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Morphology of Chronic Collagen Resorption

A Study on the Late Stages of Schistosomal Granuloma Involution

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Hepatic periovular schistosomal granulomas produced in mice with 50 cercariae per 10 weeks of infection were seen to involute following curative treatment of schistosomiasis. Residual scars remained, however, and the collagen tissue in them presented morphologic evidence of a slow, progressive degradation that lead to the almost complete disappearance of the lesions 4.5 months after chemotherapy. Ultrastructural changes indicative of collagen degradation were represented by focal lytic dissolution of collagen fibrils and/or by the transformation of such fibrils into a granular electron dense material, a picture different from that seen in more acute models of collagen resorption. In older, involuting granulomas, both type I and III collagens were found up to the end of the resorption process. The immunofluorescence staining for procollagen III and fibronectin correlated with collagen synthesis and the amount of both decreased as the granulomas involuted. Antibodies to CB-peptide (a2 $CB^{3,5}$ from type I collagen) appeared as a good marker of acute collagen degradation only. Laminin and collagen type IV were absent from the granulomas. Periportal fibrosis produced in mice with 30 cercariae per 20 weeks of infection also presented progressive degradation of collagen 2 and 3 months following curative treatment of schistosomiasis. Present findings suggest that there is not an irreversible fibrotic stage in murine schistosomiasis, that chronic collagen resorption presents peculiar ultrastructural features, that the genetic collagen types are not differentially removed, and that periportal fibrosis in schistosomiasis undergo degradation in a way after specific treatment similar to periovular granulomas. (Am J Pathol 1988, 132:389–399)

THE SCHISTOSOMAL periovular granuloma is an essentially fibrogenic lesion. As a model, the schistosomal granuloma has been used to identify the soluble factors involved in fibroblast stimulation and collagen synthesis,[1] to study the sequence of deposition of genetically different types of collagens,[2] and to investigate the pathogenesis of pipe-stem fibrosis of the liver.[3,4] The morphology of collagen formation and degradation has also been explored with this model.[5] It has been demonstrated that, soon after curative treatment of schistosomiasis, hepatic periovular granulomas start to involute. They then present an ultrastructural composite picture of fibroblast hyperplasia plus extracellular collagen breakdown and internalization or phagocytosis of collagen fragments by macrophages, fibroblasts, and myofibroblasts.[5]

The sequential morphologic changes seen in involuting periovular granuloma are similar therefore to those observed in other models, such as during the involution of the rat uterus,[6] the reversal of rat cirrhosis after discontinuation of carbon tetrachloride administration,[7] or during the rapid fibrotic turnover of carrageenin granuloma.[8] All these examples represent instances of a more of less "acute" process of collagen resorption, lasting from hours to less than a month.

When a mouse infected with *Schistosoma mansoni* is cured by chemotherapy, the granulomatous lesions in the liver shrink progressively afterwards.[9] Four to six months after treatment, fibrotic granulomas have almost disappeared, with only a few small pigmented scars remaining.[10,11] This indicates that the process of

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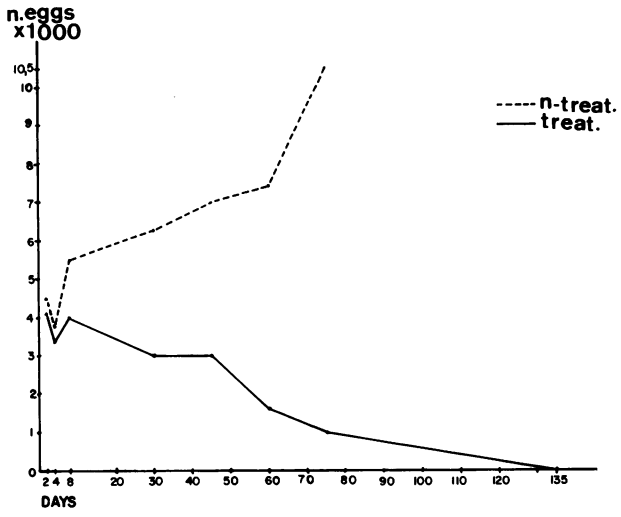

Figure 1—Number of eggs per gram of tissue in the livers of mice infected with 50 *S. mansoni* cercariae for 10 weeks and then submitted to specific treatment. Controls were not treated. Each point represents the average of the counting for 2 animals.

collagen resorption in schistosomiasis follows a slow progressive course after the initial changes of acute degradation have subsided. This later phase of collagen degradation has not been studied extensively; it is not known what factors are involved in the process of protracted fibrous resorption and its morphology needs to be investigated.

In the present investigation, the morphology of hepatic schistosomal granulomas has been sequentially analyzed in mice infected with *S. mansoni* up to 4.5 months after curative chemotherapy. The changes observed in the later stages of granulomatous involution differ from those observed in other models of collagen resorption.

Materials and Methods

Outbred albino Swiss mice of both sexes, weighing 20–22 g and maintained on a commercial balanced diet *ad libitum* were used. They were infected by the transcutaneous route with 50 cercariae of the Feira de Santana strain[12] of *S. mansoni*. After 40–45 days, they began to eliminate viable schistosome eggs in their stools. Eight weeks after exposure, half of the animals were separated and subjected to simultaneous administration of hycanthone (given in a single intramuscular injection, 80 mg/kg of body weight per animal) and oxamniquine (pure salt, administered by gastric tube in a single dose of 100 mg/kg body weight per animal), as has been previously recommended for curative treatment.[13] The nontreated animals remained as infected controls.

Two animals, male and female, were killed just before treatment. From then on, 2 treated and 2 untreated infected controls were killed 2, 4, 8, 15, 30, 45, 60, 75, 80, and 135 days after treatment. On day 135, the remaining 15 treated animals were killed. All infected controls had died by this time.

Another group of animals, infected with 30 cercariae, received the same treatment as above when they were in the 20th week after exposure, while a comparable group of nontreated animals remained as infected controls. They were killed 1, 2, and 3 months afterwards, together with 2 infected controls at each time.

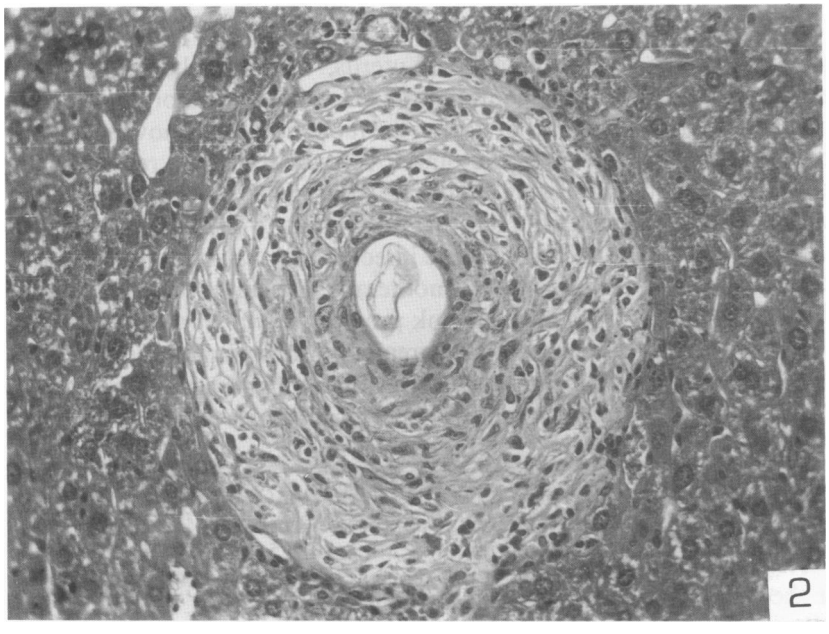

Figure 2—Aspects of one periovular granuloma 60 days after treatment. In the center there is an empty and shrunken egg shell surrounded by rather fragmented and irregular fibers, fibroblastlike and mononuclear inflammatory cells. Note the sharp demarcation line between the granuloma and hepatic parenchyma. (H&E, ×200)

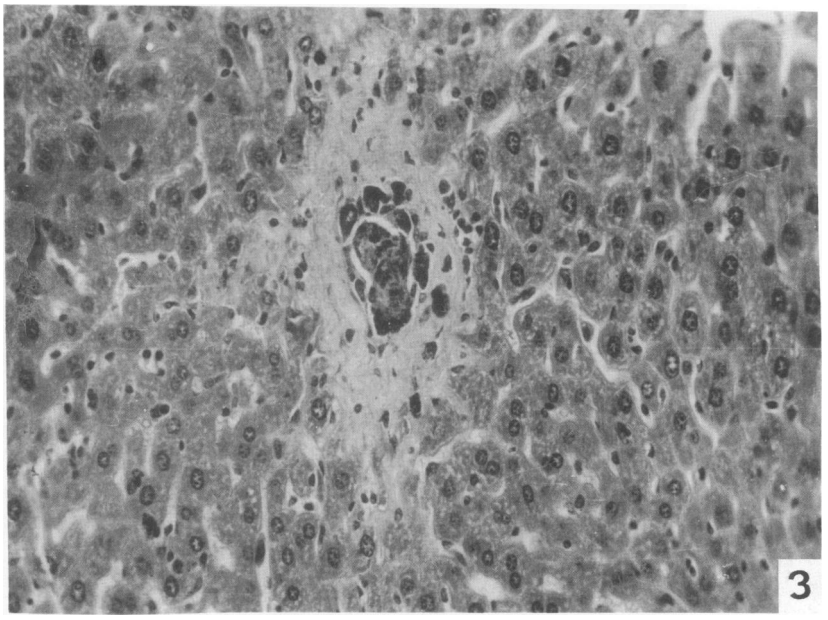

Figure 3—Remnants of a schistosome egg encircled by pigmented phagocytic cells in the center of a fibrotic area. This mouse was treated for schistosomiasis 4.5 months previously. The connective tissue in this involuting scar acquired a homogenous appearance, despite the presence of areas of different densities. (H&E, ×250)

Parasitology

The animals were killed by neck dislocation. The liver, mesenteric, and portal veins were perfused with iced saline solution for recovering and counting of adult worms, according to Duval and DeWitt's method.[14]

Fragments of liver tissue were weighed and left in 0.5% potassium hydroxide solution for digestion and counting of schistosome eggs, according to the method of Cheever.[15] The results were expressed as number of eggs per gram of tissue. Pieces of liver and intestines were mashed between 2 glass slides and used for the microscopic identification of the different types of eggs (oogram), as described by Prata.[16]

Histology

Thin slices of the liver were fixed in Bouin's fluid for 6–8 hours and then preserved in 70% alcohol.

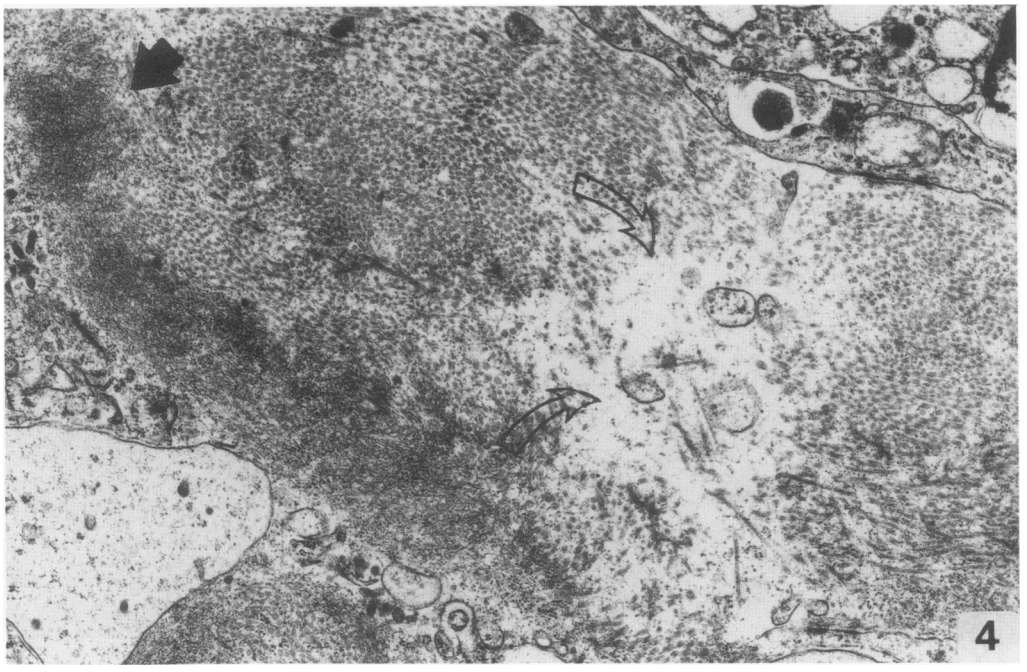

Figure 4—Collagen fibrils in a periovular granuloma 4 months after treatment of schistosomiasis showing focal areas of dissolution (open arrow) and fragmentation (lytic change) and also some dark granular transformation (closed arrow). (Electron microscopy, ×20,000)

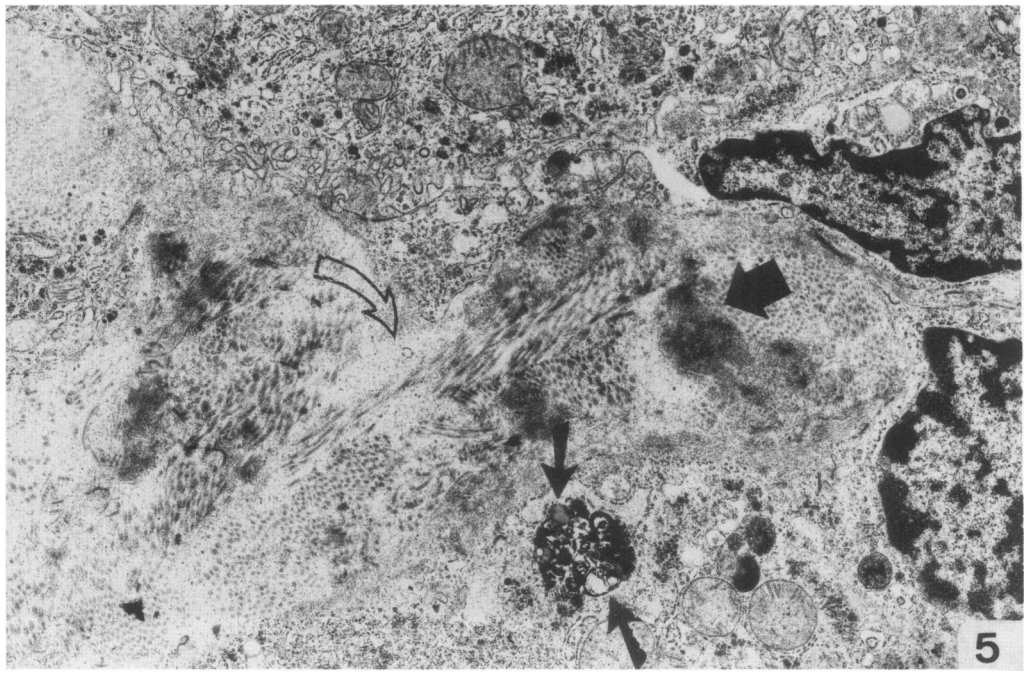

Figure 5—Involuting periovular granuloma 4 months after treatment showing lytic change (open arrow), electron dense change (closed arrow, top), and schistosomal pigment (closed arrow, bottom). (Electron microscopy, ×20,000)

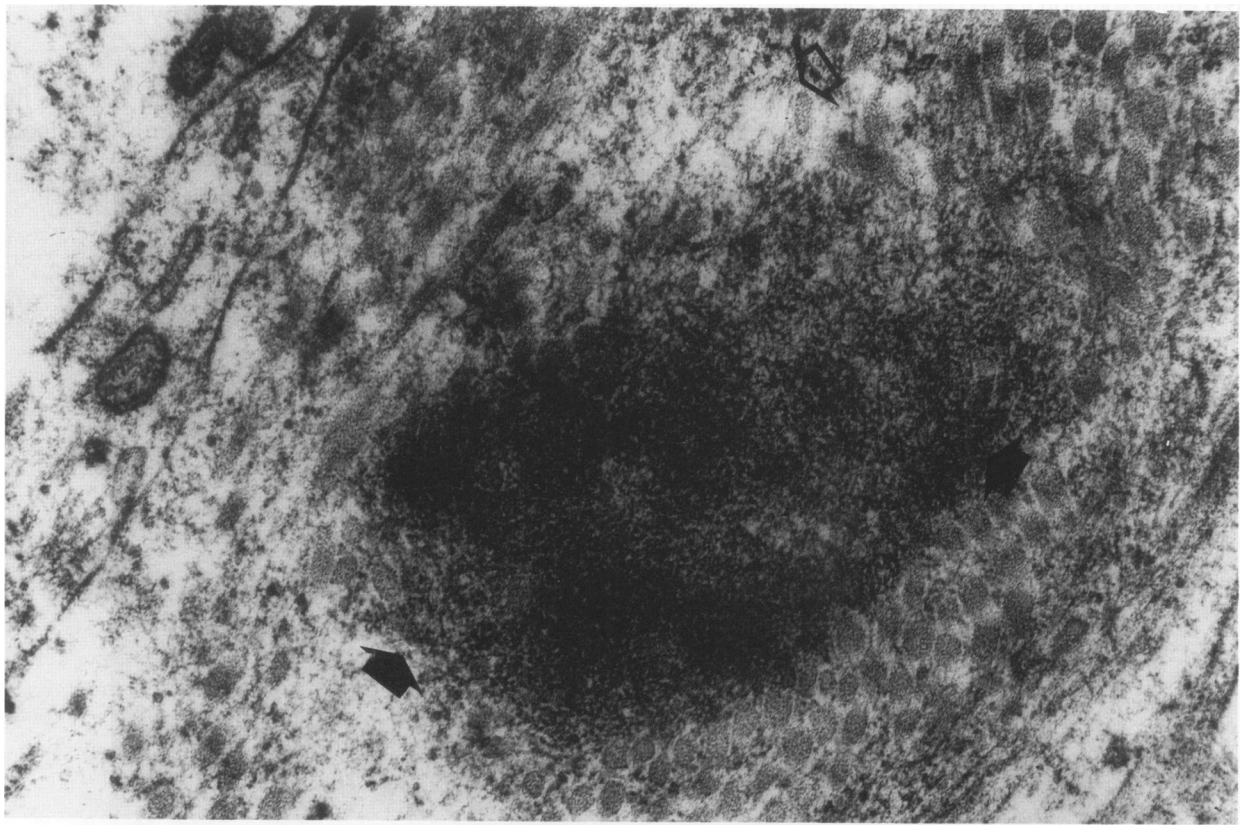

Figure 6—Focal collagen degradation seen within a periovular granuloma from an animal treated for schistosomiasis 4 months earlier. One can see the accumulation of dark and fibrilar material (electron-dense change, closed arrow) and a few clear areas of dissolution of collagen fibrils (lytic change, open arrow), the 2 main ultrastructural changes seen in such material. (Electron microscopy, ×60,000)

They were then embedded in paraffin, and the sections obtained were stained with hematoxylin and eosin (H&E), Masson's trichrome, Gomori's silver for reticulum, and the picrosirius-red method for collagen.[17]

Electron Microscopy

Tiny fragments of the liver were removed quickly after the death of the animal and fixed in cold 0,2% glutaraldehyde in cocodylate buffer. The material was postfixed in 1% osmium tetroxide solution and embedded into Spurr's resin. The blocks were cut in an automatic Reichert ultramicrotome with a diamond knife and the grids with the sections were contrasted with lead nitrate and uranyl citrate. A Zeiss EM-109 and a Phillips 300 electron microscopes were used at 60 Kw.

Immunofluorescence Microscopy

Before perfusion, small fragments of the liver were snap frozen in liquid nitrogen for a few minutes and then kept in air-tight plastic boxes at −70 C until submitted to sectioning in a cryotome at −20 C. The sections obtained were treated separately with different types of anti-sera for the demonstration of collagen (types I, III, IV, and procollagen III), laminin, fibronectin, and the CB-3,5 (I) peptide of collagen.

Collagen types I, III, Pro-III, and IV were prepared from fibrotic human liver, calf skin, and bovine lens capsule, respectively, after limited pepsin digestion and fractional precipitation with sodium chloride, according to Rhodes and Miller,[18] as modified for human liver by Chevalier et al.[19] The C-terminal a2-CB(3,5) (In) peptide was obtained from CNBr digest of human type I collagen by a combination of 2 carboxymethyl cellulose chromatographies.[20] The purity of the collagen fraction was verified by SDS-polycrylamide gel electrophoresis. Human plasma fibronectin was prepared by affinity chromatography using gelatin-sepharose 4b according to Enval and Rouslahti,[21] purified by DE cellulose chromatography, and verified by SDS-polyacrylamide electrophoresis. Laminin was provided by G. Martin (NIH, Bethesda, MD).

Antibodies to the above-mentioned purified antigens were raised in New Zealand white rabbits or goats after subcutaneous injections, every 2 weeks, of 1–5 mg of each fraction. Complete Freund's adjuvant was used for the first injection and subsequent injections were made with incomplete adjuvant. For purification of the antibodies, a screening stage including indirect immunofluorescence[22] on human liver, and ELISA, using a modified form of the technique devised by Gosslau and Barraach,[23] was carried out before purification to select batches of sera that presented the highest response to each antigen and minimal cross-reactivity with other collagen isotypes.

The test procedure was as follows: selected batches of each type of antiserum were submitted to an immunoadsorption procedure according to Timpl et al.[24] Native collagens were bound to CNBr-activated sepharose. Antibodies cross-reacting with common determinants of the different collagen types were eliminated by absorption after repeated passages through the different collagens bound to CNBr-activated sepharose. Finally, purified antibodies were obtained by an immuno-absorption elution procedure against the required collagen types using chromatographically purified collagens. Purified antibodies were tested by an ELISA microassay before use for light or electron microscopy. For purified anti-basement membrane collagen, no cross-reaction was detectable with laminin using a double immuno-diffusion test.

Results

General Data

Infection with 50 cercariae yielded 12–20 adult worms with an average of 6 worm pairs. In the treated animals, only dead worms were recovered on days 4 and 8 after treatment, but portions of disintegrating worms could be observed in mashed liver tissue as late as 45 days after treatment.

A striking and early change in the oogram was observed in the treated animals, with the number of immature eggs decreasing while that of mature and dead eggs increased, as has been described before.[16] After 15 days, only dead and disintegrating eggs were found. The results of the egg counting in the liver tissue appear in Figure 1.

Light Microscopy

As a consequence of treatment, periovular granulomas in the liver became less cellular, more fibrotic, and progressively diminished in size. Around the egg, in the center of the granuloma, there was an accumulation of polymorphonuclear leukocytes and macrophages, sometimes with giant cells engulfing parts of the egg shell. The collagen fibers in this central area appeared fragmented and scanty.

At 60 days after treatment, the collagen fibers, at first concentrically disposed around the egg in the granuloma, started to show fragmentation, while the fibroblasts and macrophages lost their parallel orien-

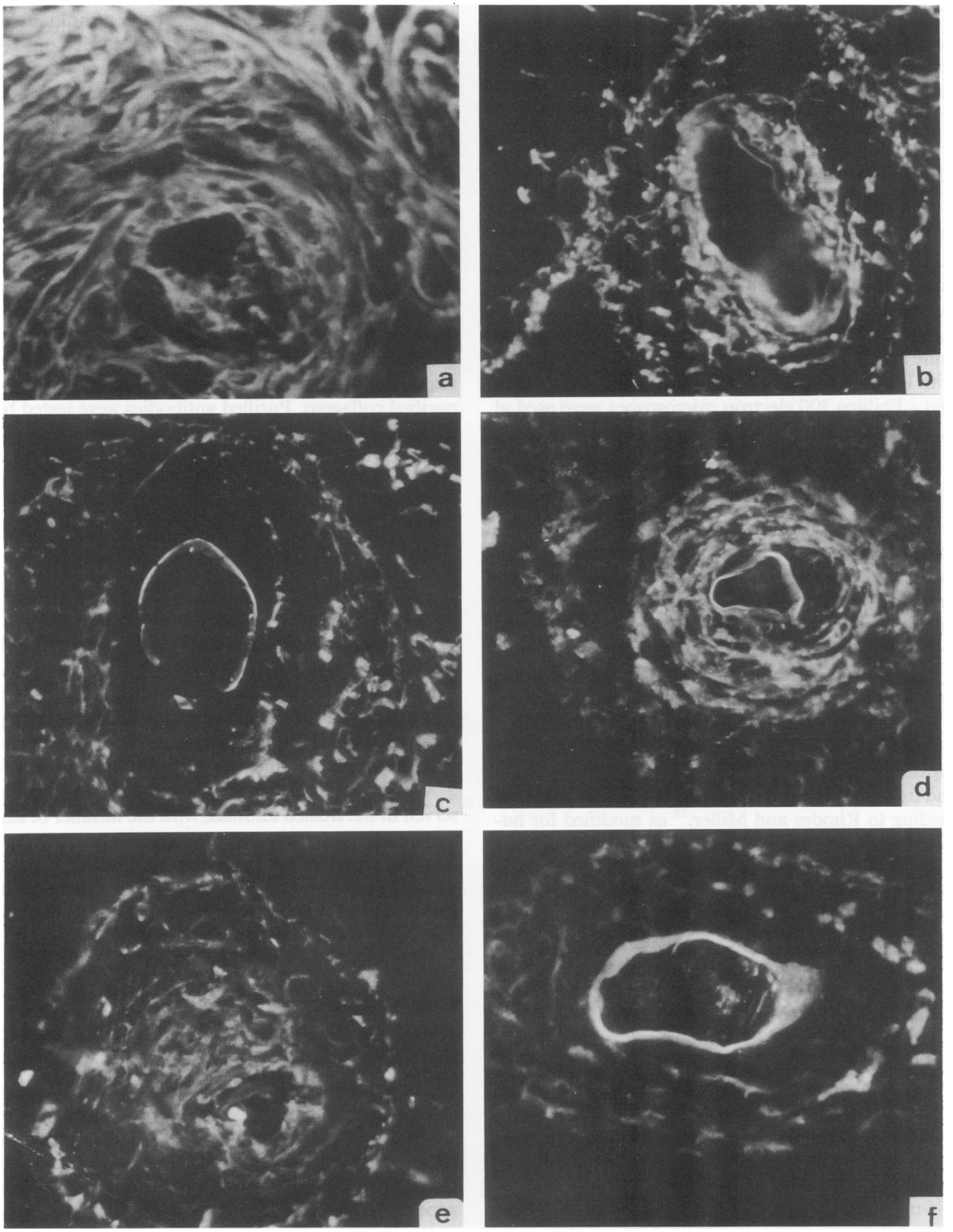

Figure 7—Behavior of Type III and Type I collagens in periovular granulomas showing posttreatment resorption. **a, b,** and **c** represent collagen Type III in untreated, 2 months and 4.5 months after treatment, respectively. **d, c,** and **f** illustrate the same events as for collagen Type I. The collagen tissue discloses fragmentation and irregular thickness of fibers as time after treatment increased. (Fluorescence microscopy, ×250)

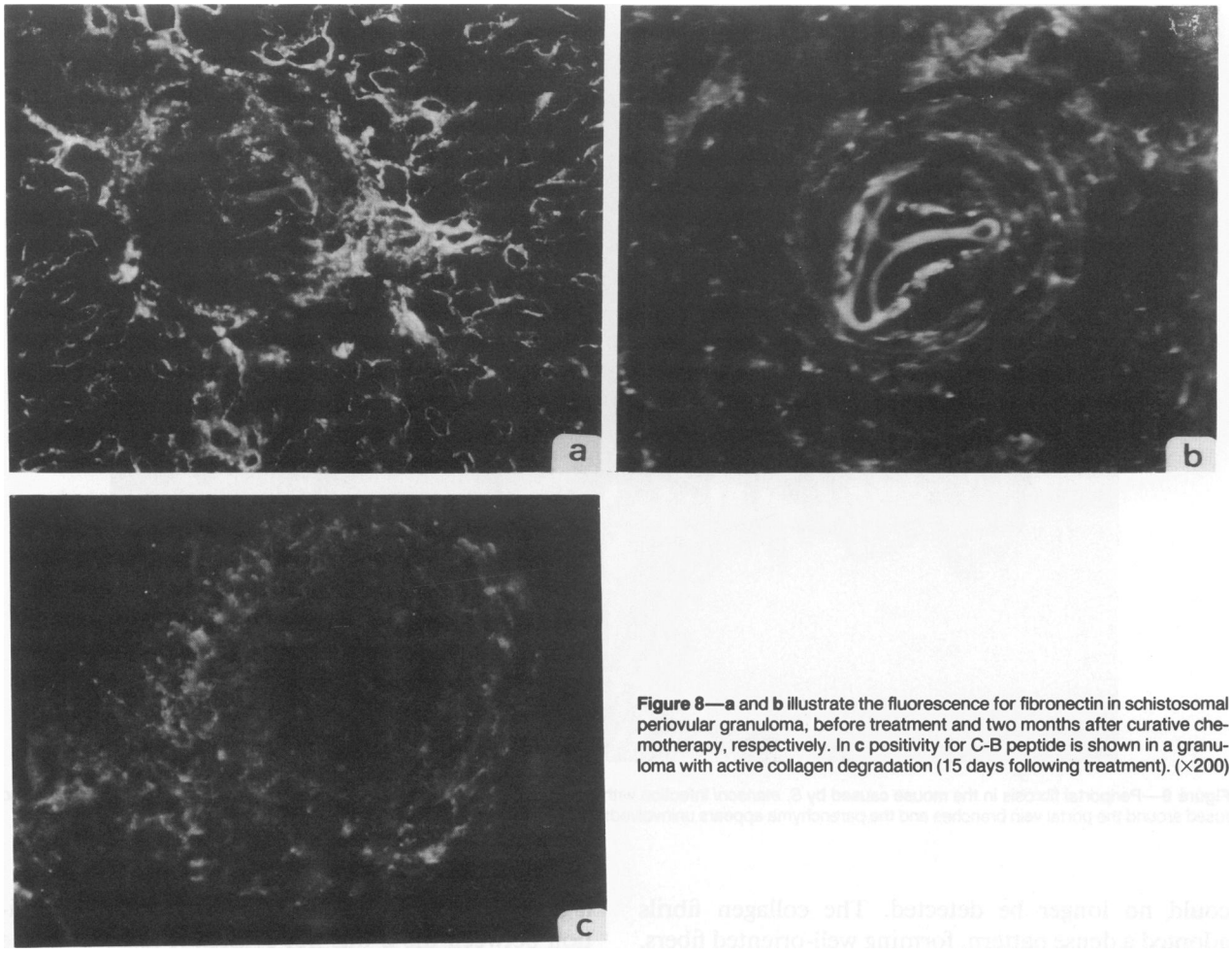

Figure 8—**a** and **b** illustrate the fluorescence for fibronectin in schistosomal periovular granuloma, before treatment and two months after curative chemotherapy, respectively. In **c** positivity for C-B peptide is shown in a granuloma with active collagen degradation (15 days following treatment). (×200)

tation (Figure 2), exhibiting a loss of polarity. In some areas, these cells appeared in great numbers and presented large, dark nuclei and a plump, clear cytoplasm. The greater the number of mononuclear cells in the involuting granuloma, the more the collagen fibers were fragmented, lacking a definite orientation. Demarcation between the granuloma periphery and the liver parenchyma was sharp, with liver cells in close apposition to the collagen fibers. With time the granulomas in the liver gradually disappeared. Four and a half months after treatment they were few and small, and were represented by a tiny pigmented scars (Figure 3), usually located in the portal areas.

Electron Microscopy

In the untreated animals, the periovular granulomas presented in several evolutional stages. The most florid and most frequently seen consisted of an accumulation of macrophages, fibroblasts, myofibroblasts, and eosinophils. Fibroblasts and myofibroblasts exhibited enlarged endoplasmic reticulum cisternae, and some of them showed phagocyticlike vacuoles containing fragments of collagen. Typical schistosomal pigment was seen within phagosomes in macrophages. Some macrophages exhibited a relatively well developed endoplasmic reticulum and interdigitating prolongations. Other granulomas appeared fibrotic, with collagen fibrils densely packed and few cells in between. A few of them showed signs of chronic collagen degradation, similar to those to be described in the treated animals.

Four and 15 days after treatment, the periovular granulomas contained numerous fibroblasts exhibiting enlarged and dilated endoplasmic reticulum and frequent internalization of collagen fragments within their cytoplasm. The extracellular collagen was fragmented and dispersed, with the fibrils presenting variable thickness and electron density. Many eosinophils appeared degranulated, and the macrophages seemed enlarged and numerous. Gradually, as the time after treatment increased, the fibroblasts acquired an elongated spindle shape, their endoplasmic reticulum became less prominent, and internalization of collagen

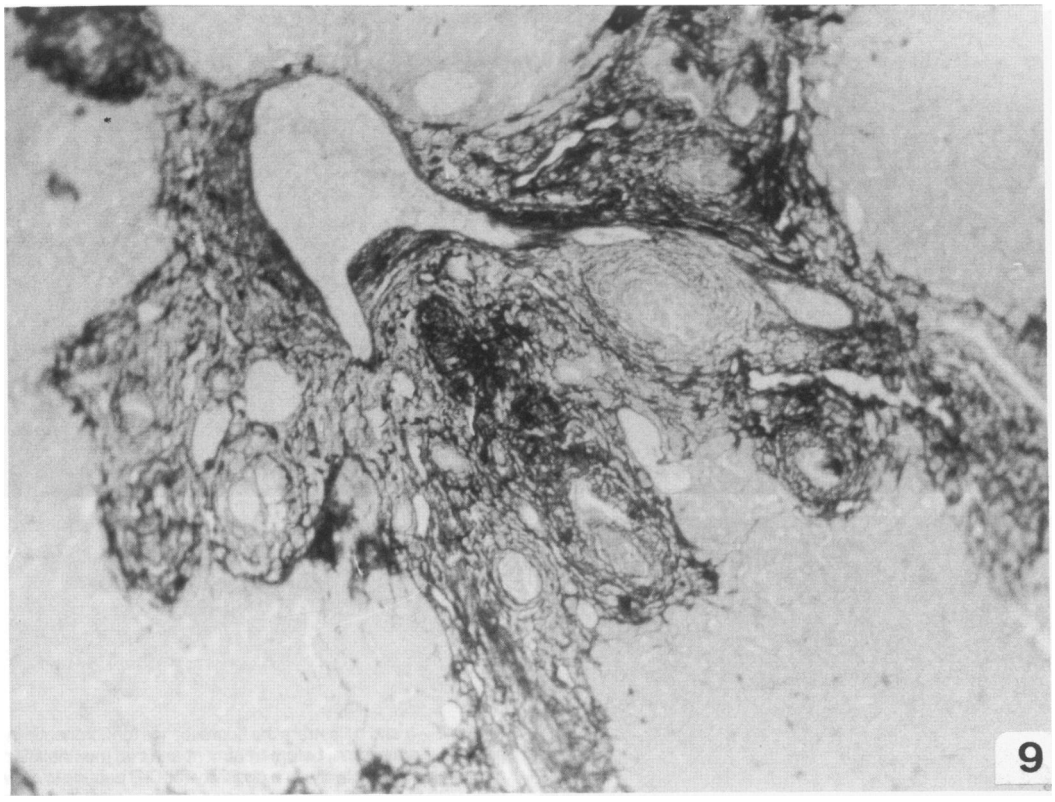

Figure 9—Periportal fibrosis in the mouse caused by *S. mansoni* infection with 30 cercariae during 20 weeks. Periovular granulomas are accumulated and fused around the portal vein branches and the parenchyma appears uninvolved: pipe stem fibrosis. (Picrosirius staining of collagen, ×100)

could no longer be detected. The collagen fibrils adopted a dense pattern, forming well-oriented fibers. The cells tended to be focally accumulated and separated by the dense collagen bundles.

Eosinophils were always present, and their granules sometimes appeared free between the collagen fibrils. After the 75th day of treatment, 2 distinct focal alterations affecting the collagen fibrils began to appear. One was focal dissolution and disappearance of collagen, leaving an empty space or "hole" in the middle of a collagen bundle (Figure 4). In some areas, partial remnants of disintegrated collagen fibrils were seen inside the empty space, suggesting transitional aspects of collagen breakdown. These focal "lytic" changes could be few or numerous, even in different samples taken from the same animal.

The other alteration consisted of the presence of fine granular or fibrillar material that appeared aggregated, forming electron-dense zones inside the collagen bundles. (Figure 5). Such electron-dense change could appear as single scattered spots or as large and irregular areas of replacement of the collagen tissue. The lytic and the electron-dense types of changes, which frequently interrupted the continuity of the collagen fibrils, could be observed side by side, but transition between the 2 was not evident (Figure 6) in the material examined.

Fluorescence Microscopy

Eight weeks after infection, the hepatic granulomas present in the untreated mice showed a predominance of fluorescent fibers of type III collagen (as well as type III procollagen) over the fibers of type I collagen. Later on, that predominance was no longer clear-cut, with both collagen fibers present, and type I collagen concentrated in the central part of the granuloma (Figure 7). Many fine fibers appeared fluorescent for fibronectin, but type IV collagen and laminin, although present along the hepatic sinusoids and around bile ducts and blood vessels, were missing in the granulomas.

Following curative treatment, type I and III collagens, as well as fibronectin, showed a progressive and similar decrease in amount (Figure 8 a,b). Fluorescence for the CB-peptide of type I collagen was weakly positive 4 and 15 days after treatment, but became negative thereafter (Figure 8 c). During the later stages of granuloma involution, 3 and 4.5 months after treat-

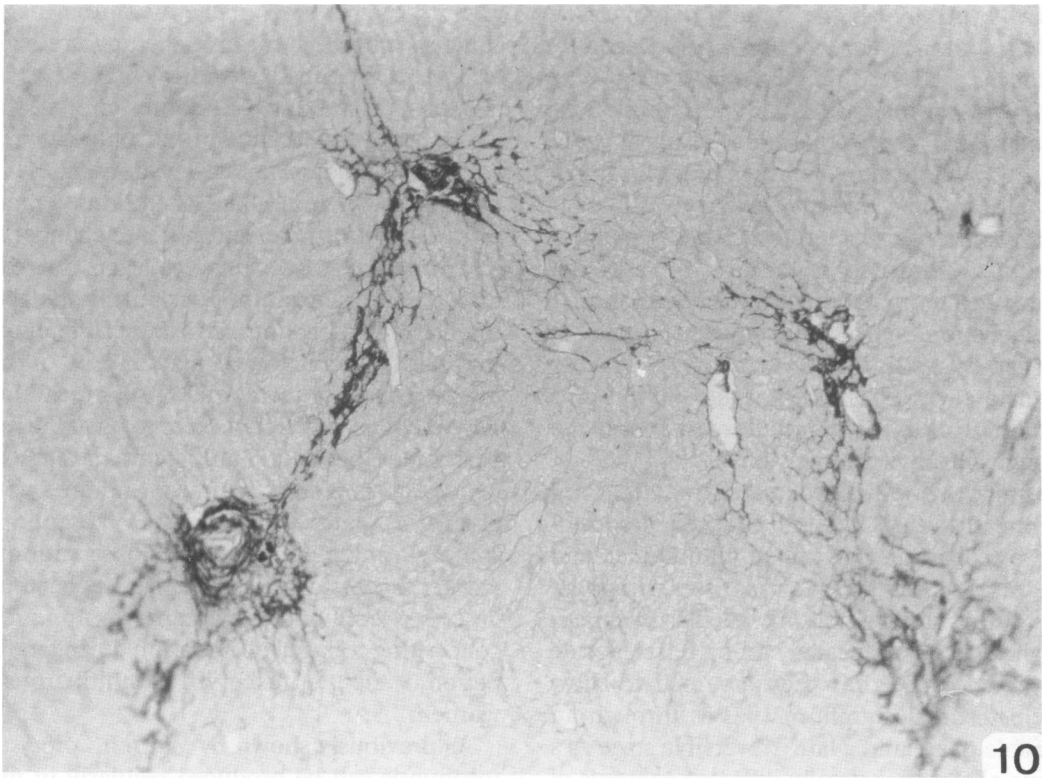

Figure 10—Periovular fibrous tissue in pipe stem schistosomiasis of the mouse shows considerable involution 3 months after specific treatment of schistosomiasis. (Picrosirius staining of collagen, ×100)

ment, the fluorescent collagen fibers appeared fragmented and loosely arranged. Even in the tiny scars seen around remnants of the egg shell, positivity for both type I and type III collagens was observed.

At the 20th week of infection, the periovular granuloma tended to accumulate within the portal spaces; only a few appeared isolated inside the parenchyma. Apparently, the granulomas were fused, forming fibrous septa or bands that sometimes connected portal spaces, simulating the pipe-stem lesion seen in human cases (Figure 9). Picro-syrius red staining revealed compact and well-oriented collagen fibers running outside and between the periovular granulomas.

The animals treated after 20 weeks of infection exhibited a progressive regression of these fibrous bands, and 3 months after treatment only fine, long strands of connective tissue could be seen in the portal spaces (Figure 10). The composition of the fibrous tissue regarding the presence of collagen types I and III and fibronectin was the same as in the periovular granulomas. After treatment, there was fragmentation, condensation, and shrinkage of fibers, but type I and III collagen and fibronectin could be detected in the fine and long connective tissue remaining in the portal spaces of the animals treated 3 months ago. No electron microscopy was performed for the fibrous changes seen in the animals treated after 20 weeks of infection.

Discussion

The excessive deposition of collagen in several types of lesions is known to undergo resorption when the provocative cause is removed. The collagen synthesis is linked with signs of collagen degradation and the final amount of fibrosis is the balanced result between collagenesis and collagenolysis.[25]

The reaction around the schistosome egg in the tissues of a permissive host gives a good illustration of these opposing changes because both signs of collagen synthesis and degradation can be seen at the ultrastructural level.[5] The macrophage, a cell known to play a key role in the process of matrix formation by secreting fibronectin and other factors that stimulate fibroblast proliferation and collagen synthesis, also secretes collagenase.[26]

After curative treatment of murine schistosomiasis, signs of collagen degradation (extracellular breakdown and internalization of collagen fragments) far exceed those of collagen formation (fibroblasts and

myofibroblasts with hyperactive endoplasmic reticulum and deposition of large amounts of collagen fibrils).[5] It has been claimed that fibrosis in periovular schistosomal granulomas may finally reach a state of irreversibility. This probably would result from a higher degree of crosslinking between collagen fibrils or from a predominance of type I over type III collagen.[27] Therefore, in older granulomas and especially in the periportal fibrosis of pipe-stem lesions,[28] the collagen becomes more ressilient to degradation and impossible to be removed. Such a concept is now being challenged by several reports showing partial or even complete reversal of hepatosplenic Manson's schistosomiasis in human subjects treated with the new and highly effective drugs available.[29,30]

It has been shown, both biochemically[27] and immunocytochemically,[5] that type III collagen predominates over type I in newly formed granulomas and that the reverse is seen later. Comparison with what happens in other similar situations, such as in experimental cirrhosis due to repeated carbon tetrachloride administration to rats,[7] has been assumed to show that the schistosomal granuloma passes through an early reversible phase and a late irreversible stage as a consequence of their genetically different contents of collagen. The present findings, however, indicate that during chronic collagen resorption both types disappear gradually and simultaneously, and that, even in the tiny residual scars left by involuting periovular granulomas, the presence of type I and III collagens can be demonstrated.

The immunofluorescent studies also confirm other published observations[31] in early schistosomal lesions in the liver of mice, that fibronectin is strongly positive within the periovular granulomas in its very active stage and decreases gradually as the granulomas involute, that laminin is absent from periovular granulomas; and that the antibody to C-B peptide[20] of type I collagen appears as a good tissular marker for collagen degradation.

Although the morphology of collagen degradation has been studied in models presenting a rapid turnover and resorption, no studies are available to explain what happens to the collagen in older cicatricial tissues. Probably the idea that collagen in such cases represents an immutable tissue has prevented inquiry into what is going on within its ultrastructure. In mice the turnover of collagen seems to be rapid, but this peculiarity may be only a difference of degree rather than of nature regarding other species.

Curative treatment of schistosomiasis prevents the arrival of new eggs in the liver and those already there are active for a maximum of about 15 days, the admitted life span for a mature miracidium. Afterwards, all granulomas are seen in the involuting stage. Earlier changes following curative therapy are striking and have been described previously.[5] These changes usually disappear about 2–3 months after treatment and are replaced by others, but morphologic evidence of collagen degradation is always present, at least on a ultrastructural level. The alterations tentatively described as lytic and electron-dense changes are different from the extracellular collagen breakdown and collagen internalization seen during the acute phase of collagen resorption, and these differences suggest that the factors involved may also be different. The fine and dense granular and fibrilar focal transformation of collagen does not seem to have the regular disposition of amyloid fibrils[32] nor the large homogeneous appearance of hyaline,[33] and does not stain for elastin, a material conspicously absent in schistosomal granuloma (unpublished observations). Apparently, the electron dense change has been previously described only once, in a paper by Junqueira et al[34] concerning collagen degradation during the dilatation period of the uterine cervix in multiparous pregnant women.

As previously shown by Warren,[3] pipe-stem schistosomiasis can be produced regularly in mice. It requires a relatively mild cercarial infection and a prolonged time of observation (at least 16 weeks) for the collagen tissue to be deposited in large amounts preferencially in and around the portal areas of the liver, forming a lesion with a strong resemblance to human pipe-stem fibrosis.[3,4] In the present investigation, that lesion was seen to undergo collagen resorption within 2–3 months after treatment, which suggests that the collagen concentrated at the portal spaces does not substantially differ from that present in periovular granuloma, at least in its capacity to undergo resorption following curative treatment of schistosomiasis.

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