Early hepatic immune response in rats infected with Fasciola hepatica

O. Tliba, Nathalie Moiré, Yves Le Vern, C. Boulard, A. Chauvin, Pierre Sibille

Vet. Res. 33 (2002) 261–270

DOI: 10.1051/vetres:2002014

Original article

Early hepatic immune response in rats infected with *Fasciola hepatica*

Omar TLIBA[a], Nathalie MOIRE[a], Yves LE VERN[b], Chantal BOULARD[a], Alain CHAUVIN[c], Pierre SIBILLE[a]*

[a]Unité de Pathologie Aviaire et Parasitologie, INRA, 37380 Nouzilly, France
[b]Service commun de Cytométrie de Flux, INRA, 37380 Nouzilly, France
[c]Unité associée INRA/ENVN Interactions hôte-parasite-milieu, École Nationale Vétérinaire de Nantes, BP 40706, 44307 Nantes Cedex 03, France

(Received 7 September 2001; accepted 7 January 2002)

Abstract – We investigated the phenotype of the T cells ($CD4^+$ and $CD8^+$) that produced Th1 (IFN-γ) and Th2 cytokines (IL-4 and IL-10) during the first two weeks of experimental fasciolosis in rats. We also followed the kinetics of the cytokine and proliferative responses of hepatic mononuclear cells (HMNC) over the same period. We found that HMNC were more numerous in the infected animals than in the controls. The percentage of $CD4^+$ cells increased significantly after infection, whereas the percentage of $CD8^+$ cells did not change. Moreover, the frequency of the cells producing (CP) cytokine changed after infection. The frequency of CP IFN-γ on 7 days postinfection (pi) was similar to that in control animals. However, the frequency of CP IFN-γ was clearly lower on day 14 pi, whereas the frequency of CP IL-4 and CP IL-10 had increased. The CP IL-10-were mostly $CD4^+$. Mitogenic stimulation (phorbol myristate acetate/ionomycin) of HMNC led to an increase in the amounts of the Th2 cytokines in the supernatant on days 7 and 14 pi, with the increase more pronounced on day 14. In contrast, IFN-γ levels also increased by day 7 pi but then decreased to below control levels by day 14. In addition, HMNC proliferation in response to mitogen followed a similar pattern to IFN-γ production. These findings suggested that, during the first 2 weeks of infection, *F. hepatica* induced a transient Th0 cytokine profile followed by downregulation of the cellular response and the induction of a Th2 cytokine profile.

***Fasciola hepatica* / rat / cytokine / liver / lymphocytes**

Résumé – Réponse immunitaire hépatique précoce chez le rat infesté par *Fasciola hepatica*. Nous avons caractérisé le phénotype des sous-populations lymphocytaires T ($CD4^+$, $CD8^+$) qui produisent des cytokines de type Th1 (IFN-γ) et Th2 (IL-4, IL-10) au cours de l'infestation expérimentale par *F. hepatica* chez le rat (phase précoce). Nous avons aussi suivi la production des cytokines et

*Correspondence and reprints
Tel.: (33) 2 47 42 77 47; fax: (33) 2 47 42 77 74; e-mail: sibille@tours.inra.fr

la réponse proliférative des cellules mononucléaires hépatiques (CMNH) après une stimulation in vitro par des mitogènes (phorbol myristate acetate) et par des antigènes spécifiques (les produits d'excrétion-sécrétion de *F. hepatica*). Le pourcentage des cellules $CD4^+$ augmente après infestation tandis que celui des cellules $CD8^+$ demeure inchangé. Après infestation, la fréquence des cellules productrices (CP) de cytokine change. Tandis qu'au 7e jour après infestation (JAI) la fréquence des CP d'IFN-γ reste identique à celles des animaux témoins, elle est nettement plus basse au 14e JAI ; la fréquence des CP d'IL-4 et des CP d'IL-10 augmente (surtout les $CD4^+$ $IL\text{-}10^+$, notamment au 14e JAI). En outre, dans les surnageants de culture des CMNH, la production de cytokines de type Th2 est accrue au 7e JAI et plus importante encore au 14e JAI. Le niveau de production d'IFN-γ augmente au 7e JAI mais décroît jusqu'à devenir inférieure à celle du témoin au 14e JAI. De plus, la réponse proliférative est plus importante au 7e JAI mais est réduite au 14e JAI. L'ensemble de ces résultats montre que durant la période précoce de l'infestation, le parasite induit une réponse transitoire de type Th0 (au 7e JAI), suivie d'une réduction de la réponse cellulaire et d'une induction d'une réponse de type Th2 (au 14e JAI).

***Fasciola hepatica* / rat / cytokine / foie / lymphocyte**

1. INTRODUCTION

Fasciola hepatica is a trematode parasite that induces both acute and chronic fasciolosis in livestock, resulting in considerable financial losses to farmers. The main pathogenic event in fasciolosis is the migration of juvenile flukes in the liver. During this migration, the migratory tracts are surrounded by an extensive local inflammatory reaction. This inflammation persists for the first six weeks of infection and involves the massive recruitment of eosinophils [26]. Hepatic lymphocytes are uniformly distributed throughout uninfected livers but are recruited to the infected areas and portal spaces following infection.

It is well accepted that the pattern of T-cell cytokine production determines the type and magnitude of the immune response [20]. The release of cytokines by lymphocytes is triggered by antigen recognition, which in turn initiates and regulates cell-to-cell communication. Granuloma formation, Ig isotype and the massive eosinophilia observed after 14 days of infection with *F. hepatica* indicate an oriented Th2 immune response [4, 17, 26]. However, before day 14 postinfection (pi), such data are unavailable and it is difficult to analyse the immune response by these means. Other tools are therefore required. Consequently, we studied the profile of cytokine production in the first two weeks of experimental fasciolosis in rats. We investigated the phenotype of the T cells ($CD4^+$ and $CD8^+$) that produced Th1 (IFN-γ) and Th2 (IL-4, IL-10) cytokines. We also monitored cytokine production in the culture supernatant and the proliferation response of hepatic mononuclear cells (HMNC).

2. MATERIALS AND METHODS

2.1. Animals and experimental infection

Inbred Wistar rats, aged about ten weeks and of both sexes (reared in our laboratory) were used. Two groups of four rats were orally infected with 60 metacercariae 7 or 14 days before sacrifice (by chloroform inhalation) [24]. One group of four healthy rats was not infected. The experiment was set up so that all the animals were killed on the same day. All animal experiments were carried out in accordance with French national guidelines for the care and use of laboratory animals.

2.2. Media and reagents

2.2.1. Media

Hanks balanced salt solution without phenol red (HBSS), RPMI 1640 medium, foetal calf serum (FCS), penicillin-streptomycin, sodium pyruvate, HEPES and L-glutamine were purchased from Life Technologies (Cergy Pontoise, France); β-mercaptoethanol was obtained from Merck (Darmstadt, Germany); 7.5% sodium bicarbonate solution, phorbol myristate acetate (PMA), ionomycin and brefeldin A were purchased from Sigma (Saint Louis, USA); Lymphoprep density gradient medium was purchased from Nycomed (Oslo, Norway). Before use, RPMI 1640 medium was supplemented with 2 mM L-glutamine, 20 mM HEPES, 100 μg penicillin-streptomycin, 20 mM sodium pyruvate, 5×10^{-5} M β-mercaptoethanol and 10% heat-inactivated FCS; HBSS was also buffered with bicarbonate (0.05%).

2.2.2. Reagents

Recombinant rat IL-10, IFN-γ and IL-4 were purchased from Becton Dickinson (Le Pont de Claix, France), Life Technologies (Cergy Pontoise, France) and R&D (Oxon, UK), respectively. Mouse anti-rat $CD4^+$(W3/25), mouse anti-rat $CD8^+$(OX8), rabbit anti-rat IFN-γ (AAR19), mouse anti-rat IFN-γ (DB-1), mouse anti-rat IFNγ fluorescein isothiocyanate (FITC)-conjugated (DB-1F) and mouse IgG1-FITC (F8-11-13) antibodies were from Serotec (Argène Varhiles, France). Mouse biotinylated anti-rat IL-4 (B11-3B), mouse anti-rat IL-10 (A5-7) and mouse biotinylated anti-rat IL-10 (A5-6B) antibodies were obtained from Becton Dickinson. Mouse anti-rat IL-4 (56567,111) antibody was obtained from R&D. Phycoerythrin-conjugated donkey anti-mouse Ig (DAM-PE) and peroxidase-conjugated goat anti-mouse (GAM- PO) antibodies were obtained from Interchim (Montluçon, France). Streptavidin-FITC, Streptavidin-peroxidase and 3, 3', 5'5- tetramethylbenzidine (TMB) were obtained from Dako (Denmark), Sigma and KPL (Realef, France), respectively.

2.3. Excretory-secretory *F. hepatica* (ES*Fh*) preparation

Live *F. hepatica* adults were recovered from the livers of slaughtered cattle. These flukes were washed five times and incubated for 3 hours at room temperature in 0.9% NaCl (1 mL/fluke). The medium was then recovered and centrifuged at 10 000 *g* for 15 min. Finally, the supernatant was filter sterilised (pore size = 0.22 μm) and stored at –20 °C.

2.4. Preparation of HMNC suspensions

Liver perfusion and cell preparation were performed as previously described [30]. Briefly, the rats were killed by chloroform inhalation and the livers were perfused with HBSS to wash out peripheral blood cells. The livers were then immediately removed. HMNC were collected by the Ficoll Hypaque method and resuspended in 5% FCS in RPMI (10^6 cells/mL). The viability of the recovered cells was > 90%.

2.5. Flow cytometry

Freshly isolated HMNC were stimulated for 18 h at 37 °C in a 5% CO_2 atmosphere in 6-well plates (Nunc, Kamstrup, Denmark) (3 mL /well) with either 10 ng/mL PMA plus 0.5 μg/mL ionomycin and 2 μg/mL Brefeldin A or in the absence of activators. After culture, the cells (400 μL per tube) were washed (5% FCS in PBS) and collected by centrifugation at 250 *g* for 7 min. The pellet was resuspended in 5% FCS in PBS and 100 μL of cell suspension was incubated with anti-rat $CD4^+$ antibody (mAb) (W3/25, 1/100) or with anti-$CD8^+$ mAb (OX-8, 1/100) on ice for 30 min. Then, the cells were washed again and stained with 100 μL of DAM-PE mAb

(1/200 dilution). For intracellular cytokine staining, the cells were fixed with 2% paraformaldehyde (w/v), permeabilised with 0.5% saponin (w/v). IL-4 and IL-10 staining was performed by incubating cells with B11-3B and A5-6 (1/300 and 1/50 respectively) for 45 min on ice followed by Streptavidin- FITC incubation for 15 min. IFN-γ positive cells were directly stained with DB-1F (Serotec, 1/20 dilution) for 30 min on ice. Finally, cytokine production was determined by two-colour flow cytometry of gated $CD4^+$ and $CD8^+$ cells in a FACStar Plus machine (Becton Dickinson). For each sample, 15 000 events were recorded.

2.6. Cytokine production and proliferation assay

Based on the results of preliminary experiments to optimise cytokine production and proliferation in response to PMA/ionomycin, a culture time of 48 h for subsequent assays was selected.

Freshly isolated HMNC were stimulated with 10 ng/mL PMA plus 0.5 μg/mL ionomycin or with 25 μg/mL ES*Fh,* or left unstimulated. Cultures were set up in a final volume of 200 μL in 96-well round-bottom plates (10^5 cells/well). The cells were incubated for 48 h at 37 °C under a 5% CO_2 atmosphere. We then collected 100 μL of each supernatant, which was stored at –70 °C until cytokine determination. The rest of the cell suspension was used for the lymphoproliferative assay.

The amounts of IFN-γ, IL-4 and IL-10 in the supernatant were analysed by standard-capture enzyme-linked immunosorbent assays (ELISA) in Nunc microplates. IL-10 and IFN-γ levels were determined according to the kit manufacturer's recommendations (Becton Dickinson and R&D, respectively). IL-4 levels were determined with a Duo Set Kit (R&D) used according to the manufacturer's protocol. The results are representative of three experiments.

After 48 h of culture, 1 μCi of [^{3}H]-thymidine (ICN, France) was added to the cell suspensions. The cells were then incubated for an additional 6 h before harvesting on glass fibre disks. Cell proliferation was expressed as mean counts per minute (cpm) for triplicate experiments and as stimulation indices (SI) = cpm of stimulated HMNC/cpm of unstimulated HMNC. Three independent experiments were carried out.

2.7. Statistical analysis

Differences between experimental groups were compared using the Kruskal- Wallis test and the level of significance was set at $P < 0.05$.

3. RESULTS

3.1. Frequency of $CD4^+$ and $CD8^+$ T cells among HMNC in *F. hepatica*-infected rats

The total numbers of cells harvested on days 7 and 14 pi were significantly higher than those in the control animals (Tab. I). The total number of cells decreased thereafter, with a smaller number of cells harvested on days 21 and 28 pi ($< 4 \times 10^5$ cells recovered per liver).

The proportion of $CD4^+$ cells was significantly lower ($P < 0.05$) than that of $CD8^+$ cells in uninfected rat livers. The proportion of $CD4^+$ T cells was significantly higher ($P < 0.05$) in infected animals than in controls while the proportion of $CD8^+$ cells did not change significantly during the observation period (Tab. I). There was no significant difference between the mean fluorescence intensity values of the $CD8^+$ and $CD4^+$ cell populations of infected and control animals, indicating that the surface expression of $CD4^+$ and $CD8^+$ was not modified (data not shown).

Table I. Percentages and numbers of $CD4^+$ and $CD8^+$ hepatic cells in *F. hepatica* infected and control rats.

		$CD4^+$		$CD8^+$	
	Total recovered HMNC ($\times 10^6$)	($\times 10^6$)	(%)	($\times 10^6$)	(%)
Control	5.7 ± 1.5	1.3 ± 0.3	22.4 ± 1.7	3.2 ± 0.3	55.9 ± 2.8
Infected:					
7 days pi	*9.5 ± 0.7	*2.5 ± 0.2	*26.0 ± 2.2	*4.8 ± 0.3	50.3 ± 2.2
14 days pi	*9.9 ± 1.8	*3.2 ± 0.6	*33.5 ± 1.3	*4.9 ± 0.9	49.5 ± 1.3

*Indicates $P < 0.05$ compared to values in uninfected animals.

Unstimulated cells and cells stimulated with ES*Fh* did not produce detectable levels of cytokines. After mitogenic stimulation, the frequency of IFN-γ^+ cells within the $CD4^+$ and $CD8^+$ subsets had not significantly changed on day 7 pi, but was significantly lower ($P < 0.05$) than that of controls on day 14 pi (Tab. II). On day 7 pi, the frequencies of IL-4- and IL-10-producing $CD4^+$ cells were significantly higher than those in the controls ($P < 0.05$). The proportions of IL-4-producing $CD4^+$ cells did not change significantly between 7 and 14 days pi. Conversely, the frequency of IL-10-producing $CD4^+$ cells continued to increase.

The frequency of IL-4-producing-cells within the $CD8^+$ subset was significantly higher ($P < 0.05$) in all infected animals than in the controls. However, IL-10-producing cells were absent from this subset ($CD8^+$) in all infected animals (Tab. II).

3.2. Patterns of cytokine secretion into HMNC supernatants (Fig. 1)

Unstimulated cells produced no detectable cytokines in culture supernatants. Stimulation with ES*Fh* resulted in a non-significant

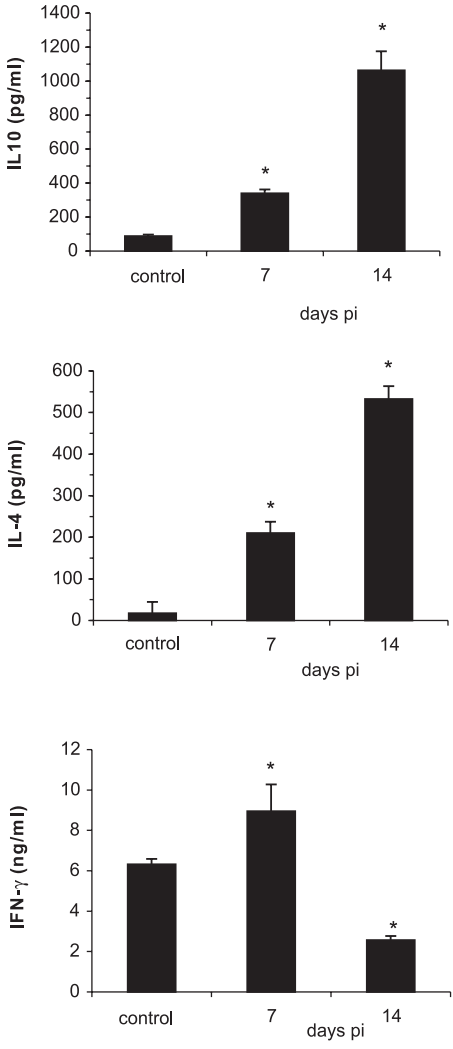

Figure 1. In vitro cytokine production by HMNC stimulated with PMA/ionomycin in control and *F. hepatica*-infected rats ($n = 4$ per group). Values represent the means ± SD. The results are representative of 3 separate experiments. *Indicates $P < 0.05$ compared to values in uninfected animals.

Table II. Percentage of cytokine-producing $CD4^+$ or $CD8^+$ hepatic cells in *F. hepatica* infected and control rats.

	Control		Infected			
			7 days pi		14 days pi	
Cytokine	$CD4^+$	$CD8^+$	$CD4^+$	$CD8^+$	$CD4^+$	$CD8^+$
IL-4	6.2 ± 0.1	11.0 ± 0.3	*9.1 ± 0.1	*14.3 ± 0.3	*10.7 ± 0.1	*15.8 ± 0.2
IL-10	2 ± 0.1	1.5 ± 0.4	*9.8 ± 0.1	0	*14.0 ± 0.1	0
IFN-γ	2.2 ± 0.1	35.6 ± 0.4	2.4 ± 0.5	38.9 ± 0.2	*1.8 ± 0.5	*18.5 ± 6.3

*Indicates $P < 0.05$ compared to values in uninfected animals.

increase in cytokine levels and was unaffected by infection at the tested doses of the ES*Fh* antigen.

The levels of IFN-γ production by HMNC following in vitro stimulation with PMA/ionomycin were significantly higher on day 7 pi than those in control animals ($P < 0.05$). As infection progressed, IFN-γ levels decreased significantly in infected animals by day 14 pi ($P < 0.05$). The levels of production of both IL-4 and IL-10 were significantly higher in infected rats on days 7 and 14 pi (for IL-4: 12.3 and 31.2 times higher respectively; for IL-10: 4 and 12.2 times higher respectively, $P < 0.05$) (Fig. 1).

3.3. Proliferative response of HMNC to mitogens and to ES*Fh* (Fig. 2)

On day 7 pi, the HMNC of *F. hepatica*-infected rats showed significantly stronger proliferative responses than did the controls following stimulation with PMA/ionomycin ($P < 0.05$). In contrast, on day 14 pi, the proliferative responses of these cells were significantly weaker than those of the controls ($P < 0.05$) (Fig. 2).

A significantly stronger proliferative response to ES*Fh* was observed in the infected cells than in the control cells on day 7 pi ($P < 0.05$), but not on day 14. Various doses of ES*Fh* antigens (25, 50, 100 μg) were tested and all gave similar results (data not shown).

4. DISCUSSION

Control over the early immune response is crucial for the establishment of the fluke in its host. Experiments in rats have shown that juvenile flukes modulate the immune response by inhibiting the early peripheral inflammatory response [2] and by delaying the hepatic inflammatory response during the first 2 weeks after infection [26]. In this study, we compared the hepatic cellular immune response in uninfected rats and rats infected with *F. hepatica* for 7 and 14 days. We investigated the production of T-cell cytokines in response to a mitogenic stimulus by flow cytometry [1, 14]. The advantage of this method over cytokine RNA monitoring [27], is its ability to examine the cytokine production of individual cells within mixed populations.

Our results indicate that a significant proportion of $CD4^+$ and $CD8^+$ HMNC from healthy rats produce IL4, IL10 and IFN-γ upon stimulation with PMA/ionomycin. Moreover, these cells proliferate in response to mitogens, such as concanavalin A and PMA/ionomycin, and specific *F. hepatica* antigens, but the levels of proliferation observed are lower than those observed with splenocytes or PBL (data not shown). To our knowledge, this is the first study focusing on cytokine production and cell proliferation in the rat liver.

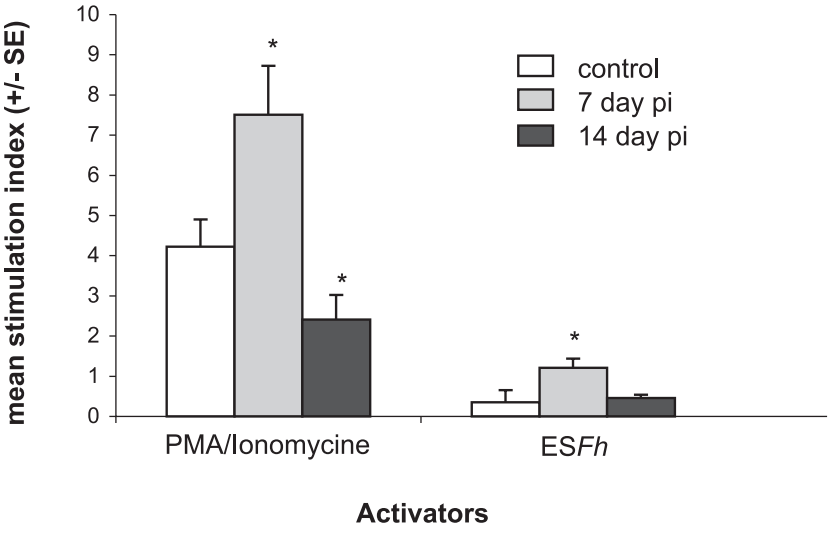

Figure 2. Proliferative responses of HMNC from *F. hepatica*-infected rats ($n = 4$ per group) to mitogens: PMA (10 ng/mL)/ionomycin (500 ng/mL) and the specific antigen, and to ES*Fh* (25 μg/mL). The values for unstimulated cells were 7200 ± 2300 cpm. The cells were harvested at the indicated time after infection (0, 7 and 14 days postinfection) and stimulated for 48 hours. Proliferation was measured by incorporation of ^{3}H-thymidine. The results are presented as the stimulation index (SI). The results are representative of 3 separate experiments. *Indicates $P < 0.05$ compared to values for uninfected animals.

After 7 days of infection, rat HMNC displayed higher levels of cytokine production than the controls. These results are consistent with RT-PCR analysis [27], which showed that levels of IL-4, IL-10 and IFN-γ mRNA increase on day 7 pi. The production by HMNC of both Th1 and Th2 cytokines suggests that these cells have a Th0 profile. Similar results were obtained in a previous study [31], which showed that the co-production of these cytokines was induced by IL-12. We have previously detected IL-12 mRNA on day 7 pi [27], which may account for the production of both Th1 and Th2 cytokines by HMNC. This is consistent with other studies on the peripheral response of mammals infected with *F. hepatica,* which was found to involve an early Th1/Th0 cytokine profile [5, 6, 18, 21, 22]. However, our results concerning IFN-γ levels are of particular interest. The concentration of this cytokine in the culture supernatants of HMNC collected from infected animals was higher on day 7 pi than that in control rats, although the frequency of IFN-γ^+ cells was similar in the two groups of rats. This suggests that other non-CD4$^+$ non-CD8$^+$ T cells may be the source of this increase. NK cells, which are abundant in the liver [16], may constitute the alternative source of IFN-γ [20] and this possibility is currently under investigation in our laboratory.

The cytokine production capacity of HMNC was modified in rats infected for 14 days. The percentage of IFN-γ-producing cells was significantly lower than that of controls on day 14 pi. This down-regulation

of IFN-γ production in the culture supernatant may result from a decrease in the frequency of IFN-γ-producing T lymphocytes ($CD4^+$ and $CD8^+$). ELISA analysis showed that more IL-4 was present on day 14 pi than on day 7 pi, despite similar frequencies and fluorescence intensities of $IL4^+$ cells on these two days. This result suggests that other non-$CD4^+$ non-$CD8^+$ T cells may be the source of this increase (e.g. contamination with granulocytes, detected evenly on day 14 pi by flow cytometry [15, 32]). As on day 7 pi, the $CD8^+$ $IL10^+$ population was absent on day 14 pi, whereas the frequency of $CD4^+$ $IL10^+$ cells had increased further. This indicates that $CD4^+$ cells are the major source of increases in IL-10 production during fasciolosis.

There are several possible reasons for the observed down-regulation of the Th1 response during the first two weeks following *F. hepatica* infection in rats. The decrease in IL-1 and IL-12 mRNA levels (produced by macrophages or dendritic cells and involved in Th1 differentiation [25, 28]) in rats on day 14 pi [27] may account for the observed decrease in IFN-γ production. In addition, IL-10 produced in massive amounts early in infestation may inhibit IL-12 production [8, 9, 12], resulting in the inhibition of IFN-γ production. Moreover, TGFβ (the mRNA for which is widely produced in early fasciolosis [27]) has been reported to inhibit IFN-γ production in an IL-10-independent manner [10, 11]. If, as suggested by the murine *S. mansoni* and *Trichinella spiralis* models, Th1 cell-based effector mechanisms limit fluke survival, the suppression of these responses may be of crucial biological importance to helminth parasites. The suppression of the Th1 response by *F. hepatica* has been reported in several previous studies [3, 23]. Induction of the "wrong" set of cytokines may inhibit other responses that are potentially more damaging for the parasite [19].

Infected animals displayed a weaker proliferative response than control animals only on day 14 pi; this may be related to changes in the distribution of leukocyte infiltrates in the livers of infected rats for 7 or 14 days [26]. On day 7 pi, the recruited immune cells were generally spread throughout the liver. In contrast, on day 14 pi, these cells were mostly localised around the migratory tracts and flukes. This is probably due to the chemoattractive molecules produced by *F. hepatica* [13], which facilitate direct contact between fluke molecules and immune cells, and may reduce overall responsiveness to mitogen. Indeed, it has been demonstrated that biologically active substances isolated from *F. hepatica* tissue may inhibit the proliferation of tumour cells in vitro [29]. The high levels of IL-10 production may also account for the decrease in proliferative response observed in HMNC on day 14 pi, similar to that observed in humans infected with *Onchocerca volvulus* [7].

In conclusion, our results indicate an early shift (7 dpi) from the Th1/Th2 response to a more pronounced Th2-regulated immune response, which may be mediated by IL10-producing $CD4^+$ cells. They also demonstrate that $CD4^+$ T lymphocytes are numerically and functionally the most important lymphoid cell population in the early immune response to fasciolosis. Further work is required to determine the location of NK cells in the liver and their pattern of cytokine production. These non-specific cells may play a crucial role in the early immune response of rats infected with *F. hepatica*.

REFERENCES

[1] Assenmacher M., Schmitz J., Radbruch A., Flow cytometric determination of cytokines in activated murine T helper lymphocytes: Expression of interleukin-10 in interferon-gamma– and in interleukin-4-expressing cells, Eur. J. Immunol. 24 (1994) 1097-1101.

[2] Baeza E., Poitou I., Delers F., Boulard C., Influence of anti-inflammatory treatments on experimental infection of rats with *Fasciola hepatica*:

Changes in serum levels of inflammatory markers during the early stages of fasciolosis, Res. Vet. Sci. 57 (1994) 172-179.

[3] Brady M.T., O'Neill S.M., Dalton J.P., Mills K.H., *Fasciola hepatica* suppresses a protective Th1 response against *Bordetella pertussis*, Infect. Immun. 67 (1999) 5372-5378.

[4] Chauvin A., Boulard C., Local immune response to experimental *Fasciola hepatica* infection in sheep, Parasite 3 (1996) 209-215.

[5] Clery D., Torgerson P., Mulcahy G., Immune responses of chronically infected adult cattle to *Fasciola hepatica* [published erratum appears in Vet. Parasitol. 65 (1–2) (1996) 169], Vet. Parasitol. 62 (1996) 71-82.

[6] Clery D.G., Mulcahy G., Lymphocyte and cytokine responses of young cattle during primary infection with *Fasciola hepatica*, Res. Vet. Sci. 65 (1998) 169-171.

[7] Cooper P.J., Mancero T., Espinel M., Sandoval C., Lovato R., Guderian R.H., Nutman T.B., Early human infection with *Onchocerca volvulus* is associated with an enhanced parasite-specific cellular immune response, J. Infect. Dis. 183 (2001) 1662-1668.

[8] Dai W.J., Kohler G., Brombacher F., Both innate and acquired immunity to *Listeria monocytogenes* infection are increased in IL-10-deficient mice, J. Immunol. 158 (1997) 2259-2267.

[9] Ding L., Shevach E.M., Differential effects of CD28 engagement and IL-12 on T cell activation by altered peptide ligands, J. Immunol. 161 (1998) 6614-6621.

[10] Espevik T., Figari I.S., Shalaby M.R., Lackides G.A., Lewis G.D., Shepard H.M., Palladino M.A. Jr., Inhibition of cytokine production by cyclosporin a and transforming growth factor beta, J. Exp. Med. 166 (1987) 571-576.

[11] Fiorentino D.F., Bond M.W., Mosmann T.R., Two types of mouse T helper cell. Iv. Th2 clones secrete a factor that inhibits cytokine production by Th1 clones, J. Exp. Med. 170 (1989) 2081-2095.

[12] Gong J.H., Zhang M., Modlin R.L., Linsley P.S., Iyer D., Lin Y., Barnes P.F., Interleukin-10 downregulates *Mycobacterium tuberculosis*-induced Th1 responses and CTLA-4 expression, Infect. Immun. 64 (1996) 913-918.

[13] Horii Y., Fujita K., Owhashi M., Partial purification and characterization of eosinophil chemotactic factors from soluble extract of *Fasciola* species, Am. J. Vet. Res. 47 (1986) 123-126.

[14] Jung T., Schauer U., Heusser C., Neumann C., Rieger C., Detection of intracellular cytokines by flow cytometry, J. Immunol. Methods 159 (1993) 197-207.

[15] Lamkhioued B., Gounni A.S., Aldebert D., Delaporte E., Prin L., Capron A., Capron M., Synthesis of type 1 (IFN gamma) and type 2 (IL-4, IL-5, and IL-10) cytokines by human eosinophils, Ann. NY. Acad. Sci. 796 (1996) 203-208.

[16] Luo D., Vanderkerken K., Bouwens L., Kuppen P.J., Crabbe E., Wisse E., The number and distribution of hepatic natural killer cells (pit cells) in normal rat liver: An immunohistochemical study, Hepatology 21 (1995) 1690-1694.

[17] Meeusen E., Lee C.S., Rickard M.D., Brandon M.R., Cellular response during liver fluke infection in sheep and its evasion by the parasite, Parasite Immunol. 17 (1995) 37-45.

[18] Moreau E., Chauvin A., Boulard C., IFN gamma and IL-10 production by hepatic lymph node and peripheral blood lymphocytes in *Fasciola hepatica* infected sheep, Parasite 5 (1998) 307-315.

[19] Nutman T.B., Kumaraswami V., Ottesen E.A., Parasite-specific anergy in human filariasis. Insights after analysis of parasite antigen-driven lymphokine production, J. Clin. Invest. 79 (1987) 1516-1523.

[20] O'Garra A., Cytokines induce the development of functionally heterogeneous T helper cell subsets, Immunity 8 (1998) 275-283.

[21] Oldham G., Immune responses in rats and cattle to primary infections with *Fasciola hepatica*, Res. Vet. Sci. 39 (1985) 357-363.

[22] Oldham G., Williams L., Cell-mediated immunity to liver fluke antigens during experimental *Fasciola hepatica* infection of cattle, Parasite Immunol. 7 (1985) 503-516.

[23] O'Neill S.M., Brady M.T., Callanan J.J., Mulcahy G., Joyce P., Mills K.H., Dalton J.P., *Fasciola hepatica* infection downregulates Th1 responses in mice, Parasite Immunol. 22 (2000) 147-155.

[24] Poitou I., Baeza E., Boulard C., Analysis of the results obtained using a technique of experimental primary infestation with *Fasciola hepatica* in the rat, Int. J. Parasitol. 23 (1993) 403-406.

[25] Sinigaglia F., D'Ambrosio D., Panina-Bordignon P., Rogge L., Regulation of the IL-12/IL-12R axis: A critical step in T-helper cell differentiation and effector function, Immunol. Rev. 170 (1999) 65-72.

[26] Tliba O., Sibille P., Boulard C., Chauvin A., Local hepatic immune response in rats during primary infection with *Fasciola hepatica*, Parasite 7 (2000) 9-18.

[27] Tliba O., Sibille P., Boulard C., Chauvin A., Early hepatic cytokine mRNA expression in experimental rat fasciolosis, Vet. Parasitol. (in press).

[28] Trinchieri G., Interleukin-12: A cytokine produced by antigen-presenting cells with immunoregulatory functions in the generation of T-helper cells type 1 and cytotoxic lymphocytes, Blood 84 (1994) 4008-4027.

[29] Tsocheva N., Toshkova R., Filchev A., Inhibition of proliferation of tumour cell cultures by biologically active substances isolated from the

tissues of *Fasciola hepatica* and *Fasciola hepatica*- infected rat liver, Folia. Parasitol. 39 (1992) 387-390.

[30] Watanabi H., Ohtsuka K., Kiruma M., Ikarashi Y., Ohmori K., Kusumi A., Ohteki T., Seki S., Abo T., Details of an isolation method for hepatic lymphocytes in mice, J. Immunol. Methods 146 (1992) 145-154.

[31] Windhagen A., Anderson D.E., Carrizosa A., Williams R.E., Hafler D.A., IL-12 induces human T cells secreting IL-10 with IFN-gamma, J. Immunol. 157 (1996) 1127-1131.

[32] Woerly G., Roger N., Loiseau S., Capron M., Expression of Th1 and Th2 immunoregulatory cytokines by human eosinophils, Int. Arch. Allergy Immunol. 118 (1999) 95-97.