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Cellular responses in the small intestine and liver of Fasciola hepatica-infected rats

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Introduction

Helminths have been reported to stimulate pronounced polyclonal IgE-responses, consisting of both parasite-specific and parasite-non-specific IgE, resp. (JARRETT and MILLER 1982). Recently, PFISTER et al. (1983) also reported similar responses in *Fasciola hepatica* infected rats. Studies using predominantly the rat *Schistosoma mansoni* model and associated **in-vitro** tests have revealed that, depending on the stage of infection, parasite-specific IgE or specific IgE containing immune complexes is a prerequisite in Antibody-Dependent Cell-Mediated Cytotoxicity reactions (ADCC; BUTTERWORTH et al. 1974; CAPRON et al. 1975; CAPRON et al. 1982). Support that IgE is also active **in-vivo** has been provided by the induction of a selective IgE suppression in *Trichinella spiralis* infected rats (DESSEIN et al. 1981). In the immunosuppressed animals, there was a decreased larval encystment together with reduced peripheral and tissue eosinophilia. The effector cells in ADCC to helminthic infections are eosinophils, neutrophils and macrophages (CAPRON et al. 1982). Mast cells which also increase during helminthic infections, are not of themselves cytotoxic to parasites, however they are capable of potentiating the cytotoxic activities of eosinophils in the rat *S. mansoni* model (CAPRON et al. 1978 a, b). By interacting with anaphylactic antibodies, mast cells release ECF-A (Eosinophil Chemotactic Factor of Anaphylaxis) which may increase both the eosinophil population and the expression of eosinophil Fc receptors, resp.

A striking feature of many gastrointestinal nematode infections including *Nippostrongylus brasiliensis, Trichostrongylus colubriformis, Trichinella spiralis* etc. is an accumulation of intestinal or mucosal mast cells (MMC; MILLER and JARRETT 1971; ROTHWELL 1975; RUITENBERG and ELGERSMA 1976; BEFUS et al. 1979). According to ASKENASE (1980), the occurrence of mucosal anaphylaxis in reinfected animals and the interaction of MMC with parasite-specific IgE is thought to take part in the immune expulsion of nematodes. MMC contain a neutral protease, rat mast cell protease II (RMCP II), which is antigenically distinctive and it may be measured as an indicator of MMC activity using the ELISA test (WOODBURY et al. 1978; MILLER et al. 1983). Consequently, MILLER et al. (1983) demonstrated that in *N. brasiliensis* primed rats, RMCP II was systemically released after receiving a challenge infection, indicating MMC activation. It was later shown by WOODBURY et al. (1984) that MMC were also active during the immune elimination of primary infections of *N. brasiliensis* and *T. spiralis.*

Goblet cell proliferation has also been described in various gastrointestinal parasitic infections (MILLER and NAWA 1979). WALKER et al. (1977) found evidence that the immune system promotes the synthesis and release of intestinal mucus and in subsequent work, MILLER and NAWA (1979) suggested that specifically sensitized T-cells stimulate the differentiation and proliferation of goblet cells (GC). A further analysis in

N. brasiliensis infections revealed that the mucus secreted by GC of immune rats may trap adult worms and act as barrier to infection by larvae (MILLER et al. 1981). In conclusion, it appears to be an orchestrated cooperation of various cell types together with IgE in the immune response to helminthic infections. It seems that the intestinal mucosa plays a particularly significant role in the interactions of gastrointestinal nematode infections. The observation of tissue eosinophilia in the intestinal wall of *F. hepatica* infected rats (DOY et al. 1981 b) is similar to the host responses cited above with other helminths and suggests that the immune mechanisms in fascioliasis may be similar on the intestinal level. As yet, the time course of eosinophilic infiltration into the intestinal wall and the prevalence, kinetics and possible role of intestinal MMC and goblet cells in *F. hepatica* infections have not yet been studied. In this paper we present some preliminary results on the cellular reactions in the intestinal wall which are part of a comprehensive study on the immunopathogenetic mechanisms in *F. hepatica* infections.

Material and Methods

Animals / Infection

Fourteen groups of five rats/group (outbred CIBA-GEIGY-strain, male, 150 g) have been infected with 20 metacercariae of *F. hepatica* */rat as shown in table 1. The infection has been performed by gastric probe and general ether anaesthesia. Eleven groups of five rats served as controls.

TABLE 1:
Experimental design (Infection, killing incl. blood and organ sampling)

	Day 0	Day 7	Day 28	Day 35
Monoinfection	20 Mc	I	–	I
Reinfection	20 Mc	–	20 Mc	I
Control	–	I	–	I

20 Mc = Infection with 20 metacercariae of *F. hepatica*
I = Killing of one group incl. serum- and organ-sampling

Blood sampling / Killing

Rats have been anaesthetized, bled retroorbitally for haematological, biochemical and serological analyses and thereafter killed. Collected sera have been stored at −20° C until use.

Post-mortem / Fixation of tissue

Small intestine and liver

Four successive intestinal segments of five cm of length, beginning from the pylorus have carefully been released from their contents and washed repeatedly with PBS using a syringe. Thereafter, the isolated segments have been perfused with Carnoy's

* Kindly donated by Dr. F. Speiser, Swiss Tropical Institute, 4051 Basel, Switzerland

fixative, processed further using the Swiss-Roll-technique (BEXTER, 1978), placed in Carnoy's fixative for 24 hours and eventually embedded in paraffin. Two slices of liver (1–2 mm each) from *F. hepatica* affected lobes (corresponding lobes from controls) have also been removed and processed as above.

Histology

The embedded small intestinal tissue was prepared to give sections (5–6 μm) of a longitudinal axis of the intestine and of a right angle to the surface of the mucosa. Liver tissue sections (5–6 μm) have been prepared and subsequently stained with HE and Astrablue-Saffranin.

Stainings

Haematoxylin / Eosin (HE) for the counting of eosinophils, neutrophils, plasma cells and lymphocytes.

Perjodic-Acid-Schiff (PAS) for the counting of goblet cells.

Astrablue-Saffranin for the staining of MMC. According to ENERBAECK (1966) and MILLER and WALSHAW (1972) it provides a distinctive staining of MMC. The distinction is based on the development of blue granules which contain weekly sulphated mucopolysaccharides. The granules of connective tissue mast cells (CTMC) with strongly sulphated mucopolysaccharides stain red.

Microscopy / Cell counts

Small intestine

The small intestinal tissue sections have been examined using a projector-microscope (Reichert Austria, 125× for MMC; 620× for other cells). The MMC counts have been performed on a 10×10 cm field (42 dots, distance 16,5 mm) using a totalisator (Mod. 630, Fistronic AG, Zürich). The other cells have been counted manually on the same field (cf. above).

The number of cells has been calculated according to the formula:

$$x = NZ \times \frac{1}{FxNFx\,\frac{Pin\ (Pex)}{NF \times 42}}$$

x = Number of cells/mm^2 mucosa (MMC, Eos., etc)
epithelium (goblet cells)
NZ = Total of counted cells
NF = Total of fields
Pex = Number of dots outside the surface to be counted: MMC, Eos.
Pin = Number of dots inside the surface to be counted: goblet cells
F = Size of the original field (i. e. 125 × = 0,64 mm^2, 620 × = 0,026 mm^2)

Liver

Liver tissue sections have been analysed semiquantitatively for MMC. The average of liver MMC/rat has been calculated from the numbers of MMC of ten equal fields of liver tissue sections as defined by:
a) the presence of a hepatic trias in the center and
b) an optical field as delimited by a 400 × magnification.

Results

Small intestine

The counts of MMC, eosinophils and goblet cells after primary and secondary infections are presented in Fig., 1, 2.

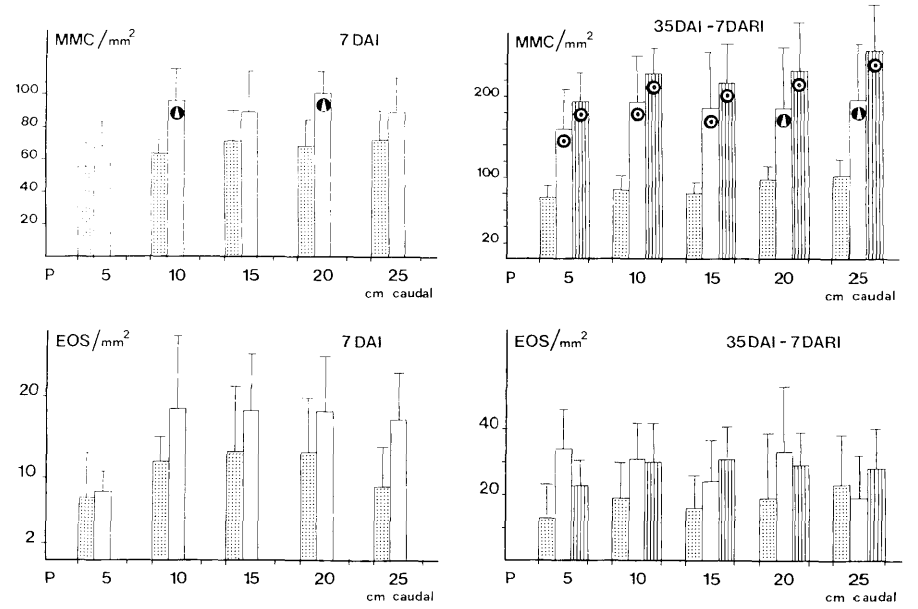

Fig. 1: Mucosa Mast Cells and Eosinophils in the small intestine of *F. hepatica* infected rats.

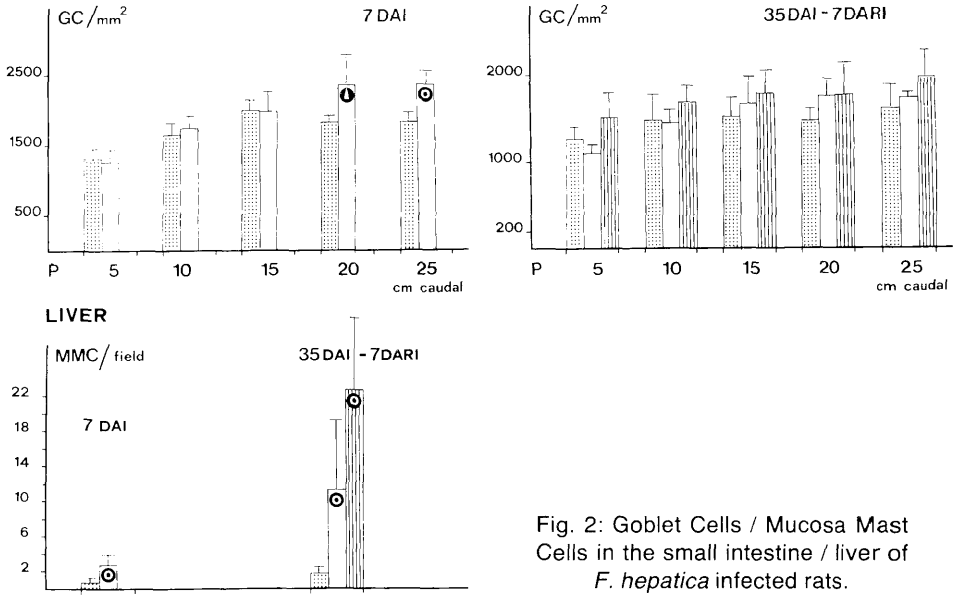

Fig. 2: Goblet Cells / Mucosa Mast Cells in the small intestine / liver of *F. hepatica* infected rats.

Legend to Fig. 1 and Fig. 2:

MMC = n Mucosal Mast Cells/mm^2 Mucosa
EOS = n Eosinophils/mm^2 Mucosa
GC = n Goblet cells/mm^2 Epithelium
MMC / field = mean n MMC/10 optical fields (400×)
DA(R)I = days after (re)infection (20 metacercariae)
P = Pylorus
cm caudal = cm caudal of Pylorus


= mean + standard deviation of control rats


= mean + standard deviation of primoinfections


= mean + standard deviation of reinfections


= $p < 0,05$ of the group vs control group


= $p < 0,01$ of the group vs control group

The results were analysed using the F-test.

MMC

It is clearly evident that intestinal MMC increased within 7 days after primary infection of rats with *F. hepatica*. This phenomen became more pronounced 5 weeks after primary infection. An even greater increase in MMC occurred in rats 7 days after reinfection. The levels of MMC along the longitudinal axis of the intestinal wall in control rats remained more or less constant.

Eosinophils

The time sequence in the changes of eosinophil levels was similar to that of MMC. Eosinophils of infected rats increased within 7 days of infection when compared to controls and persisted for 5 weeks after the first infection, although some decrease was observed in the more distal part of the intestine. Generally higher eosinophil counts were obtained in reinfected animals. There is no preferential site of eosinophil accumulation in the various segments of the intestines, on the contrary, a general increase of eosinophils occurs with the age of the infection. Lower increases of eosinophils can also be seen in control rats, which raises to the question whether the presence of eosinophils is age dependent.

Goblet cells

Very little difference is seen in the goblet cell population 7 days after primary infection. Nevertheless, there are some differences between infected rats and controls in the more caudal areas of the small intestine of the two groups. At 5 weeks after primary infection, the differences have almost completely disappeared even though reinfected rats generally show slightly higher numbers of goblet cells in all investigated intestinal segments.

Liver

The results of the semiquantitative MMC counts in the liver trias are presented in Fig. 2.

The results demonstrate a slight increase of MMC in the hepatic trias of infected animals 7 days after primary infection. The difference between infected and control rats became much greater 35 days after primary infection and a still further enhancement of MMC was obtained 7 days after challenge.

Discussion

Small intestine

A number of gastrointestinal nematode infections induce marked intestinal MMC-responses which are dependent on the integrity of the host T-lymphocyte system (BEFUS and BIENENSTOCK 1982; JARRETT and HAIG 1984). To our knowledge, in trematode infections however, very little information exists regarding intestinal MMC proliferation, primarily from studies in sheep infected with *F. hepatica* (MURRAY et al. 1968). The increase of intestinal MMC in rats seven days after a primary infection (7 DAI) with *F. hepatica* observed in our studies is striking (Fig. 1). From the data we have obtained there could be an **in-vivo** release of parasite-derived factors which cause a mucosal mastocytosis, as it has been shown **in-vitro** by HAIG et al. (1982) in the *N. brasiliensis* model. The cellular changes are also correlated with the detection of increased RMCP II-levels in the small intestinal tissue of the rats (PFISTER et al., in prep.). Chronologically, the gut MMC proliferation response 7 DAI is consistent with the results reported by WOODBURY et al. (1984) in *N. brasiliensis* infected rats, which possessed MMC-hyperplasia from day 6 after infection. Although the *N. brasiliensis* and the *F. hepatica* infections differ considerably, the almost uniformly distributed increase of MMC (over the examined intestinal segments) after primary infections suggests sensitization after a primary infection and perhaps the presence of a local immune response.

Up to day 35 of infection, the number of MMC rose steadily reaching twice the levels of 7 DAI which suggests that there may be a continuous stimulation of these cells. However, at the present time it is not clear whether continuous antigen-release is necessary by migratory larvae or whether MMC-hyperplasia is triggered by a single event. The highest MMC-levels have been deteced in rats 7 days after reinfection (7 DARI) which suggests very high MMC activity.

This correlates with the presence of a high level of parasite-specific serum IgE which peaks at about 4 weeks after a primary fluke infection (PFISTER et al. 1983). It is not certain whether mast cells interact exclusively with parasite-specific IgE (JESKA 1985). Also, increased MMC and serum IgE after reinfection correlate well with local intestinal anaphylaxis in *F. hepatica* primed rats (DOY et al. 1981 a). However, no direct interactions between MMC and *F. hepatica* have been reported. To the contrary, in nematode infections, a systemic secretion of RMCP II during the expulsion of both, primary and secondary infections clearly demonstrates that MMC are active during the reaction (MILLER et al. 1983; WOODBURY et al. 1984). However, in fascioliasis, since expulsion does not occur at 4–5 weeks after infection when there is observed increase in MMC, the phenomenon may not be directly related to expulsion, but it may reflect the entry of preadult flukes into the bile ducts.

Similarly to the MMC, the intestinal eosinophils were stimulated within 7 DAI and reached levels that were generally higher than those in control rats although statistically not significant and an increased number of eosinophils was still detectable 5 weeks after primary infection (Fig. 1). There was no evidence that these eosinophil levels further increased after reinfection (7 DARI). Additional experiments are needed to determine whether this observation can be confirmed, however, our findings are consistent with those by DOY et al. (1981 b) which indicate that there is an intestinal eosinophilia in *F. hepatica* infected rats. The presence of eosinophilia in the small intestine does not prove the occurrence of immunologically mediated reactions, however, the observations may suggest that an immune feature exists as was shown in ADCC-reactions with other trematodes (CAPRON et al. 1982) and they are consistent with the findings by DOY et al. (1981 b).

The number of goblet cells, unlike MMC and eosinophils, does not change significantly between infected, reinfected and control rats, except that the reinfected animals (7 DARI) had slightly higher mean goblet cell counts than uninfected controls. In the small intestine, goblet cells also show a tendancy to increase from cranial to caudal. As yet, the function of goblet cell hyperplasia in fascioliasis is poorly understood and needs further investigation. It may be that these cells play a role in local defense during a *F. hepatica* infection and reinfection, resp. In *N. brasiliensis* and other nematode infections it has been shown that mucus may trap parasites and thereby prevent completion of the infectious process (MILLER et al. 1981).

Liver

Two observations were dominant in the involvement of the liver during the reactions of rats to early *F. hepatica* infections: Firstly, it is obvious that in the liver the MMC response is already present 7 DAI, at the time-period when the juvenile flukes just begin to enter the liver tissue. This observation reflects MMC activation prior to the migration of juvenile flukes across the liver. To date, the presence of MMC in the hepatic trias has been associated primarily with the presence of adult flukes in the bile ducts, and their subsequent elimination, resp. (MURRAY 1972). However, it remains to be further evaluated whether the association of juvenile flukes and MMC is an expression of systemic or of local immune response. Secondly, there is a dramatic increase of MMC in rats 35 DAI, and particularly 7 DARI. It was also found that at the time of MMC increase, there is a simultaneous secretion of RMCP II (PFISTER et al., in prep.). Since *F. hepatica* infections of the liver are usually associated with a considerable infiltration of eosinophils and macrophages, additional studies will help to clarify the interactions of MMC and phagocytic cells in the pathogenetic mechanisms of fascioliasis.

Summary

Rats infected with 20 metacercariae of *Fasciola hepatica* have shown highly increased mucosal mast cell (MMC) levels in the small intestine seven days after primary infection. Up to 35th day after infection, the number of gut MMC rose steadily, reaching twice the levels of seven day infections. There could be a possible continuous release of parasite-derived factors which stimulate an intestinal mucosal mastocystosis as was shown **in-vitro** in the rat *N. brasiliensis* model. The highest MMC-levels have been detected in rats seven days after reinfection. Although the differences between infected and control rats were not significant, the infected rats showed generally higher intestinal eosinophil counts. Also, the number of intestinal goblet cells did not appear

to change between infected and uninfected animals. In the liver, a MMC response had already appeared on day seven after infection which clearly reflects an activation of these cells prior to the liver entry of juvenile flukes. Thirty-five days after primary infection and seven days after reinfection, there is a dramatic increase of MMC in the hepatic trias which strongly supports the idea of an active involvement of these cells in the local inflammatory and defense reactions.

Zusammenfassung

Aspekte der zellulären Immunabwehr bei F. hepatica-Infektionen

Sieben Tage nach Erstinfektion (7 DAI) weisen experimentell mit *Fasciola hepatica* infizierte Ratten (20 Mc/Ratte) eine deutliche Vermehrung der Mukosa-Mastzellen (MMC) im Dünndarm auf. Die MMC-Werte sind 35 Tage nach Erstinfektion (35 DAI) im Vergleich zu den 7 DAI-Infektionen doppelt so hoch. Dies könnte auf eine kontinuierliche Freisetzung von parasitären Faktoren zurückzuführen sein, welche die intestinale MMC-Hyperplasie stimulieren, wie dies **in-vitro** im Ratten-*N. brasiliensis*-Modell gezeigt wurde. Die noch höheren MMC-Werte 7 Tage nach Reinfektion (7 DARI) geben die sehr starke Aktivität dieser Zellen in der Darmmukosa nach erneuter Antigenstimulation wieder. Obwohl die intestinalen Eosinophilenzahlen zwischen infizierten Ratten und Kontrolltieren statistisch nicht in allen untersuchten Darmabschnitten signifikant verschieden waren, wiesen die ersteren im allgemeinen höhere durchschnittliche Eosinophilenwerte auf.

Betreffend Becherzellgehalt ergaben sich innerhalb der untersuchten Darmabschnitte keine nennenswerten Unterschiede zwischen infizierten und nicht infizierten Ratten. Die Gesamtheit dieser Ergebnisse bestätigt das Vorkommen einer zellulären Reaktion in der Darmwand der mit *F. hepatica* infizierten Ratte. Die bereits 7 DAI feststellbare signifikante MMC-Antwort in der Leber widerspiegelt eine Aktivierung dieser Zellen vor der eigentlichen *F. hepatica* Lebermigration. Fünf Wochen nach Erstinfektion bzw. eine Woche nach Reinfektion kommt es zu einem massiven MMC-Anstieg in den Glisson'schen Dreiecken. Eine derartige Vermehrung weist auf eine aktive Rolle dieser Zellen in den lokalen Entzündungs- und Abwehrmechanismen hin.

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