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Pathological and immunohistochemical evaluation of the effects of interferon gamma (IFN-γ) and aminoguanidine in rats experimentally infected with *Fasciola hepatica*⋆

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Abstract: The present study was aimed at pathologically and immunohistochemically evaluating the effects of interferon gamma (IFN-γ) and aminoguanidine (AG) in rats experimentally infected with *Fasciola hepatica*. A total of 44 Wistar rats were divided into 4 groups. With the exception of the controls (fourth group), the remaining rats were infected orally with 25 metacerceria of *F. hepatica*. The first group was administered 0.2% AG in drinking water daily, in order to block nitric oxide (NO) production. The second group was administered 250 units of IFN-γ daily, in order to stimulate NO synthesis. The third group was administered a placebo only. At the end of the 2 month experimentation period all the rats were killed under ether anesthesia and necropsied. Migrating tracts, necrosis, and enlargement of the main bile duct were the predominant lesions in infected livers. The parasite burden was lower in the IFN-γ–treated rats than rats in the other groups. Acute migrating tracts were occluded with numerous erythrocytes, necrotic hepatocytes, and neutrophils and surrounded by mononuclear cell infiltration interspersed with eosinophils. Chronic migrating tracts were generally repaired with fibrous connective tissue and surrounded by chronic inflammatory infiltrate. Immunohistochemistry detected $CD3^+$ T and $CD79\alpha cy^+$ B lymphocyte infiltration, λ IgG $^+$ plasmocytes and PCNA-positive cells in the infected livers, hepatic and mesenterial lymph nodes, and Peyer's patches. Given the low numbers of parasites and the limited repair in the liver of the animals administered IFN-γ and the severity of the lesions in the livers of the animals administered AG, it was concluded that IFN-γ positively affected the immune system and that AG blocked NO production in the animals.

Key words: Aminoguanidine, fasciolosis, immunohistochemistry, interferon gamma, pathology, rats

Fasciola hepatica ile deneysel olarak enfekte edilen ratlarda interferon gamma (IFN-γ) ve aminoguanidin etkisinin patolojik ve immunohistokimyasal değerlendirilmesi

Özet: Bu çalışmada, *Fasciola hepatica* ile deneysel olarak enfekte edilen ratlarda interferon gamma (IFN-γ) ve aminoguanidin'in (AG) etkisi patolojik ve immunohistokimyasal olarak değerlendirildi. Çalışmada 44 Wistar rat dört gruba ayrıldı. Kontrol grubu hariç (4. grup), diğer üç grup (1-3. grup) 25 adet *F. hepatica* metaserkeriyle enfekte edildi. Birinci gruptaki ratlara, nitrik oksit (NO) sentezini bloke etmek için, % 0,2 AG içme suyuna ilave edilerek günlük olarak verildi. İkinci gruba, NO sentezi ve pro-inflamatuvar yanıtı uyarmak için, 250 ünite IFN-γ günlük olarak verildi.

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Üçüncü gruptaki ratlar enfekte kontrol grubu olarak değerlendirildi. Yaklaşık iki aylık deneme periyodu sonunda, tüm ratlar eter anestezisi altında öldürülerek nekropsileri yapıldı. Nekropside, enfekte gruplarda karaciğerde göç yolları, nekroz ve safra kanalında genişleme görüldü. Toplanan parazit sayısının IFN-γ verilen grupta diğerlerine göre nispeten düşük olduğu belirlendi. Akut göç yollarının çok sayıda eritrosit, nekrotik hepatositler ve nötrofil lökositler ile dolduğu; eozinofil lökositler ve mononükleer hücre infiltrasyonu ile çevrelendiği saptandı. Kronik göç yollarının genellikle fibröz bağdoku ile onarıldığı ve kronik yangısel infiltratla çevrelendiği gözlendi. İmmunohistokimyasal olarak enfekte ratların karaciğer, hepatik ve mezenteriyal lenf düğümleri ile peyer plaklarında CD3 $^{+}$ T and CD79αcy $^{+}$ B lenfosit infiltrasyonu ile λ IgG $^{+}$ plazmositler ve PCNA pozitif hücreler tespit edildi. İnterferon gamma verilen ratlarda parazit sayısının düşük olması ve fibröz bağdoku onarımının şekillenmesi; AG verilen ratlarda ise şiddetli karaciğer lezyonlarının oluşmasına dayanılarak, interferon gammanın ratlarda bağışıklık sistemini olumlu yönde etkilediği ve AG'in NO sentezini bloke ettiği sonucuna varıldı.

Anahtar sözcükler: Aminoguanidin; fasiolazis; immunohistokimya; interferon gamma; patoloji; rat

Introduction

Fasciola hepatica, the common liver fluke, is a cosmopolitan trematode found in the liver and bile ducts of many mammal species, especially cattle, sheep, and goats. The parasite induces both acute and chronic fasciolosis in livestock and causes considerable economic loss on farms. In particular, dependence on expensive antihelmintics; liver condemnation; production loss due to mortality; lower production of meat, milk and wool; reduced weight gain; and impaired fertility constitute the main economic losses for breeders (1-3).

The main pathogenic event in fasciolosis is the migration of juvenile flukes in the liver. After infection juvenile flukes migrate into the liver to reach the gall bladder and cause mechanical damage in the parenchyma of the organ, which results in necrosis, hemorrhages, fibrosis, and cirrhosis (1,2,4). Hepatic lesions produced by *F. hepatica* in various host species are generally similar and associated with the number of ingested metacercariae. During migration the migratory tracts are surrounded by an extensive local inflammatory reaction. This inflammation persists for the first 6 weeks of infection and involves a massive infiltration of eosinophils (4). The immune response to the parasite varies among host species; rats and cattle develop partial resistance, but sheep are generally unable to mount such a response (5,6). The humoral and cellular immune response to the parasite appears to play an important role in the rejection of *F. hepatica* infection and in the development of effective resistance to re-infection (7,8).

Nitric oxide (NO), a nitrogenous free radical secreted by a variety of mammalian cells, is produced from the amino acid L-arginine by the enzymatic action of nitric oxide synthase (NOS). Inducible nitric oxide synthase (iNOS) isoform is primarily responsible for NO production by activating lung macrophages. Its expression is induced by several agents including bacterial lipopolysaccarides or cytokines, such as IFN-γ, IL-I, or TNF-α (9,10). In addition to the physiological roles of NO as a pulmonary vasodilatator, neurotransmitter, and inhibitor of platelet aggregation, the radical promotes the cytotoxic and microbiocidal activities of macrophages (9-13). IFN-γ has long been recognized as a signature pro-inflammatory cytokine, produced mainly by natural killer (NK), CD4 $^{+}$ T helper cells (Th1), and a subset of CD8 $^{+}$ T cells, which play a central role in inflammation and autoimmune diseases. Among interferons, IFN-γ is the most potent macrophage-activating factor and the only known cytokine with the capacity to induce iNOS in macrophages by itself (13,14). Aminoguanidine (AG) has commonly been reported as selective inhibitor of iNOS (12,13,15,16).

It has been reported that macrophages from *F. hepatica*-infected rats were able to kill newly excysted juvenile liver flukes with antibodies and reactive nitrogen intermediates (17,18). To date, no research has been undertaken to determine whether rat macrophages activated by NO kill the parasites in vivo or whether NO affects the pathology of rat fasciolosis. Thus, the present study investigates pathologically and immunohistochemically the liver,

hepatic and mesenterial lymph nodes, and intestines of rats experimentally infected with *F. hepatica* and treated with either IFN-γ or AG in order to induce iNOS expression and a pro-inflammatory response, or to inhibit iNOS expression and NO production, respectively.

Materials and methods

Animals and experimental design

A total of 44 Wistar rats were randomly divided into 4 groups of 10 animals each. With the exception of the animals in the fourth group (control), the remaining rats were infected orally with 25 metacerceria of *F. hepatica*, which were maintained in the laboratory. The animals in the first group were administered 0.2% AG in drinking water daily; the rats were also injected intraperitonally with 5 mg AG twice a week. In the second group, the rats were injected intraperitonally with 250 U IFN-γ daily for 15 days and then at 3 day intervals, in order to stimulate NO synthesis and a pro-inflammatory response. The animals in the third group were administered with a placebo only. The remaining 4 rats were divided into 2 pairs and added to groups 1 and 2 as internal controls for the effects of AG and IFN-γ. Apart from being uninfected, these animals were treated in the same way as the rest of their group. Throughout the experiment rats were fed standard pellet feed, given tap water ad libitum, and kept in cages. At the end of the 2 month period of experimentation, the rats were killed by cervical dislocation under ether anesthesia and necropsied.

Histopathology

Tissue samples taken from the liver, hepatic and mesenteric lymph nodes, intestines, kidneys, and lungs were processed routinely and stained with hematoxylin-eosin (H&E) and Turnbull's blue for hemosiderin and examined under the light microscope.

Immunohistochemistry

Serial sections from the tissues were stained immunohistochemically using the avidin-biotin-peroxidase complex (ABC) technique (19) for $CD3^+$ T and $CD79\alpha cy^+$ B lymphocytes, IgG lambda light chain (λ IgG), and proliferating cell nuclear antigen (PCNA). Details of the primary antibodies used are given in Table 1. Sections prepared from paraffin blocks in a 4 μm thickness were dewaxed in xylene and hydrated through graded alcohols. Endogenous peroxidase activity was blocked with 3% hydrogen peroxide in distilled water for 30 min. The sections were incubated with tris-buffered saline (TBS) for 5 min and subsequently put in citrate buffer saline (pH 6.0) in a microwave oven for 20 min for antigen retrieval. After they were washed with TBS for 5 min the slides were incubated with normal rabbit (CD79αcy, PCNA) or goat (λ IgG) serum at room temperature

Table 1. Details of primary antibodies used for immunohistochemical analysis.

Primary antibodies	Pre-treatment	Primary antibody dilution	Incubation conditions	Origin (commercial reference)
Polyclonal rabbit anti-human IgG lamda light chain (λ IgG) ‡	Microwave oven *	1 in 1500	Overnight at 4 °C	Novocastra (NCL-LAMp)
Polyclonal rabbit anti-human CD3 ‡	Microwave oven	1 in 150	Room temperature	Dako (Catalog no. N 1580)
Monoclonal mouse anti-human CD79αcy ‡	Microwave oven	1 in 25	Room temperature	Dako (Catalog no M 7051)
Monoclonal mouse anti-rat PCNA ‡	Microwave oven	1 in 2000	Overnight at 4 °C	Chemicon (Clone PC10)

‡: The antibodies have been shown to cross-react in sheep in the manufacturer's data sheets, except λ IgG, for which we found an intense reaction in plasma cells;

*: microwave oven pre-treatment consisted of immersion of the sections in 10 mM sodium citrate buffer pH 6.0 and irradiation in a 800 W microwave oven for 20 min.

(RT) for 60 min. The sections were then incubated with monoclonal mouse anti-human CD79αcy, polyclonal rabbit anti-human λ IgG, and monoclonal mouse anti-rat PCNA primary antibodies, according to the manufacturer's recommended procedures. The sections were washed 3 times for 5 min each with TBS and then incubated with biotinylated goat anti-rabbit IgG (λ IgG) and biotinylated rabbit anti-mouse IgG (CD79αcy, PCNA) at a dilution of 1/200 in TBS for 60 min at RT (secondary antibodies supplied by Dako, Carpinteria, USA). After further washing with TBS all the sections were treated with streptavidin peroxidase complex (ABC; Dako, Carpinteria, USA) at a dilution of 1/300 for 30 min at RT. Detection of $CD3^+$ T cells was undertaken with polyclonal rabbit anti-human CD3 antibody, biotinylated linked anti-mouse and anti-rabbit immunoglobulin (Dako LSAB2™ system, Dako), and Streptavidin HRP (Dako LSAB2™ system, Dako), each overlaid onto the sections for 30 min at RT. Immunolabeling was obtained using 3,3-diaminobenzidine (DAB) or 3-amino-9-ethylcarbazole (AEC) as the chromogen. Mayer's hematoxylin was used as the counterstain. Primary antibodies were omitted from negative control sections, which were incubated with either TBS or diluted normal serum from the species in which the primary antibody was raised. Marker expression was recorded by a semiquantitative grading scheme based upon the percentage of cells that was labeled in 3 representative fields examined with the ×40 objective of the microscope [negative (-) = 0%, low (+) = 1%-10%, moderate (++) = 11%-59%, or abundant (+++) ≥ 60%].

Results

Gross lesions

Similar gross lesions were observed in the livers of the infected groups. Tortuous migratory tracts, 1-2 mm in diameter, and hemorrhages were seen in the livers after the second week post-infection (WPI) (Figure 1). The livers of the *F. hepatica*-infected rats often revealed irregular surfaces with small nodular or pale necrotic areas. The main bile ducts evidently appeared to be enlarged, and varying numbers of flukes were recovered from them. The parasite burden was lower in the IFN-γ-treated group than in the AG-treated and IC groups. In addition, in 1 rat with severe liver damage from the IC group, the kidneys showed marked enlargement along with many hemorrhagic migratory tracts on both capsular and cut surfaces. The gross, histological, and immunohistochemical findings, and parasite burdens of the rats from all infected groups are shown in Table 2.

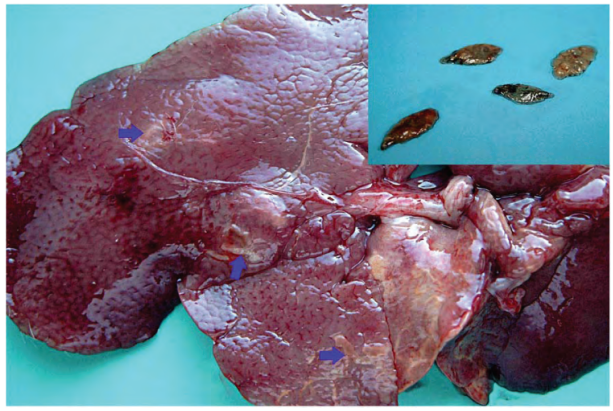

Figure 1. Migratory tracts (arrows) in the liver of IFN-γ-treated rat and recovered flukes (inset).

Histopathological changes

Acute or chronic migrating tracts were the most predominant finding in the livers from all of the infected rats. In the animals which died in the second and third weeks after infection, lesions characteristic of acute fasciolosis were seen. These consisted of acute or subacute migratory tunnels filled with a large amount of blood, fibrin, and cellular debris (Figure 2). The inflammatory infiltrate surrounding the migratory tunnels varied from sparse to moderate in rats and mainly comprised heavy infiltration of eosinophil and neutrophil leukocytes, a few macrophages, plasmocytes, and lymphocytes. The number of acute migratory tunnels was high in rats dead of infection in the early period (2-3 WPI), and neutrophils were the predominant cells within the inflammatory infiltrate.

Chronic migratory tracts were seen at 7-9 WPI. These were mainly characterized by moderate to severe increase of fibrous connective tissue and were surrounded by eosinophils, lymphocytes, macrophages, multinucleated giant cells, and plasma cells that often contained Russell bodies (Figure 3). Fibrinous perihepatitis, hemosiderin-laden macrophages that were corrected as hemosiderin by

Table 2. Numbers of flukes recovered, gross and microscopic changes, and the results of immunohistochemical staining for CD3 $^{+}$ T and CD79αcy $^{+}$ B lymphocytes and λ IgG $^{+}$ plasma cells in the liver, hepatic lymph nodes, and Peyer's patches of *F. hepatica*-infected and control rats.

Lesions and infiltrating cells	Group 1 [aminoguanidine (AG) treatment]								Group 2 [interferon gamma (IFN-γ) treatment]							Group 3 (*F. hepatica* infected control)							Control	
	R1	R2	R3	R4	R5	R6	R7	R10	R1	R2	R4	R5	R6	R7	R11	R1	R2	R3	R4	R5	R6	R7	R1	R2
Numbers of flukes recovered	2	4	1	--	2	1	6	4	3	1	-	1	4	1	3	3	1	9	5	4	5	4	--	--
Gross migratory tracts	+	++	+	-	+	+	+++	++	+++	++	-	+	+++	+	-	++	++	+++	+++	+++	+++	+++	--	--
Acute migratory tracts	-	-	-	-	-	+	-	++	-	+	-	-	-	-	-	-	-	-	-	+	-	++	--	--
Chronic migratory tracts	++	++	+	+	+	-	++	+	+	-	++	-	+++	+++	++	+++	+++	+++	++	-	+++	+	--	--
Bile duct hyperplasia	++	++	+++	-	+	+++	+	+	+	+	+	-	-	++	+	++	+++	+++	+	+	+++	-	--	--
Portal fibrosis	+++	+++	+++	+++	++	+	-	+	+	+	++	-	+	++	+	++	++	+++	+	-	+	+	--	--
Hemosiderin-laden macrophages	++	+++	++	+	+	++	-	+	++	+	++	+	+++	+	-	++	++	+++	+	+	+++	+	+	--
Eosinophil infiltration	++	++	-	++	+	+	+++	++	+	+	+	+	++	+++	-	+++	+++	+++	+	+++	+++	+	--	--
λ IgG $^{+}$ cells in the liver	++	++	+++	+	+	+	+++	+	+++	++	++	+	++	++	+++	+	+++	++	++	+++	+++	++	--	--
CD3 $^{+}$ T cells in the liver	++	++	+	++	+	+	+++	++	+++	++	+++	++	+++	+++	+++	++	+	+++	+++	+	++	+	--	+
CD79αcy^{+} B cells in the liver	+	++	+	+	+	+	++	++	++	++	+	++	++	+	++	+	+	++	+	++	+	+	--	--
λ IgG $^{+}$ plasma cells in the HLN	++	++	+++	+++	++	+++	++	++	++	++	+++	++	+++	+++	+++	++	+++	+++	+++	+++	+++	+++	+	+
CD3 $^{+}$ T cells in the HLN	++	+++	+	+++	++	+++	+++	+++	+++	++	+++	+++	+++	+++	++	+++	+++	+++	+++	++	+++	++	+	++
CD79αcy $^{+}$ B cells in the HLN	++	+++	++	+++	+++	++	+++	++	+++	++	++	++	+++	+++	+++	+++	++	++	++	+	+	++	+	+
λ IgG $^{+}$ cells in the PP	+++	++	+++	++	+	+++	+++	+++	+	+	++	++	++	+++	+++	++	+++	+++	+++	++	+++	++	+	+
CD3 $^{+}$ T cells in the PP	+++	+++	+++	+++	++	++	+++	+++	+++	+	+++	++	++	+++	+++	+++	+++	+++	+++	++	++	+	++	++
CD79αcy $^{+}$ B cells in the PP	+	+	+	++	++	++	+	+++	+	+	++	+	+++	+	+++	+	+	++	+++	++	+	++	++	+

Severity of lesions: -: nil; +: mild; ++: moderate; +++: severe; antigen amount (as defined in Materials and methods): -: negative; +: low; ++: moderate; +++: abundant; HLN: hepatic lymph node; PP: Peyer's patches.

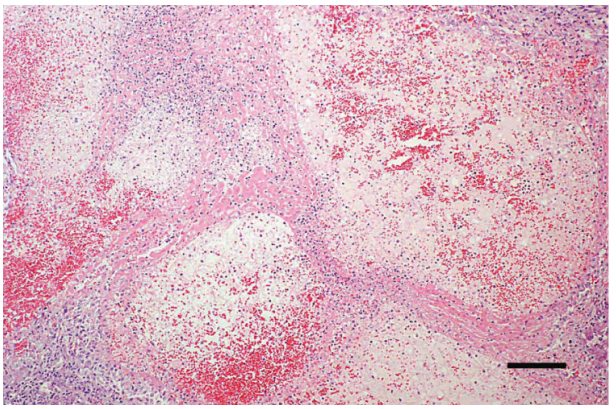

Figure 2. Acute migratory tunnels filled with blood and surrounded by inflammatory infiltrate (IFN-γ group); H&E, Bar: 543 μm.

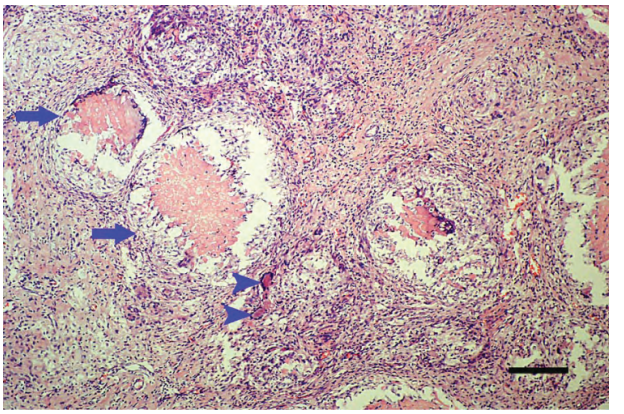

Figure 3. Chronic migratory tunnels (arrows) surrounded by fibrosis, inflammatory infiltrate, and multinucleated giant cells (arrow heads) (IC group); H&E, Bar: 543 μm.

Turnbull's blue staining, and Kupffer' cell activation were also detected as important and common findings in the liver parenchyma. Even though fibrosis of the tracts was seen in all infected rats, inflammatory infiltrate was more severe in the IC rats than in the other groups. In some rats from the IC group small mineralized granulomas composed of multinucleated giant cells and macrophages, along with vascular necrosis and fibrotic nodules in the lumen of vessels, were observed. The main histological lesions of the bile ducts were periductal fibrosis and severe epithelial hyperplasia, accompanied by a moderate to abundant infiltrate of eosinophils, macrophages, plasma cells, and lymphocytes. Occasionally lymphoid follicle formation with germinal centers was detected around the chronic migratory tunnels and in the submucosa of the bile ducts. In some cases the bile ducts and hepatic parenchyma contained a few fluke eggs. In addition, in the livers of 2 rats from the IC group, a few flukes were detected within the hemorrhagic tunnels with the attachment of a few leukocytes to tegument at 5 WPI.

The main histopathological changes in the hepatic and mesenterial lymph nodes of the infected rats were mild follicular hyperplasia along with an increase in plasmacytes, macrophages, and eosinophils in the medullary areas. Slight hemorrhages were also found in the medullary region of the nodes. The small intestines of the infected rats showed epithelial necrosis and lymphoid infiltration in the lamina propria and submucosa. Kidneys of infected rats often revealed tubular necrosis and focal perivascular lymphoid infiltrations. In a rat from the IC group, 1 kidney revealed hemorrhagic migratory tunnels surrounded by inflammatory infiltrate and moderate fibrosis. The lungs of the infected groups showed no marked changes, with the exception of a fluke surrounded by heavy infiltration of inflammatory cells in the parenchyma at 9 WPI.

Immunohistochemical changes

Diffuse $CD3^+$ T lymphocyte infiltration was observed in infected livers at 4 WPI, with an increase in their numbers at 8 WPI. $CD3^+$ T lymphocytes were detected as an element of the inflammatory infiltrate, particularly around the migrating tracts in the liver (Figure 4). Occasionally, $CD3^+$ T lymphocytes were distributed in the sinusoids, portal areas, and lamina propria of the bile ducts, or formed small lymphoid aggregates at the periphery of chronic migrating tracts and bile ducts. Furthermore, labeling of CD3 epitopes showed grouped cells in the periphery of lymphoid follicles, and a few cells were seen inside the follicles around the bile ducts. Examination of the hepatic and mesenterial lymph nodes of the infected rats revealed high numbers of $CD3^+$ T lymphocytes at the paracortical area surrounding the lymphoid follicles in the cortex, with fewer positive cells in the germinal centre of follicles (Figure 5). $CD3^+$ T cells were often seen as grouped or scattered cells in the layers of the small intestines and at the periphery of

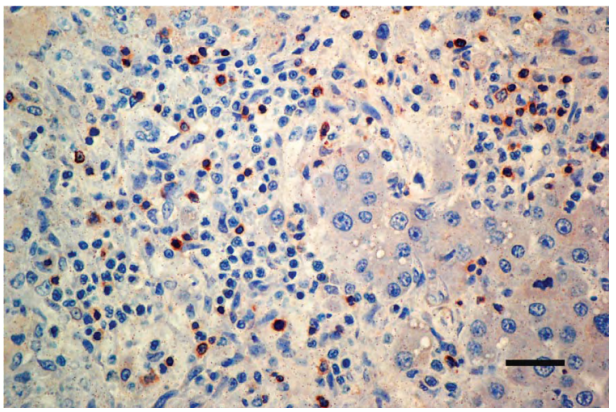

Figure 4. CD3⁺ T lymphocyte infiltration around the migratory tract in the damaged liver (IFN-γ group); IHC, Bar: 51 µm.

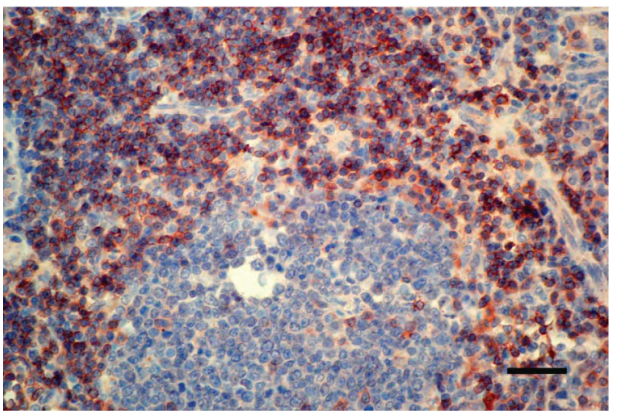

Figure 5. Numerous CD3⁺ T lymphoctes in the paracortex of the hepatic lymph node (IFN-γ group); IHC, Bar: 51 µm.

Peyer's patches. The proportion of positive cells was slightly higher in rats treated with IFN-γ than in rats from the AG-treated and IC groups (Table 2).

Moderate or scarce numbers of CD79αcy ⁺ B lymphocytes were scattered in small groups around the migrating tracts (Figure 6) and bile ducts, or as grouped cells inside the lymphoid follicles in the lymph nodes. The majority of B lymphocytes in the lymphoid follicles were found to be strongly labeled for CD79αcy ⁺ B epitopes (Figure 7). Positive immunolabeling for the CD79αcy antibody increased openly in the injured livers with high numbers of flukes. A few B cells showed a positive reaction for the CD79αcy antibody in the interfollicular areas and medullary cords of the lymph nodes. Numerous CD79αcy ⁺ B cells were found in the peripheral area of follicles in the Peyer's patches, with high numbers of positive immunolabeled B cells in the germinal centers. There were fewer CD79αcy ⁺ B cells than CD3⁺ T lymphocytes in both damaged livers and the lymph nodes.

Plasma cells and B lymphocytes showed a cytoplasmic immuno positive reaction for λ IgG antibody in the livers, hepatic and mesenteric lymph nodes, and Peyer's patches of *F. hepatica*-infected rats. λ IgG ⁺ plasma cells were found as early as 3 WPI and were predominantly localized around the chronic migrating tracts (Figure 8) in the lamina propria and submucosa of the main bile ducts and in the portal areas. In the hepatic and mesenterial lymph nodes high numbers of λ IgG ⁺ plasmocytes were mainly found in the medullary region, with sparse positive cells in the cortex (Figure 9). In cases of chronic fasciolosis, numerous Russell bodies of globoid shape and varying size revealed an evidently severe immunopositive reaction for λ IgG in both the lymph nodes and around the chronic migratory tunnels. There were no marked differences in the numbers of λ IgG ⁺ cells among the infected groups (Table 2). In all of the *F. hepatica*-infected groups the rats that died in the early period of experimentation revealed a few scattered λ IgG ⁺ plasma cells in the portal areas and submucosa of the main bile duct. The control rats also revealed only a few λ IgG ⁺ plasma cells in the medullary region of the lymph nodes. Inflammatory infiltrate in the layers of the small intestines and Peyer's patches also showed a few cells positive for λ IgG.

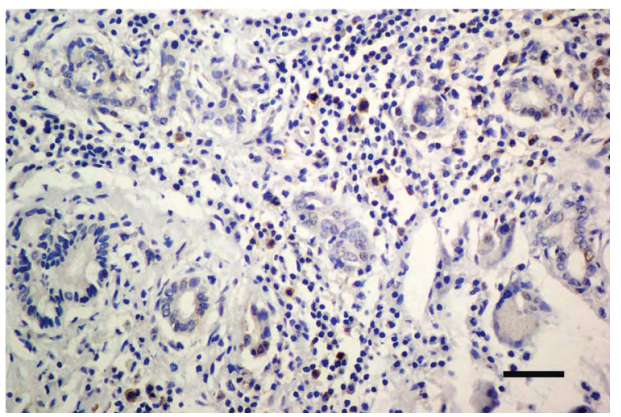

Figure 6. Low numbers of CD79αcy ⁺ B lymphocytes around the migratory tract in the damaged liver (IFN-γ group); IHC, Bar: 51 µm.

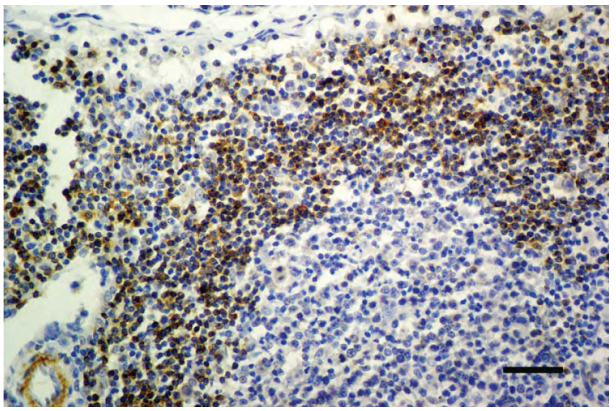

Figure 7. CD79αcy $^{+}$ numerous B lymphocytes in the parafollicular area of the mesenterial lymph node (IC group); IHC, Bar: 51 μm.

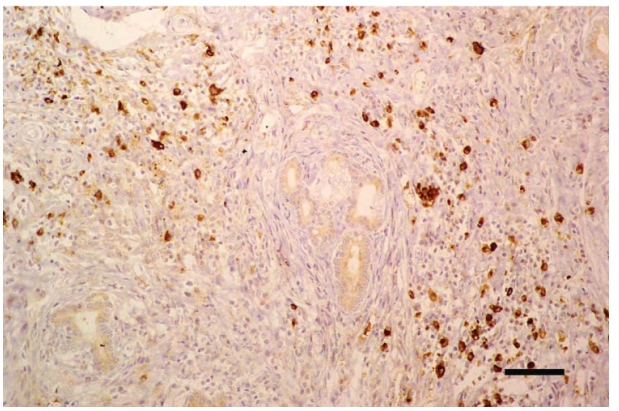

Figure 8. Numerous λ IgG $^{+}$ plasma cells surrounding the proliferated bile ducts in the infected liver (AG group); IHC, Bar: 166 μm.

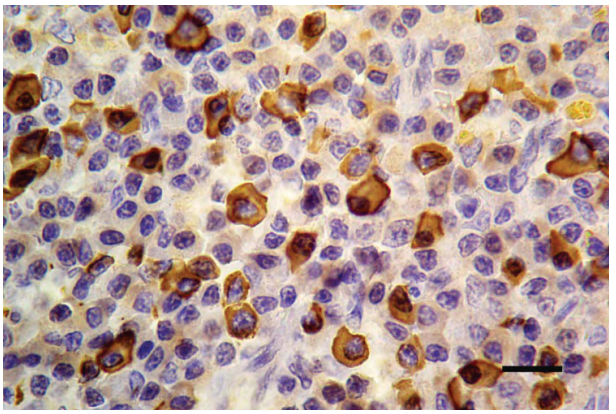

Figure 9. Numerous λ IgG $^{+}$ plasma cells in the hepatic lymph node (AG group); IHC, Bar: 11 μm.

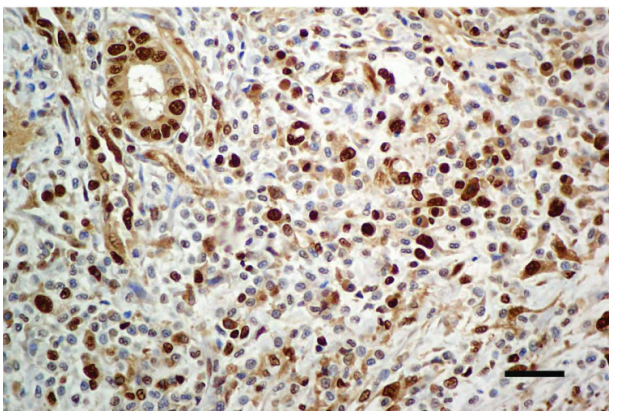

Figure 10. Diffuse PCNA nuclear positive reaction of the cells around the migratory tracts in the liver of rat with chronic fasciolosis (IC group); IHC, Bar: 51 μm.

When PCNA immunostaining was used to evaluate the proliferative activity of cells high numbers of nuclear positive cells were detected in both the liver and the lymph nodes of infected rats. In the infected liver the immunopositive reaction was commonly seen in the nuclei of hepatocytes, fibroblasts, and bile duct epithelium (Figure 10). Numerous hepatocytes were clearly detected in the various stages of mitosis. In particular, the lymphocytes around the chronic migratory tracts and bile ducts and within the germinal centers of the lymphoid follicles in the hepatic and mesenterial lymph nodes and Peyer's patches revealed an intense positive reaction for PCNA.

Discussion

Necropsy findings were characterized by acute and chronic migratory tunnels and irregularities in the surface of the liver consistent with previously documented results in *F. hepatica*-infected rats (1,4,20). It has been well established that the main pathogenic event of fasciolosis is the migration of juvenile flukes in the liver and that, during the migration, the migratory tracts are surrounded by an extensive local inflammatory reaction (1,21). In the present study the mean numbers of flukes recovered from the infected livers at necropsy were higher in the AG-treated and IC groups than in IFN-

γ-treated rats. Similarly, Brunet (9) reported that in the absence of NO, iNOS-/- mice and mice treated with aminoguanidine showed an increased parasite burden. The low parasite burden in IFN-γ-treated rats might be caused by a toxic effect of NO upon the parasite because of its oxidant properties and its ability to react with iron-containing compounds. The cytotoxic properties of NO may depend on the production of peroxynitrite anions from the reaction between NO and superoxide anions. It has also been reported that NO production occurred in both protozoon and helmintic infections and that in the case of protozoal infections, macrophages activated by IFN-γ derived from parasite-specific T cells were able to destroy intracellular parasites through the protection of NO (9,11,14). Some authors (17,18,22) have found in vitro that peritoneal macrophages from *F. hepatica*-infected rats were able to kill newly excysted juveniles (NEJ) of the liver fluke by a mechanism involving antibodies and the production of reactive nitrogen intermediates. Thus, in the present study, the low numbers of flukes recovered in the IFN-γ-treated rats may be due to the parasiticidal effects of peroxynitrite. The high numbers of flukes recovered in the AG group might also reveal the blocking of NO synthesis as a result of AG administration.

The present study found that the microscopy of the *F. hepatica*-affected livers was mainly characterized by acute and chronic migratory tracts. Hemorrhagic sinuous migratory tunnels surrounded by leukocytic infiltrations were detected in the early period of infection (2-3 WPI) in rats. This has also been reported as characteristic of acute fasciolosis in sheep (21,23) and goats (6). It has been reported from experiments in rats that juvenile flukes modulate the immune response by inhibiting the early peripheral inflammatory response (16) and delaying the hepatic inflammatory response during the first 2 weeks after infection (1). Hemosiderin-laden macrophages often infiltrated the area surrounding the tunnels and portal spaces, occluding the tunnels with blood, necrotic debris, and fibrin, as reported by others (6,21,23-25). Consistent with our results, eosionphils, neutrophils, and macrophages have been implicated as effector cells in resistance to the fluke in sensitized rats (16,25). It has also been reported that while pronounced eosinophilia is observed in the peripheral blood of rats at 3 WPI, the infected liver reveals massive eosinophil infiltration and degranulation (25-28). It is likely that eosinophil infiltration in the damaged liver may be due to the non-specific degranulation of mast cells in the connective tissue of the triads, or to toxic products from the parasite. It is possible that these cells may participate in the defense against the parasite in rats, as stated by Chauvin et al. (26) and Bossaert et al. (27). In 2 rats, flukes with attachment of a few inflammatory cells to the tegument were found within the hemorrhagic tunnels. Tliba et al. (1) have documented that during the first week of infection inflammatory infiltrate was not observed to be in contact with the juvenile flukes and have suggested that cellular responses did not reach the parasite in the early period of infection.

Chronic migratory tracts were seen at 4-5 WPI, and their development increased, peaking at week 8 postinfection, consistent with the results reported by Poitou et al. (20). Chronic fasciolosis was generally characterized by severe periportal and periductal fibrosis, along with diffuse mononuclear cell infiltration at the periphery of the migratory tracts, as reported in primary and/or secondary *F. hepatica* infection of sheep (21,23), goats (6,24), and calves (27). The inflammatory infiltrates were common in rats with a parasite burden, indicating that the local immune response might be related to the number of flukes, the amount of excretion-secretion antigens released, and to the number and size of the lesions in the liver, as reported by Tliba et al. (1). Multinucleated giant cells and severe bile duct hyperplasia were also evident in some rats with chronic fasciolosis, as has been found in goats (6). This finding was supported by the persistence of lymphoid follicle formation in the areas surrounding the migratory tracts and bile ducts. A marked hyperplasia of the lymphoid follicles and medullary cords in the hepatic and mesenterial lymph nodes was detected in the infected rats, suggesting an intense local immune response against *F. hepatica.* These results coincide with findings previously reported in rats (1) and goats (7). Our study also found many mitotic figures in the liver and positive immunolabeling of hepatocytes, fibroblasts,

and lymphoid cells for PCNA. This indicates a capacity for regeneration and healing in the damaged liver, except for areas of lesion which developed into cirrhosis, consistent with results reported by Tliba et al. (1), who found that the regeneration of damaged liver cells in rats infected with *F. hepatica* was completed about 8 WPI.

Immunohistochemical labeling of $CD3^+$ T and CD79αcy $^+$ B lymphocytes, and λ IgG $^+$ plasma cells in the hepatic lesions, main bile ducts, hepatic and mesenterial lymph nodes, and Peyer's patches indicated development of local cellular and humoral immune responses in *F. hepatica*-infested rats. This is consistent with findings reported in rats (1), calves (27), sheep (21,23,29), and goats (6,24). It is accepted that T cells are important in resistance to subsequent infections by the liver fluke in rats (25) and that T cells may also be involved in helping and selecting specific antibody responses and in activating eosinophils (28). It was noticeable that the differences between the infected groups were more related to the extent of the infiltrate than to its cellular composition, with an insignificant increase in the IFN-γ-treated and IC groups compared to the AG-treated and control rats. A partial increase in $CD3^+$ T cells in the IFN-γ-treated group might have been caused by the administration of IFN-γ. Likewise, a close relationship between lymphocyte proliferation and IL-2 production has been reported in cattle infected with the fluke (5). The inflammatory infiltrate in the areas surrounding the hemorrhagic tracts at 2 WPI was distributed throughout the liver. It was composed mainly of polymorphnuclear leukocytes, macrophages, and a few $CD3^+$ T lymphocytes, in accordance with previous findings in rats (4) and goats (6,24). High numbers of $CD3^+$ T cells were found around the old migratory tracts and in the portal spaces of rats with chronic fasciolosis. This was probably due to the chemoattractive molecules produced by flukes, consistent with findings in sheep chronically infected with flukes (23). Poitou et al. (20) reported that while the percentage of circulating T lymphocytes decreased during experimental rat fasciolosis, T cell accumulation increased in the infiltrate surrounding the migratory tracts in the liver. Moreover, a positive correlation was evident between lymphocyte proliferation, parasite burden, and the degree of hepatic damage, as reported by Clery et al. (30). In contrast to $CD3^+$ T cells, low numbers of CD79αcy $^+$ B lymphocytes were found in the areas surrounding the migratory tracts and the bile ducts, even though a diffuse reaction of B cells was detected in the lymph nodes and Peyer's patches. These results are in agreement with those found in sheep (21), goats (7), and cattle (30) infected with the liver fluke.

The marked increase in λ IgG $^+$ plasmocytes, in addition to B cells in the hepatic lesions and the lymphoid organs, is indicative of a strong local humoral immune response in rats, as reported by others (23,24,26,28). It is thought that in damaged livers and related lymph nodes, B cell infiltration and differentiation into IgG-bearing plasma cells may occur as a response to continuous stimulation by parasite antigen in chronic cases (4,28). Infiltration by λ IgG $^+$ plasma cells increased from 3 WPI to 8 WPI, as documented previously in sheep (23), goats (24), cattle (28), and rats (1). Although an intense local immune response was found in the livers of *F. hepatica*-infected rats, it has been reported that this immune response did not prevent hepatic damage in subsequent infections and that it did not kill either immature or mature flukes in the hepatic parenchyma (20,23,24,29).

In conclusion, an intense local immune response against *F. hepatica* was found in the damaged livers, hepatic and mesenterial lymph nodes, and Peyer's patches, where cellular and humoral responses with numerous $CD3^+$ T and CD79αcy $^+$ B cells and λ IgG $^+$ plasma cells predominated. Although there was no marked difference in the severity of hepatic lesions between the infected groups, the amount of inflammatory infiltrate was found to be somewhat higher in the IFN-γ-treated rats than in rats from the AG and IC groups. The number of flukes recovered from the IFN-γ-treated rats was lower than the numbers taken from the other infected groups, which might indicate a parasiticidal effect of NO. The increased parasite burden in AG-treated rats might also reflect the inhibition of iNOS expression and NO production by AG.

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