for CMTI III by Wilusz et al.[6]: Arg-Val-Cys-Pro-Arg-Ile-Leu-Met-Lys-Cys-Lys-Lys-Asp-Ser-Asp-Cys-Leu-Ala-Glu-Cys-Val-Cys-Leu-Glu-His-Gly-Tyr-Cys-Gly.

The peptides were synthesized by the solid-phase procedure[8] using, in the case of the 28-peptide Boc-Tyr(2,6-Cl-Bzl)-Pam-resin (capacity 0.15 meq/g) and in the case of the 29-peptide Boc-Gly-O-CH_2-resin (capacity 0.20 meq/g). All deblocking, rinsing and coupling steps were carried out using an automatic peptide synthesizer (Beckman, Model 990). N-Boc-protection was used throughout the synthesis with the following side chain protecting groups: Arg(NO_2), Asp(OBzl), Cys(Acm), Gln(Xan), Glu(OBzl), His(Tos), Lys(2-Cl-Z), Ser(Bzl) and Tyr(2,6-Cl-Bzl). Double couplings in CH_2Cl_2 were performed using DCC. Deblockings were achieved using 33% TFA/CH_2Cl_2 with 2% dimethylsulphide. For neutralization 10% TEA/CH_2Cl_2 was used. After completing the synthesis, the Acm-peptides were cleaved from the resins with liquid HF at 0 °C in the presence of anisole. The Acm-peptides were purified on a Sephadex G-25F column. Both peptides gave the required amino acid composition on amino acid analysis. Then the Acm-groups were removed with mercuric acetate according to a previously described procedure[9]. The reduced peptides were desalted on a Sephadex G-10 column and reacted with an equimolar mixture of oxidized and reduced glutathione in Tris-HCl buffer (pH 8.5) at 35 °C for 20 h[10].

The oxidized 28-peptide based on the sequence as suggested by Nowak et al.[5] did not show any antitrypsin activity in the assay according to Erlanger et al.[11].

Yields obtained in the preparation and purification of synthetic 28- and 29-peptides

Product	Yield (%)
Crude Acm-28-peptide	53
Purified Acm-28-peptide (Sephadex G-25F, twice)	20
Reduced 28-peptide (removal of Acm groups, Sephadex G-10)	70
Crude Acm-29-peptide	42
Purified Acm-29-peptide (Sephadex G-25F, twice)	23
Reduced 29-peptide (removal of Acm-groups, Sephadex G-10)	67
Synthetic CMTI III (oxidation, affinity chromatography)	10

The oxidized 29-peptide with the sequence suggested by Wilusz et al.[6] was purified on a column of immobilized anhydrotrypsin. The peptide had the same antitrypsin activity as native CMTI III and gave identical electrophoretic and immunological[12] patterns, as well as similar UV and CD spectra. The synthetic peptide was modified with a catalytic amount of trypsin to the CMTI III* in the same manner as the native CMTI III.

Our results indicate that Wilusz et al.[6] presented the correct sequence for trypsin inhibitor CMTI III.

1 Acknowledgments. This work was supported by the Swedish Natural Science Research Council (K-GF 3020-107) and the Polish Academy of Sciences (Research grant No. MR I 12.1.6.1).

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7 Abbreviations. The symbols of amino acids and peptides are in accordance with the IUPAC-IUB Commission on Biochemical Nomenclature. All amino acids except glycine are in the L-configuration. Pam: the phenylacetamidomethyl group, Xan: the xanthyl group, TFA: trifluoroacetic acid, TEA: triethylamine, DCC: dicyclohexylcarbodiimide.

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Unisexual murine schistosomiasis: portal hepatitis in subacute infections[1]

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Summary. Liver changes induced by unisexual male infection with *S. mansoni* were studied in mice during 2–20 weeks post-infection, in order to distinguish changes related to released worm substances from changes related to schistosome egg deposition. In subacute unisexual infection the venous wall appears as the target for inflammation which remains focal for a long time and affects limited segments of the main portal veins. Schistosomal pigment deposited in the lobule does not induce inflammatory or fibrogenic reactions.

Key words. Unisexual schistosomiasis; liver pathology; portal hepatitis.

Most tissue changes in schistosomiasis are related to the immunopathological response of the host to the parasite[2]. Although extensive studies have shown that the determining factor in development of hepatosplenic schistosomiasis is the host granulomatous response to schistosome eggs which are trapped in the liver[3,4], it is generally accepted that development of the disease results in the synergistic action of multiple antigens from both the worm and the egg[5].

In schistosomiasis, there have been numerous reports indicating permanent circulation of toxic or antigenic substances produced by schistosomula or adult worms[6–8], allergens and hormones[9,10]. It has been suggested that secretions, excretions and breakdown products from living worms enter the liver via the portal circulation and could be at least partly responsible for hepatic changes[11,12]. Surprisingly, little attempt has been made to differentiate these immunopathological changes in the liver into those exclusively related to schistosome egg deposition and those related to released worm substances.

In the present study, we report on the hepatic changes induced in mice by living worms following unisexual male infection with *S. mansoni*.

Material and methods. Unisexual infection: The first group (n = 130) of Swiss albino SWR mice, weighing 18–20 g, was infected by 300 cercariae of *Schistosoma mansoni* (PR strain)

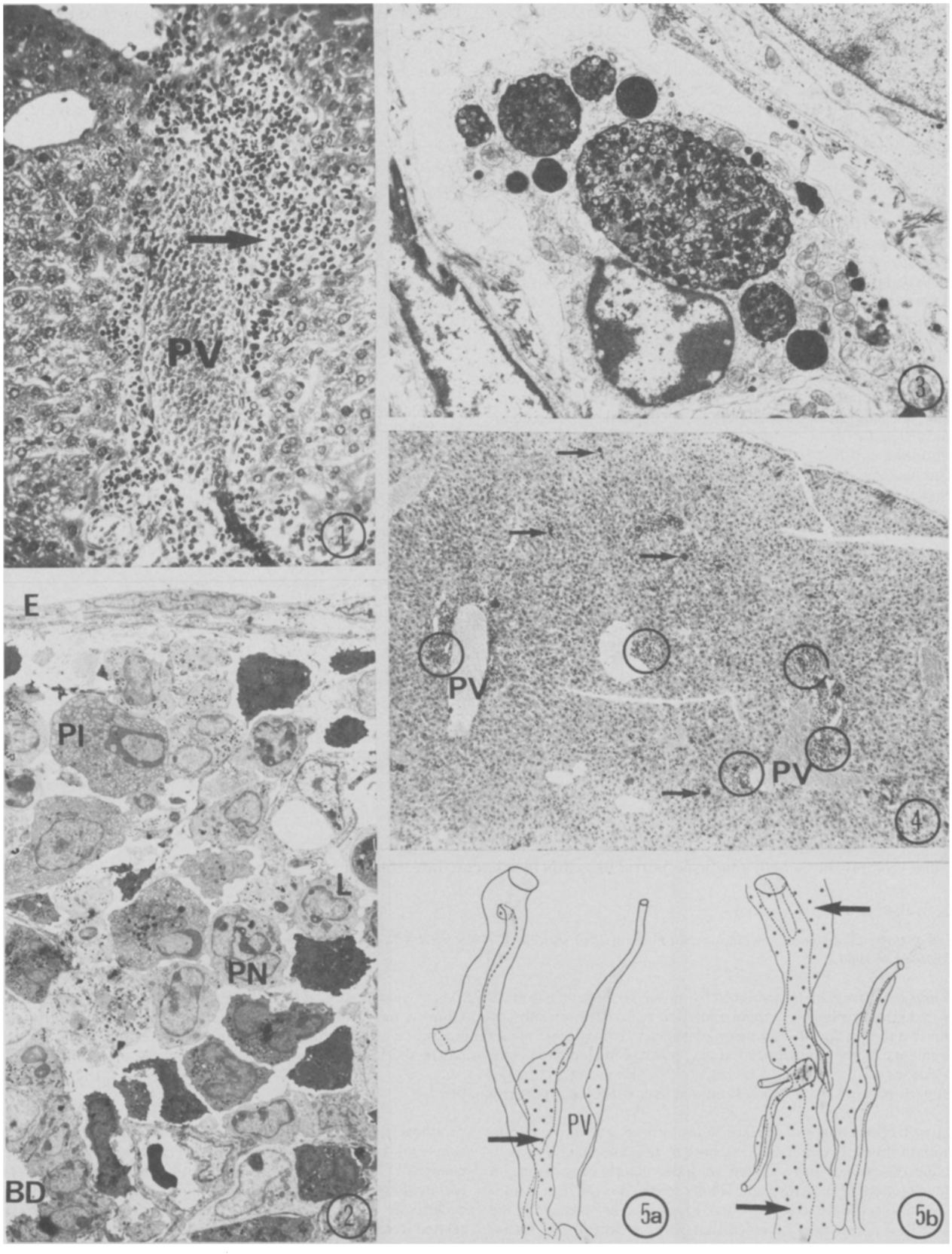

Figure 1. Venous and perivenous portal inflammatory infiltrate 5 weeks after unisexual male infection (Masson Trichrome). × 500. PV: portal vein; → main intraparietal inflammatory focus.

Figure 2. Electron microscopic appearance of subendothelial and intraparietal portal inflammatory infiltrate 5 weeks after unisexual male infection. × 2300. BD: bile duct; E: endothelial cell; L: lymphocyte; Pl: plasma cell; PN: polymorphonuclear leucocyte.

Periportal changes in the livers of mice unisexually infected with *S.mansoni* (U) compared with bisexually infected controls (B)

Weeks after infection	(1)		Macrophages (M)		Lymphocytes (Ly)		Plasma cells (Pl)		Eosinophils (Eo)		Neutrophils (Ne)		Fibroblasts (F)		Fibrosis		Prominent cell type	
	U	B	U	B	U	B	U	B	U	B	U	B	U	B	U	B	U	B
0 week	0%	0%	–	–	–	–	○/–	○/–	○	○	○	○	○	○	○	○		
< 5 weeks	< 10%	< 10%	–	–	–	–	○/–	○/–	○	○	○/–	○/–	○	○	○	○	M	M
5–10 weeks	≦ 50%	≧ 80%	+/++	+/++	+	+	+	++	–	+++	+	+	○	+	○	–	M	Eo
15 weeks	≦ 70%	≦ 100%	++	++	++	++	++	++	–	+	+	+	–	++	○	++	M Ly Pl	M F
20 weeks	≧ 80%	100%	++	++	++	+/++	++	++	○	–	+	+	–	++	–	+++	M Ly Pl	M F

(1) Presence of periportal inflammation; amount percent estimated on 50 portal spaces. ○ absent, – rare, + few, ++ numerous, +++ very numerous.

obtained from one snail (Biomphalaria glabrata), exposed to one miracidium[3]. Pure male or female schistosome infections were performed, only male infection was selected for this work. Mice were sacrified at 2, 3, 5, 10, 15 and 20 weeks after infection in batches of 10 to 15 animals. A second group (n = 25) was infected simultaneously with cercariae of both sexes to serve as a bisexual infected control. A third group (n = 13) was maintained as the uninfected control. Mice from the second and third groups were sacrified at 2, 3, 5, 10, 15 and 20 weeks in batches of two animals.

Worm recovery: The perfusion method of Duvall and Dewitt[14] was used for worm recovery and numeration.

Light microscopy. Liver specimens were fixed and treated by standard histological methods for light microscopy. Silver impregnation was performed following Gordon-Sweet's method. Serial sections of two blocks were performed on a segment 176 µm thick for planimetric reconstruction of the portal vascular tree.

Electron microscopy: For EM, livers were fixed in osmium tetroxide (2%), buffered in sodium cacodylate (0.3 M) at 4 °C for 1 h. After ethanol dehydration and embedding in Epon, ultrathin sections were stained with uranyl acetate and lead citrate, and examined using a Philips EM 300 microscope.

Results. Unisexual (male) infected mice (see table). 2–3 weeks after infection, limited modifications were observed in liver tissue which included small portal (< 5% of portal spaces) foci of inflammatory cells associated with the venous wall (see below) and Kupffer cell hyperplasia with intracellular pigment deposition.

5 weeks after infection, no macroscopic change was noted. Light microscopy showed portal and periportal inflammatory infiltrate, affecting up to 20% of the portal spaces and comprising a mixed cell population of monocytes, macrophages (slightly predominant) and polymorphonuclear leucocytes and lymphocytes (figs 1 and 2). No change was noted within the lobule affecting hepatocytes except for reticulo-endothelial and perisinusoidal cell hyperplasia. Electron microscopy of Kupffer cells and perisinusoidal cells confirmed the presence of secondary lysosomes which were loaded with electron dense deposits of typical schistosomal pigment, associated with other irregular, dense substances.

10 weeks after infection, macroscopically the livers were brown or black. The sinusoids exhibited marked Kupffer and sinusoidal cell hyperplasia with obvious deposition of schistosomal pigment in these cells, macrophages and Kupffer cells (fig. 3). Portal and periportal inflammatory infiltrates affected 50% of the portal spaces. Transverse sections of the portal veins revealed that the infiltrate did not appear circumferential, the affected portal venous wall and some portal tracts showing only a slight inflammatory infiltrate which was focal (fig. 4). The cell composition of the infiltrate was similar to the 5-week unisexual infections.

20 weeks after infection, inflammatory infiltrate affected more than 80% of the portal tracts. Focal or submassive necrosis resulting from embolization of dead worms was occasionally observed.

Worm recovery: About 10% (9% ± 0.85) of living adult worms were recovered in the different series of animals.

Portal vein visualization by serial section and planimetric reconstruction: Figures 5a and b demonstrate the irregular distribution of the infiltrate along the portal venous axis. Some portal segments were devoid of inflammatory infiltration. Irregularity of the portal luminal size unrelated to the presence of inflammatory infiltration was also noted in one segment.

Bisexually infected mice (see table). Before the 5th week after bisexual infection, i.e. before egg deposition, light microscopy of the livers showed the same limited modifications as those observed in unisexually infected mice.

5 to 10 weeks after infection, more than 80% of the portal spaces were affected by the inflammatory reaction and numerous egg granulomas were observed. A considerable number of eosinophils were present both around egg granulomas and about the portal spaces. The number of eosinophils decreased up to the 10th week after infection, whereas lymphocytes macrophages and plasma cells were more numerous.

A marked portal fibrotic reaction was observed up to the 15th week after infection. It affected all the portal tracts and showed a cell population essentially composed of macrophages and fibroblasts.

Discussion. Compared to granulomatous inflammation around the schistosome eggs, or fibrosis, portal hepatitis is not generally considered to be a prominent feature in human and experimental hepatosplenic schistosomiasis. This is probably because it is difficult, if not impossible, to distinguish it from the periovular portal lesions which are always present in bisexual infections. Therefore Andrade[2] observed 'pathogenic importance has only rarely been ascribed to it ... However, the portal hepatitis of schistosomiasis seems to be more than a simple portal infiltration'. Previous reports have mentioned portal infiltrates following unisexual infection in mice or bisexual infection before egg production[15–17]. Our results indicate that portal inflammatory

Figure 3. Enlarged macrophage containing pigment (phagosomal and residual form) from an intralobular cluster. × 6000.

Figure 4. Portal inflammatory foci (○) 20 weeks after unisexual male infection (Masson Trichrome). × 120. PV: portal vein; → perisinusoidal clusters of schistosomal pigment.

Figures 5a and b. Three-dimensional representation based on spatial drawing after planimetric reconstruction of portal veins 20 weeks after infection, showing the segmental and irregular inflammatory jacket around portal veins. PV: portal vein; → inflammatory areas.

infiltration can be detected by light microscopy during the very early course of unisexual male infection: 2–5 weeks after infection, 5–20% of the main portal venous walls showed one or more foci of inflammatory cells.

Compared to bisexual infected controls, during the first 10 weeks:

– the periportal inflammatory reaction was less important after unisexual infection,

– the cell population was dominated by macrophages and lymphocytes in the unisexual infection, while eosinophils were the prominent cells in the bisexually infected controls.

This may be explained by the fact that, in murine schistosomiasis mansoni, eggs deposited in the liver induced a chronic cell-mediated granulomatous reaction in which eosinophils are predominant[3].

Their role in the destruction of eggs has been previously demonstrated[18, 19, 20]. It has been suggested that the miracidial antigenic material induce the production by the lymphoid cells present in the granulomas of a lymphokine which attracts eosinophils to the close vicinity of the eggs[21, 22]. Furthermore, a high mol.wt eosinophil chemotactic factor recently purified from *Schistosoma mansoni* eggs has been claimed to act in bisexual infection[23]. However, in the absence of egg deposition, i.e. unisexual infection, portal infiltrate remains to be explained. It is known that during the first weeks after infection schistosomula and adult worms release numerous substances which are carried by the blood to the liver; the mesenteric and portal veins being the natural habitat of *Schistosoma mansoni*. Except for pigment, the exact destination and effect of each substance is not clearly demonstrated, in spite of documented and interesting trials[24, 25]. It seems most probable that they display in situ effects which differ, depending on the biochemical nature of the substance.

Schistosomal pigment derived from the host blood is known to be inert and does not provoke inflammatory or fibrogenic reactions[2, 26, 27]. In unisexual infections pigment is found, from the early stages, in the lobule, trapped in the reticulo-endothelial and Kupffer cells. It has been demonstrated that intracellular pigment deposition is roughly proportional to the number of worms present[26, 27]. It indicates, in our case, a normal intravascular development of the male worms confirmed by the normal recovery rate of adult specimens. As in bisexual infection, cell pigment deposition occurred rapidly and clusters of macrophages were observed in the sinusoids. No inflammatory or fibrogenic reaction was detected however. Compared to adjacent parenchyma, a significant increase in pigment deposition was not found in foci of portal hepatitis and other factors must probably be involved.

Recently, different types of proteinase activity, including an endoprotease with trypsin-like activity, have been demonstrated in the secretory products of schistosomula[28]. This is of particular interest with respect to the pathogenesis of the very early portal changes. In fact, 8 days after infection schistosomula are present in the portal venules. There, they start to ingest erythrocytes and grow actively until the time of the mesenteric migration, as a young adult, on the 26th day. Our observations clearly indicate that during this period, the first detected inflammatory foci affect the portal venous wall and spread subsequently to the adjacent connective tissue and the whole portal tract, including bile ducts and artery. It is possible that the proteolytic product released by the schistosomula and worms could affect the venous endothelial cells as the first target. Since such endothelial changes do not necessarily require inflammatory cells[29], an endothelial disruption or a permeability change may occur. Subsequent diffusion of the multiple schistosome circulating products within the portal venous wall would trigger the host inflammatory response, namely the portal hepatitis.

During the early stage and up to 20 weeks, the inflammatory reaction remains limited to the portal area without any tendency to invade the hepatocyte limiting plate. Reconstruction of a segment of the portal vascular tree showed that the cellular infiltrate did not display a circumferential distribution around the vessel but rather a segmental one. This favors an initial local phenomenon, rather than a diffuse vasculitis which would involve the whole portal venous tree. At this stage, fibrosis was not prominent and except for pigment deposition, no changes were observed within the lobule. This last point attracted our attention; no inflammatory focus was detected outside the portal veins. However, schistosomal products are carried along the sinusoids since endothelial and Kupffer cells show intracellular lysosomal pigment deposition.

It would seem reasonable to propose that the portal venous endothelial cell possesses specific microdomains on its luminal surface which are sensitive to schistosome products, these characteristics being absent from the limiting sinusoidal endothelial cells. Micro-heterogeneity of the endothelial cell population and segmental distribution along the microvascular tree of binding sites for specific proteins has been clearly demonstrated in the liver[30].

Fine endothelial labeling of binding sites and subsequent discriminative localization of schistosome products in vivo may confirm this hypothesis.

1 This work was supported by a contract MIR B 5383 L 0953 and assistance from the Société d'Hépathologie Expérimentale.

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