1 (WC1) (MAb 19-19) was also used; this WC1 is a membrane protein, previously described as T19, present on 90 % of T lymphocytes expressing the γδ chains of the T Cell Receptor (Mackay *et al.*, 1991). B lymphocytes were identified by the presence of cell surface Ig using an anti-Ig Light Chain MAb (Serotec Realeff, France). Because of high background staining of hepatic parenchyma with this antiboby, an anti-IgM MAb (Serotec Realeff, France) was used as well. All MAb solutions were culture supernatants.

For immunohistochemical staining, the technique previously described by Cordell *et al.* (1984), as modified by Pépin *et al.* (1992), was used. Tissues sections were fixed in acetone at –20 °C for 10 min, air dried for 20 min and rehydrated for 15 min with Tris-buffered saline (TBS, 0.05 M Tris, 0.15M NaCl, pH 7.6). Sections were incubated with MAb for 30 min in a humid chamber (1:20 dilution in TBS for anti-Ig Light Chain and 1:6 dilution for the other MAb solutions). After three washes in TBS, sections were covered with a 1:30 dilution of rabbit anti-mouse Ig (Dako, Denmark) for 30 min in a humid chamber. After three washes in TBS, sections were incubated with a 1:60 dilution of Alkaline Phosphatase anti-Alkaline Phosphatase complex (APAAP; Dako, Denmark) for 30 min in a humid chamber. After washing twice in TBS and twice in Tris HCl 0.1M pH 8.2, the slides were stained with the filtered substrate [20 mg Naphtol AS-TR phosphate (Sigma), 2 ml dimethylformamide (Prolabo), 30 µg levamisole (Sigma), 100 ml Tris HCl 0.1M pH 8.2 and 100 mg Fast Red TR salt (Sigma)] for 20 min. Slides were washed in water for 10 min, counterstained with hematoxylin (Sigma) for 30 to 60 sec and mounted in glycerol gelatin.

RESULTS

Hepatic lymph node

The HLN from each control animal was macroscopically normal. Microscopic examination showed no abnormalities except an erosion of the capsule which probably was a processing artefact. In infected sheep, HLN hypertrophy was observed in three of four animals of group C2 and in all animals of Groups C3, C4 and C5. In all cases of HLN hypertrophy, we observed numerous microscopic follicles in the cortical zone which distorted the paracortical zone and the medulla. The follicles were composed of numerous B lymphocytes (Ig Light Chain+ and IgM+) and, in the center, some OvCD5+, OvCD4+ cells. T lymphocytes in the paracortical zone were either OvCD4+ or OvCD8+. OvWC1+ T lymphocytes were less numerous. In the medulla, B lymphocytes (Ig Light Chain+ and IgM+) and OvCD4+, OvCD8+ or OvWC1+ T lymphocytes were observed. Numerous eosinophils were also present.

Hepatic parenchyma

There was no evidence of macroscopic or microscopic liver damage in control animals. Using immunohistochemical staining, OvCD4+ or OvCD8+ T lympho-

cytes were distributed throughout the hepatic parenchyma. Few OvWC1+ T lymphocytes were detected.

In infected animals, numerous macroscopic, tortuous migratory tracts were observed, particularly in the right lobe of the liver towards the diaphragm. Flukes were present in the bile ducts of the animals necropsied at DPI 56. On microscopic examination, only three juvenile flukes were observed in hepatic parenchyma (in two animals at DPI 28 and in one animal at DPI 56). Microscopic examination of the hepatic parenchyma around these juvenile flukes revealed similar changes. The parasites were partially surrounded by an infiltration of inflammatory cells (Fig. 1 *a*), particularly macrophages, neutrophils and eosinophils (Fig. 1 *b*). Immunohistochemical staining showed that OvCD5+, OvCD4+ lymphocytes were present only at the periphery of the inflammatory infiltrate (Fig. 1 *d*). Some IgM+ cells were observed in the inflammatory infiltrate. The tegument of all three juvenile flukes were covered by IgM (Fig. 1 *c*).

The most striking feature of the hepatic parenchyma was the numerous migratory tunnels caused by juvenile flukes. In microscopic examination they appeared as focal areas of necrosis containing hepatocytes and eosinophils, surrounded by an area of infiltrating inflammatory cells (Fig. 2 *a*), particularly many macrophages, eosinophils and lymphocytes (Fig. 2 *b*). Eosinophils and lymphocytes were located primarily at the periphery of the lesions and were particularly numerous adjacent to the portal tracts. Some IgM+ cells were present in the area of infiltration, and IgM was present in the necrotic core (Fig. 2 *c*). OvCD5+ lymphocytes (Fig. 2 *d*) were numerous in the periphery of the lesion; they were primarily of the OvCD4+ subpopulation (Fig. 2 *e*). Few OvCD8+ lymphocytes were observed (Fig. 2 *f*).

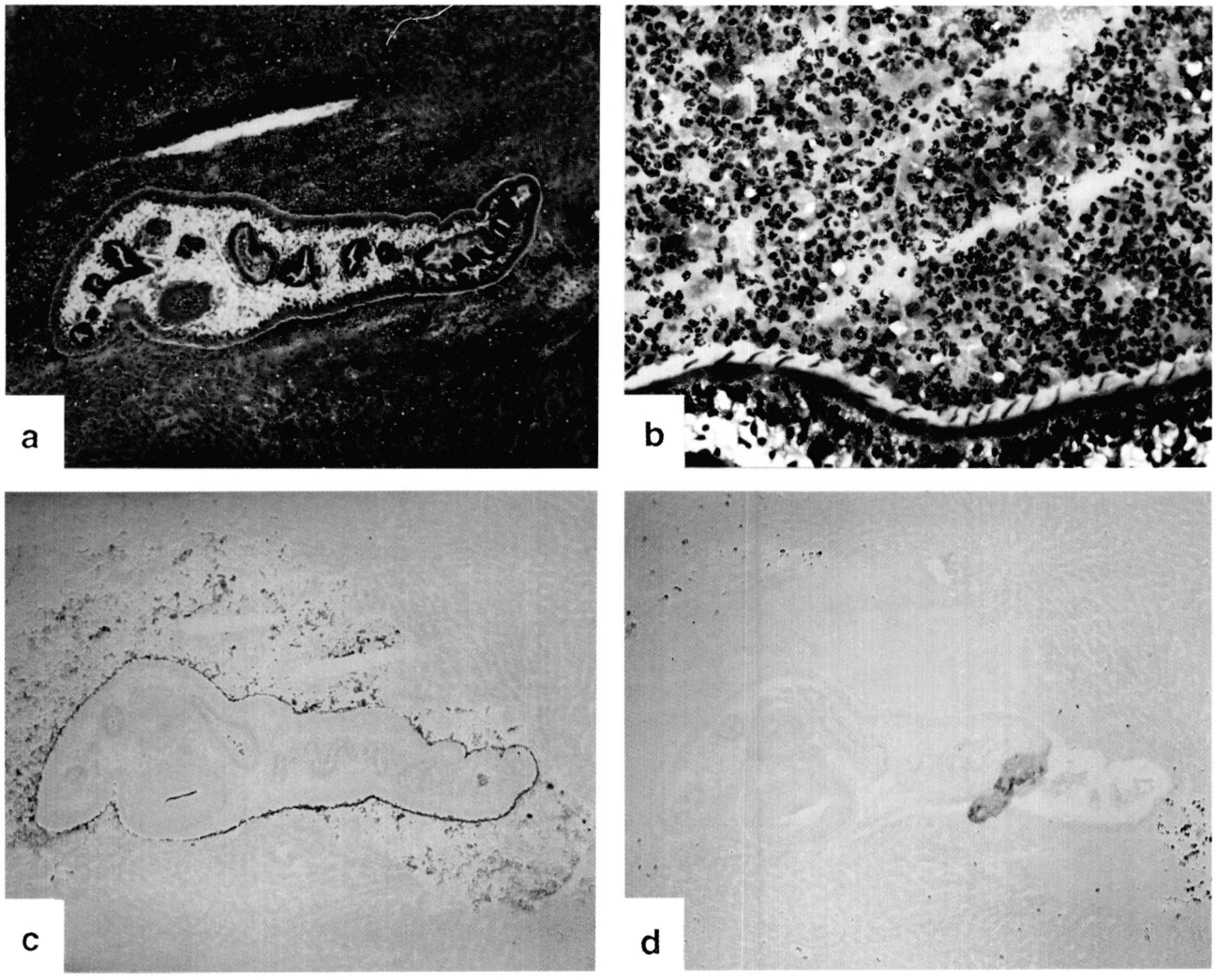

Fig. 1. – Four week old juvenile fluke in hepatic parenchyma. (*a*) Hemalum-Eosine (× 6), (*b*) May-Grünwald Giemsa (× 25), (*c*) IgM+ (× 6), (*d*) OvCD5+ (× 6).

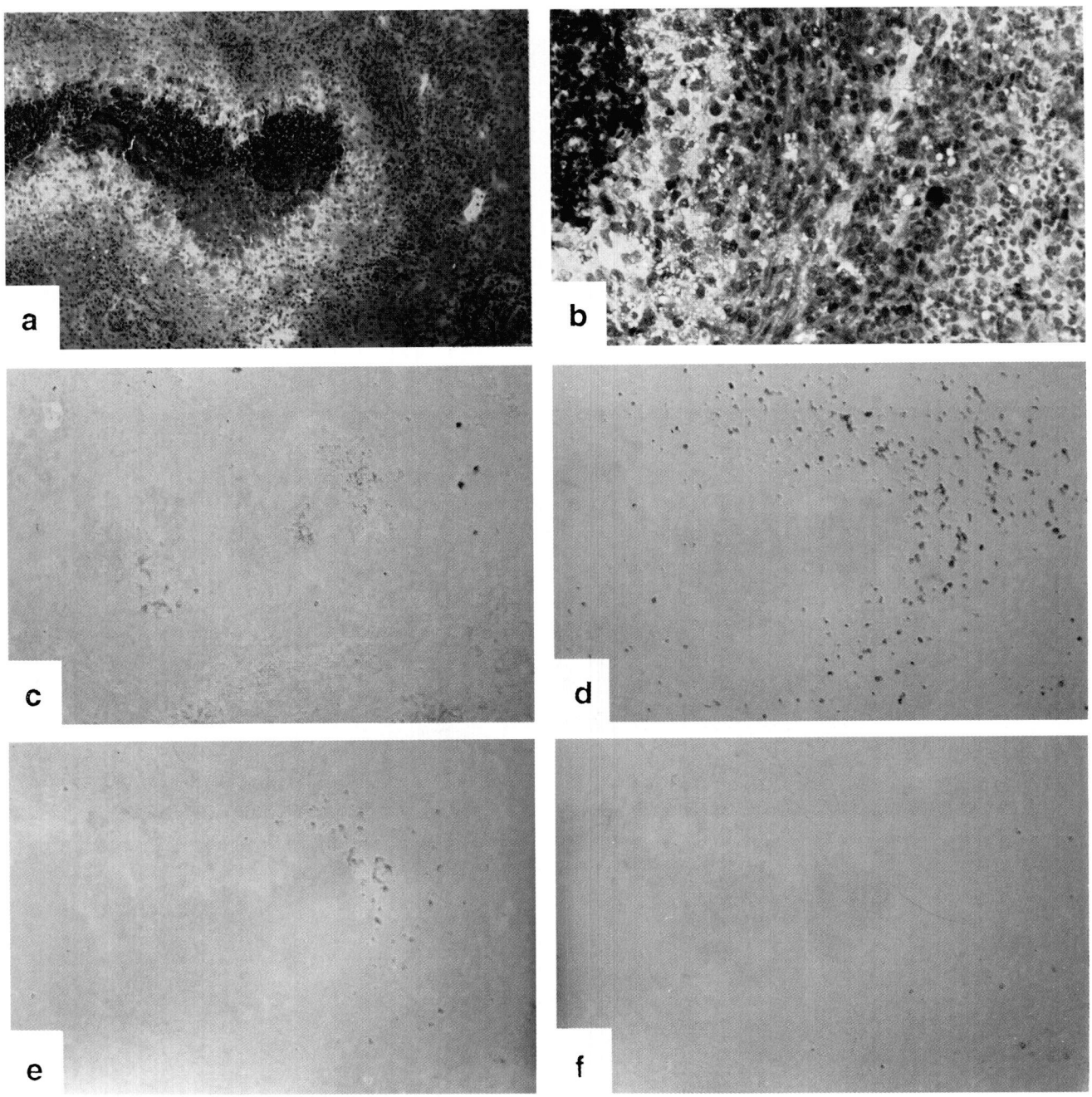

Fig. 2. – Granulomatous lesion in hepatic parenchyma at DPI 28. (*a*) Hemalum-Eosine (× 13), (*b*) May-Grünwald Giemsa (× 40), (*c*) IgM^+ (× 13), (*d*) $OvCD5^+$ (× 13), (*e*) $OvCD4^+$ (× 13), (*f*) $OvCD8^+$ (× 13).

The structure of these granulomatous lesions was similar in all infected sheep but their size increased from DPI 14 to DPI 56. Also in all infected animals, portal tracts were infiltrated by numerous eosinophils and T lymphocytes, notably OvCD4+ cells. In addition, in some portal tracts of animals necropsied in DPI 42 and 56, lymphoid follicles were observed with a central core of B lymphocytes (IgM+) surrounded by T helper lymphocytes (OvCD5+, OvCD4+). In one animal of group C5, a granuloma with a fibrous capsule was also present. In this case, OvCD8+ lymphocytes seemed more numerous and some OvWC1$^+$ cells were present within the granuloma.

DISCUSSION

Only three juvenile flukes were observed in the sample of hepatic parenchyma from infected sheep examined histologically although numerous migratory lesions were observed. Meeusen *et al.* (1995) detected several young flukes in the first few centimeter under the liver capsule ten days after primary infection, but did not observe juvenile flukes in the parenchyma ten days after a secondary infection, during which flukes are believed to migrate more rapidly (Sandeman & Howell, 1981; Meeusen *et al.*, 1995; Chauvin *et al.*, 1995). In this experiment, used samples were dissected only from liver lesions, from which the juvenile flukes may have migrated into healthy tissue. The responses of the sheep at DPI 28 and DPI 56 seem similar to those observed by Meeusen *et al.*, 1995; Chauvin *et al.*, 1995). In this experiment, used samples were dissected only from liver lesions, from which the juvenile flukes may have migrated into healthy tissue. The responses of the sheep at DPI 28 and DPI 56 seem similar to those observed by Meeusen *et al.* (1995), who found no leucocyte infiltration around juvenile flukes 10 days after primary infection. In our experiment, at DPI 28 and 56, juvenile flukes were in contact with the leucocyte infiltration but also in contact with healthy parenchyma. In addition the T lymphocytes were located only at the periphery of the leucocyte infiltration. This suggests that *F. hepatica* may depress the local inflammatory and immune responses facilitating its migration through healthy parenchyma. Baéza *et al.* (1994) noted an early reduced systemic inflammatory response in rats infected with *F. hepatica.*

Local immune responses can be correlated with general immune responses. For example, many authors have described parasite-specific antibodies to *F. hepatica* produced soon after experimental infection of sheep. In the present experiment, there was an early and marked development of lymphoid follicles in the hepatic lymph nodes and in the hepatic parenchyma at DPI 42 and 56. Poitou *et al.* (1993) observed an increase in B lymphocyte numbers in the spleen of experimentally infected rats. An early and intense local eosinophilic infiltration was observed both in our experiment and 10 days post-infection by Meeusen *et al.* (1995); it also seems to be correlated with the systemic eosinophilia previously described by Chauvin *et al.* (1995). The origin of this local and systemic eosinophilia has been attributed to an « Il5-like » effect of the *F. hepatica* excretory-secretory products (FhESP) by Milbourne & Howell (1990, 1993), who observed a systemic eosinophilia in rats injected with FhESP, as well as *in vitro* eosinophil differentiation of bone marrow cells cultured with FhESP. It is possible that this « Il5-like » phenomenon is induced by an « Il5-like » substance produced by *F. hepatica* or by the secretion of Il5 by T helper lymphocytes stimulated by parasitic antigens. The role of T lymphocytes in eosinophilia induction was previously demonstrated by Flagstadt *et al.* (1972), who was unable to detect an eosinophilia following experimental infection with *F. hepatica* in T lymphocyte-deficient animals.

In granulomatous lesions, the inflammatory response is regulated by lymphocytes which circulate between tissue and blood. During fasciolosis, the dominant subpopulation of T lymphocytes between DPI 14 and 56, in both hepatic parenchyma and hepatic portal tracts consisted of OvCD4+ lymphocytes. Meeusen *et al.* (1995) demonstrated a similar pattern 10 days post-infection but subsequently observed, four months after infection, that OvCD8+ cells became more numerous. Poitou *et al.* (1993) demonstrated that the number of CD8+ cells was decreased in infected rat spleen during the hepatic migration of juvenile flukes and returned to normal level in WPI 7, 8 or 9, when flukes reached the bile ducts (which occurs sooner than in ruminants). These data suggest that the local immune response is regulated by OvCD4+ lymphocytes during the hepatic migration of juvenile flukes, and that immune regulation is different when adult flukes are present. The different observed phenomena, particularly a high number of Th lymphocytes, dramatic eosinophilia, B cell proliferation and high levels of antibody production, notably IgE, observed in rats by Pfister (1983) and Poitou *et al.* (1993), suggest a Th2 regulation of the immune response to *F. hepatica* as proposed by Brown *et al.* (1994). These authors observed that BoCD4+ T cell clones, isolated from infected cattle and stimulated with *F. hepatica* antigens, secreted cytokines with Th0 or Th2 profiles. The low number of OvCD8+ cells observed in our study is also a characteristic of Th2 regulated immune responses (Gajewski & Fitch, 1991).

Such a Th2 regulation of the immune response to *F. hepatica* is compatible with the development of an

Antibody Dependent Cell Cytotoxicity (ADCC), frequently suggested as an effector mechanism against tissue helminths. Some mechanisms of immune evasion to ADCC have already been described in *F. hepatica*. In particular, this parasite releases a cathepsin-like protease (Chapman & Mitchell, 1982; Carmona *et al.*, 1993) which cleaves immunoglobulins. In addition, Duffus & Francks (1980) and Hanna (1980) described a rapid turn-over of the outer glycocalyx of juvenile flukes. In our experiment, juvenile flukes were found to be covered by IgM. While eosinophils do not express the Fcμ receptor (McEwen, 1992), IgM deposition on fluke tegument could inhibit eosinophil adhesion. This has been observed with *F. hepatica* by Glauert *et al.* (1985). Further studies on Ig isotypes secreted around juvenile flukes are necessary to explore this antibody-blocking evasion mechanism, which has also been described in schistosomes (Capron *et al.*, 1987; Dunne *et al.*, 1987). While *F. hepatica* seems able to evade ADCC mechanisms, which may be regulated by Th2 cytokines, further studies are needed to explore the role of the parasite in regulation of the immune response. Particularly, since Th1/Th2 balance seems to be essential in determining parasite rejection or maintenance (Sher *et al.*, 1992), the role of *F. hepatica* in the differentiation of T lymphocytes should be further investigated.

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