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Hepatoma Formation in ddY Mice with Chronic *Schistosomiasis japonica*

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Three hundred and ninety-five four-week-old SPF female ddY mice were each exposed to 5 or 6 cercariae of *Schistosoma japonicum* (Japanese strain) on their shaved abdomens and were maintained in a conditioned clean environment and fed on sterilized food and water. Fecal examinations at 8 to 10 weeks postinfection (PI) revealed 169 mice to be infected. More than half of them died within 30 weeks PI and 70 mice that survived to the 50th week PI were sacrificed. At autopsy, we could find no schistosome eggs in the liver or intestinal wall of 9 mice, and they were excluded. Out of 61 mice which showed *S. japonicum* eggs in their livers, 48 had single or multiple hepatoma, while no tumor was observed in the livers of the 60 control mice. The tumors were yellowish-white in color with distinct boundaries and the centers of the tumors were depressed in some cases. The size of the tumors varied from 1 to 20 mm in diameter. Most of the tumors retained the normal trabecular pattern, but in some cases the trabeculae were thickened, having wide vascular spaces. The tumor cells were PAS-negative and showed varieties of pleomorphism. The sizes of cells and nuclei varied greatly. These findings suggested some causal relationship between *S. japonicum* infection and the hepatoma formation in the host's liver. In the chronic course of schistosomiasis japonica in the endemic areas, *S. japonicum* infection probably plays a role in hepatoma formation of patients.

Key words: Hepatocarcinogenicity — Flat worm — ddY mouse — Parasitic disease — *Schistosoma japonicum* (schistosomiasis)

Schistosoma japonicum is a digenetic trematode found in the portal and mesenteric veins of its hosts. Within the blood vessels, the thread-like female schistosomes lie within clefts (canalis gynaecophorus) of the male worms, remaining in a coupled state for many years during which time they produce about 3,000 eggs daily. Less than half of the discharged eggs pass out through the venules, the tissues and into the lumen of the intestines. More than half of these eggs remain in the intestinal wall or liver of the definitive host. Schistosomiasis is mainly induced by granuloma formation around the remaining eggs in the tissues and is not augmented by the adult worms.[1]

Epidemiologically vesical cancer has been known as a result of *S. haematobium* infection in Africa and the Middle East,[2] but the relationship between carcinoma (hepatoma, colorectal carcinoma) and schistosomiasis japonica or mansoni is still under debate.[3–9]

In experimental models, *S. japonicum* or *S. mansoni* infection intensified the carcinogenic potencies of some chemical carcinogens in hepatocellular carcinoma formation.[10–14] However, there are few papers dealing with the direct influence of schistosomiasis japonica or mansoni on tumor formation in murine models.[12, 15]

We succeeded in developing hepatoma in ddY mice with chronic schistosomiasis japonica without the feeding of any chemical carcinogen.

MATERIALS AND METHODS

Four-week-old specific pathogen-free female ddY mice were purchased from the Shizuoka Laboratory Animal Center (Hamamatsu, Shizuoka). We have been keeping the Japanese strain (origin: Yamanashi Prefecture) of *S. japonicum* in our laboratory in mice and *Oncomelania hupensis nosophora*.

The mice were anesthetized by an intraperitoneal injection of pentobarbital and then 5 or 6 cercariae of *S. japonicum* (Japanese strain) were placed on the shaved abdomens. The mice were then reared in conditioned clean environment with sterilized cages and received a sterilized CE-2 diet (CLEA

Japan Inc., Tokyo) and sterile water *ad libitum*. The feces were examined for schistosome eggs 3 times from 8 to 10 weeks postinfection (PI) and only the positive mice were used for this experiment. At 50 weeks PI the surviving animals were sacrificed by ether anesthesia. After laparotomy, adult worms were collected from the portal and mesenteric veins. The viscera were removed and the liver and spleen were weighed. The liver and lungs were investigated carefully in order to find tumors and metastases. For pathological examination the organs were fixed in 10% neutral formalin or Bouin's fluid. These fixed organs were routinely embedded in paraffin, sectioned at 4 μm thickness, and stained with hematoxylin and eosin (HE), and periodic acid Schiff (PAS) stain, as well as by the elastica Van Gieson method and Azan Mallory method.

Results

Out of 395 mice, 163 experimental animals became infected with *S. japonicum*, an infection rate of 41.3%. As a control group, 61 mice were anesthetized by the same method and were reared in the same conditioned environment. Out of 163 mice with *S. japonicum* infection, more than half died within 30 weeks PI due to intestinal obstruction and/or intestinal bleeding resulting from the granulomatous lesions around schistosome eggs. A total of 70 infected mice survived to 50 weeks PI (survival rate of 42.9%) and were sacrificed (Table I). However, in 8 mice adult worms could not be found in the portal veins and no eggs were seen in the livers and intestines. One mouse had only 1 male worm in the portal vein. We excluded those 9 mice which showed no eggs in their livers and intestines from the group of infected mice. In another ten mice, we could not find adult worms in the portal veins, but discovered schistosome eggs in the livers and intestinal walls. We therefore did an oncogenetic study on 61 mice with schistosome eggs. Most of these mice harbored one or two pairs of adult worms in their portal veins (Table II). Hepatosplenomegaly was significant in mice with chronic schistosomiasis japonica, though their mean body weight was almost the same as that of the control group. The mean weight of the livers in the group of infected mice was 3.115±SE 0.099 g (control mice: 1.630±0.037g), the largest being 5.184g. The liver was dark brown with a rough glossy surface. The mean weight of the spleens was 887±63 mg (control mice: 165±10 mg) and the largest was 2,680 mg.

Table I. Number of ddY Mice with Chronic Schistosomiasis japonica

Infected group[a]	
No. of mice	395
No. of infected mice[b]	163 (41.3%)
No. of survivors[c]	70 (42.9%)
Control group	
No. of mice	61
No. of survivors	60

a) Each of the 4-week-old ddY mice was exposed to 5 or 6 cercariae of *S. japonicum*.
b) The infection was recognized by fecal examination from 8 to 10 weeks PI.
c) The infected mice were maintained until 50 weeks PI.

Table II. Recovery[a] of *S. japonicum* Adult Worms at 50 Weeks Postinfection

No. of adult worms	No. of mice
0	18
schistosome eggs (+)	(10)
schistosome eggs (−)	(8)
1 male	1[b]
1 pair	38
2 pairs	12
3 pairs	1
Total	70

No. of schistosome egg (−) mice: 9.
a) The worms were recovered from the portal vein of the examined mice.
b) Schistosome eggs were not discovered in the liver or intestine.

Table III. Incidence of Liver Tumors in ddY Mice Infected with *S. japonicum* at 50 Weeks PI

	Infected group	Control group
No. of examined mice	61	60
No. of mice with liver tumors	48 (78.7%)	0 (0%)

Out of the 61 infected mice, 48 (78.7%) had single or multiple tumors in their livers (Figs. 1 and 2), while no tumor was found in the livers of control mice (Table III). The tumor size varied from 1 to 20 mm in diameter. Most were yellowish white and some were

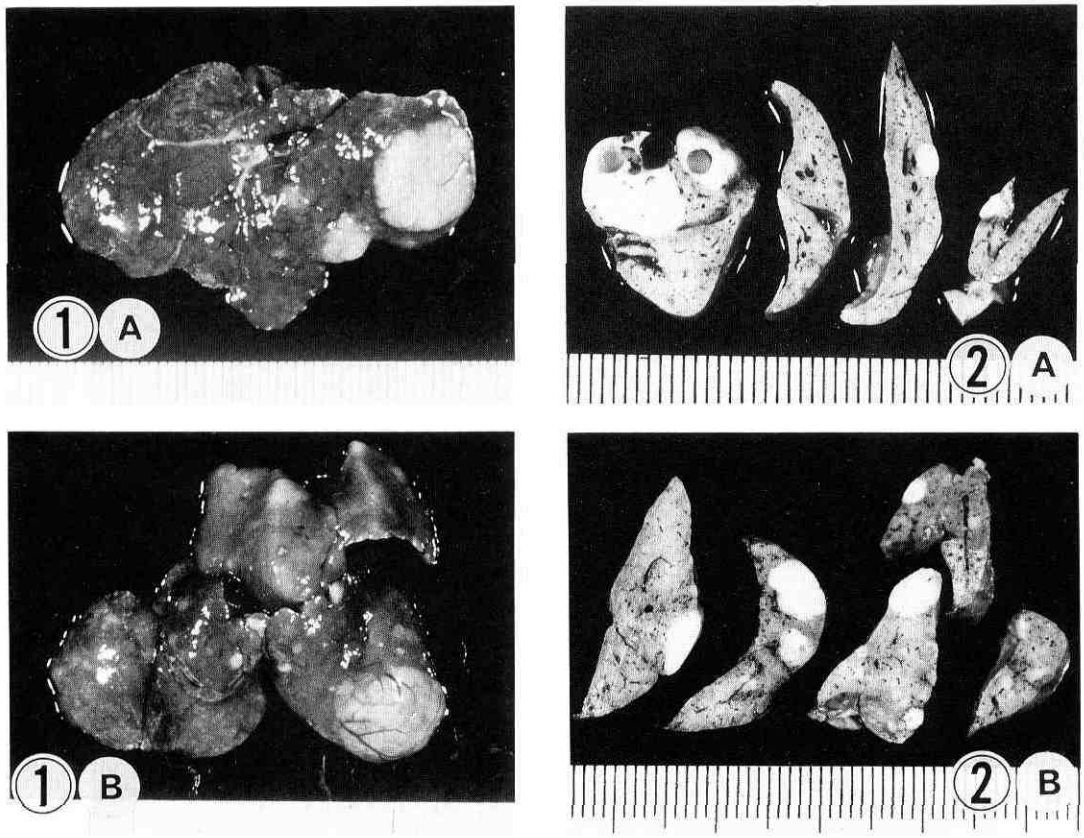

Fig. 1. Macrophoto of multiple hepatoma in a ddY mouse with chronic schistosomiasis japonica at 50 weeks PI. A: the center of tumor in the left lobe is depressed. B: new vessels are seen on the surface of the tumor.
Fig. 2. On the cut surface of the liver, the boundary of the tumor is distinct and cysts are seen in the tumor.

reddish brown. The boundaries of these tumors were distinct and the vessels on the surface of tumors were clearly seen. Some of the tumors were depressed in their centers and in 9 cases hyperplastic tumors contained cystic areas like hemangioma on the cut surface (Figs. 2 and 3).

Although the cytoplasma of tumor cells was more acidophilic (Fig. 4), the demarcation of tumors was not so clear in the HE-stained sections as in the gross findings. The hyperplastic tumors pressed against the surrounding liver tissues, but did not infiltrate (Fig. 5). The vessels of the portal tracts and central veins of the surrounding tissues became stretched around the rim of the expanding tumors. On the specimens dyed with elastica Van Gieson's stain, the collagen fibers appeared at the boundaries of tumors. Some of the tumor cells in the tumor center were not stained by the PAS method (Fig. 6). The orderly trabecular pattern was retained in most of the tumors, but in 9 cases the trabeculae were thickened and irregular in form and surrounded by wide sinusoids (Fig. 7). The trabeculae showed little endothelial cells lining the vascular space. The hyperplastic tumors were composed of pleomorphic cells. In 38 cases of the 61 mice, parenchymal cells in the tumors contained hyaline droplets (62.5%) (Fig. 8). Nuclei of tumor cells showed irregularity (Fig. 9). Some of the tumor cells had inclusion bodies in the nuclei. Besides tumor formation, in some cases a

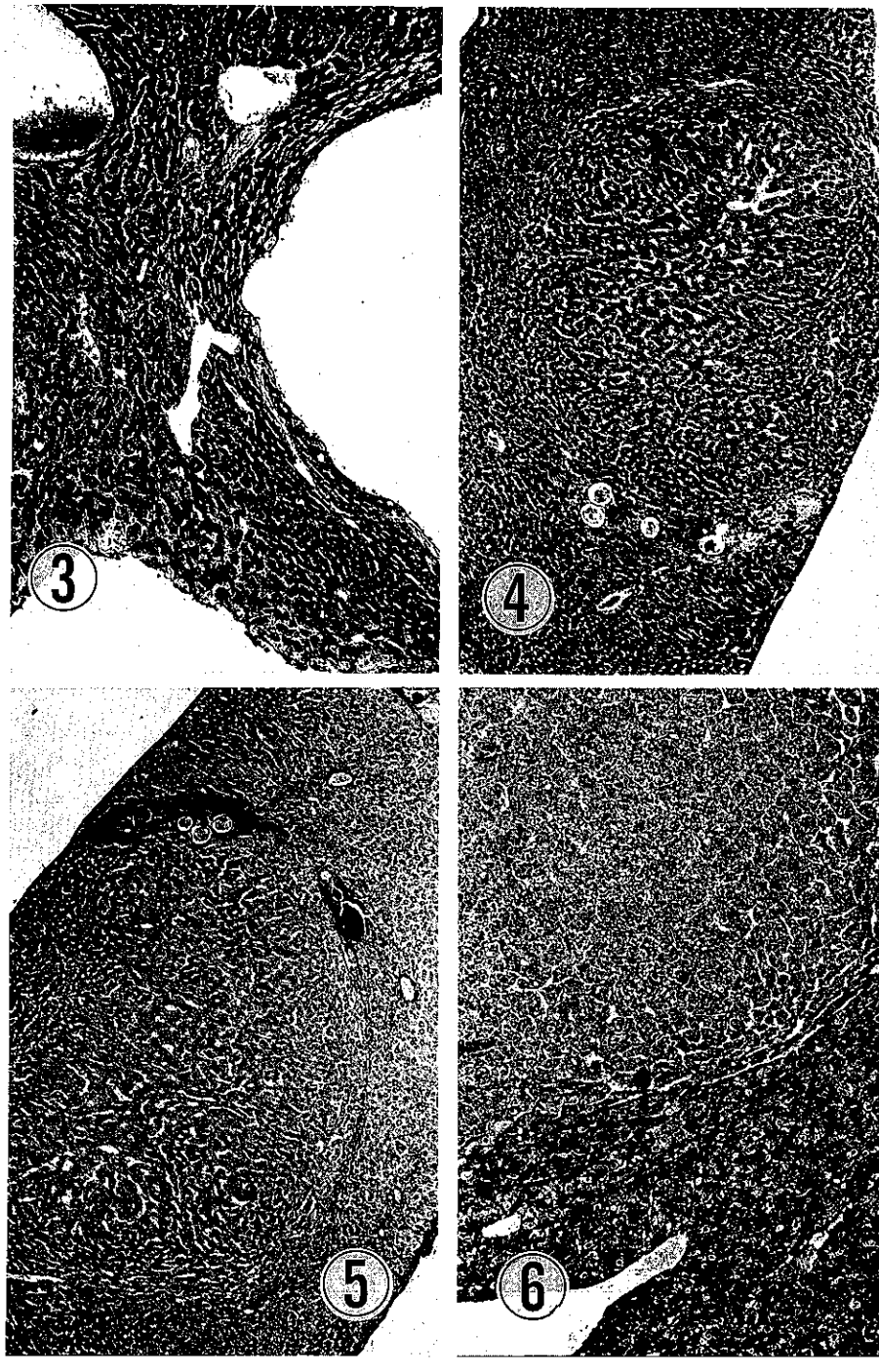

Fig. 3. Blood lakes compress the surrounding parenchymal cells. (HE, ×40)
Fig. 4. Acidophilic cytoplasm of cells in the tumor focus. (HE, ×40)
Fig. 5. The tumor focus is divided from the surrounding liver cells by collagen fibers. (Elastica van Gieson, ×40)
Fig. 6. The tumor cells are not stained with PAS stain. (×40)

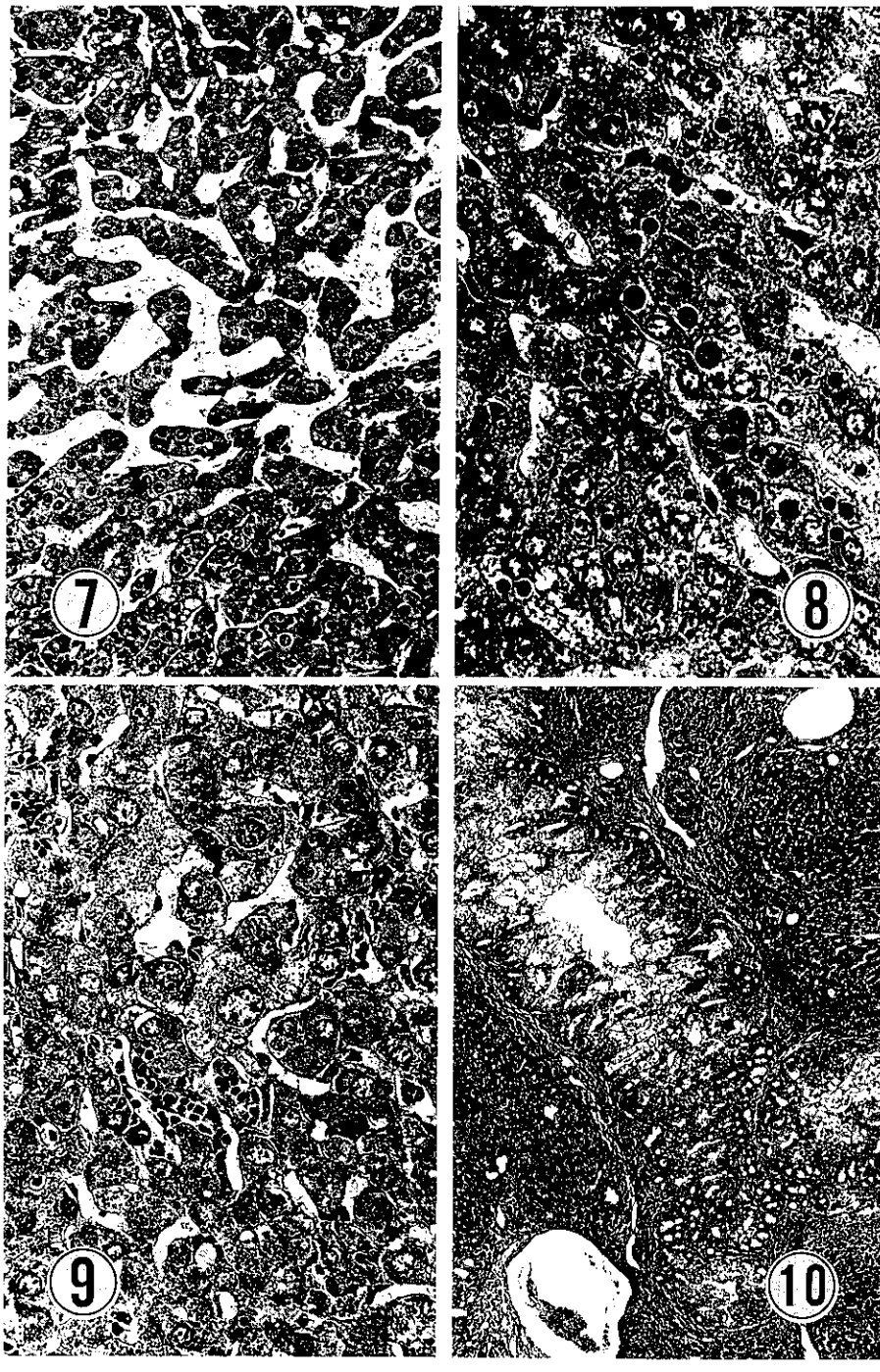

Fig. 7. High magnification of hepatocellular carcinoma. (HE, ×200)
Fig. 8. Hyaline droplets in tumor cells. (HE, ×400)
Fig. 9. The size of nucleus in tumors is variable. (HE, ×400)
Fig. 10. Hypertrophy of intrahepatic bile duct. (HE, ×40)

dramatic increase of bile duct as in cholangioma was noticed (Fig. 10) and in other cases the number of lymphoid follicles increased in the periportal area. Most of the schistosome eggs were seen in the liver surrounding hepatoma and rarely in the tumors. The granuloma around eggs varied in structure at various times PI. The granuloma around relatively fresh eggs was large and consisted of lymphocytes, eosinophiles and fibroblasts. The granuloma around old eggs was small, and consisted mainly of connective tissues or showed no cell reaction. No tumor or metastasis was observed in their lungs and other organs. Another finding was the regional hypertrophy of the small intestinal walls.

Discussion

The etiological relationship between schistosomiasis japonica and primary liver cell carcinoma has long been in debate since Kusama's report in 1907.[16] Some investigators have reported a significantly high incidence of liver cell carcinoma in the endemic areas of *S. japonicum*,[3, 9, 17–19] but statistically the occurrence of carcinoma and schistosomiasis in the endemic areas was not significantly correlated.[7, 20]

Schistosomiasis is not a direct initiator or a carcinogen, but plays a role as a promoter or a cocarcinogen to produce neoplasms.[10, 11, 13, 14] Based on the two-stage theory of carcinogenesis, we may speculate that damage to the liver in childhood due to this promoter may alter reactivity to hepatotoxic agents later in life.[21] Recently, it has been suggested that hepatic involvement of schistosomiasis japonica or mansoni makes patients more susceptible to viral hepatitis and more likely to become Hbs Ag carriers.[22] As a result, the incidence of hepatoma in patients with chronic schistosomiasis was significantly higher than that of other cases.[4, 9, 22]

The murine models of schistosomiasis japonica or mansoni have proved that the hyperplastic response in the host liver renders the liver cells susceptible to chemical carcinogens.[10–12, 14] The partial hepatectomy of mice also enhanced the carcinogenic effect of hycanthone to produce hepatocellular carcinoma.[23] These results suggest that liver cell proliferation due to schistosomiasis intensifies the carcinogenic effect. A similar process may occur in man in the endemic areas of schistosomiasis, though a marked difference exists between the liver lesions in humans and those in murine schistosomiasis.[24]

Warren and DeWitt[25] first reported that mice were good experimental models to analyze hepatosplenic diseases in humans. However, it is very difficult to rear mice long enough to produce carcinoma in their organs. On the other hand, spontaneous hepatoma can be easily investigated in some strains of mice. The need for a proper classification of liver tumors in murine models cannot be overestimated.[26, 27] Very few studies have appeared on carcinoma in murine models combined with chronic schistosomiasis mansoni. Cheever[15] observed hepatoma in 2 of 21 C57BL/6Jn mice infected with *S. mansoni* at 60–90 weeks PI, whereas no similar tumors were found in 32 controls. Haese *et al.*[12] observed 1 hepatoma and 10 hyperplastic nodules in 66 CFW mice infected with *S. mansoni*. However, these experiments used too small a number of mice to establish a correlation between schistosomiasis and hepatoma development. In the case of murine schistosomiasis japonica no studies have dealt with the relationship of the hepatoma production and chronic schistosomiasis japonica. *S. japonicum* female worms produce 10 times as many eggs as *S. mansoni* and the eggs of *S. japonicum* tend to aggregate and easily calcify. These characteristic features make symptoms of schistosomiasis japonica much more acute than those of schistosomiasis mansoni.[28] Keeping experimental animals infected is more difficult with *S. japonicum* than with *S. mansoni*.

We infected ddY mice with as few cercariae of *S. japonicum* as necessary to get one or two pairs of adult worms and then maintained them in a clean conditioned environment to prevent any contamination. Under these circumstances, 61 ddY female mice survived and 48 mice produced hepatoma at 50 weeks PI. Though some strains of mice have a tendency to produce spontaneous hepatoma, Suzuki *et al.*[29] and Yamamoto *et al.*[30] observed that the incidence of spontaneous hepatoma in ddY mice was very low. In the experimental animals with schistosomiasis japonica, the incidence of hepatoma in our study was highly significant compared with those in the data of

Cheever[15] or Haese *et al.*[12] This observation may be related to the difference in symptoms between *S. japonicum* and *S. mansoni.*

It may be suggested that schistosomiasis japonica itself acts as an initiator to produce hepatocellular carcinoma in murine models. Of course, we must consider the difference of pathophysiology between humans and murine models. However, we cannot ignore the apparent relationship between schistosomiasis and carcinogenicity.

To resolve the relationship between parasitic disease and carcinoma is more difficult than to determine the connection between chemical compounds and carcinoma. The real effects of schistosomiasis japonica in producing hepatoma are not clear. There are many possibilities, including mechanical damage of liver cells by adult worms and/or schistosome eggs, and waste products from worms and/or eggs. Pathological examination showed that schistosome infection damages parenchymal liver cells and induces focal necrosis from a very early stage (unpublished data). Such repeated damage of parenchymal cells in chronic schistosomiasis may increase the chances of malignancy.

We have just started to examine the correlation between carcinoma and schistosomiasis japonica. Our next task will be to examine the relationship in other strains of mice, to investigate early histopathological changes of liver parenchymal cells, and to investigate the histopathology of hepatoma at more than 50 weeks PI.

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REFERENCES

1) Warren, K. S. The secret of the immunopathogenesis of schistosomiasis: *in vivo* models. *Immunol. Rev.*, **61**, 189–213 (1982).
2) Elsebai, A. I. Parasites in the etiology of cancer — bilharziasis and bladder cancer. *Cancer J. Clin.*, **27**, 100–106 (1977).
3) Tsutsumi, H., Ozasa, T., Matsunaga, M., Ito, T., Ninomiya, F. and Ninomiya, K. Relationship between cancer of the colon and the liver, and schistosomiasis japonica. *J. Kurume Med. Assoc.*, **34**, 319–328 (1971) (in Japanese).
4) Nakashima, T., Okuda, K., Kojiro, M., Sakamoto, K., Kubo, Y. and Shimokawa, Y. Primary liver cancer coincident with schistosomiasis japonica. *Cancer*, **36**, 1483–1489 (1975).
5) Cheever, A. W. Schistosomiasis and neoplasia. *J. Natl. Cancer Inst.*, **61**, 13–18 (1978).
6) Mott, K. E. Possible relationship of *Schistosoma* infection and liver carcinoma. *Trans. R. Soc. Trop. Med. Hyg.*, **72**, 552–553 (1978).
7) Edington, G. M. Schistosomiasis and primary liver cell carcinoma. *Trans. R. Soc. Trop. Med. Hyg.*, **73**, 351–352 (1979).
8) Amano, T. Clinicopathological studies on the gastro-intestinal schistosomiasis in the endemic area of Yamanashi prefecture, with special reference to the carcinogenicity of schistosome infection. *Jpn. J. Parasitol.*, **29**, 305–312 (1980) (in Japanese).
9) Kojiro, M., Kakizoe, S., Yano, H., Tsumagari, J., Kenmochi, K. and Nakashima, T. Hepatocellular carcinoma and schistosomiasis japonica. *Acta Pathol. Jpn.*, **36**, 525–532 (1986).
10) Shigefuku, T. An experimental study on the relation of changes in the liver caused by parasites and the development of cancer of the liver. *Exp. Med. J.*, **27**, 356–365 (1943) (in Japanese).
11) Domingo, E. O., Warren, K. S. and Stenger, R. J. Increased incidence of hepatoma in mice with chronic schistosomiasis mansoni treated with a carcinogen. *Am. J. Pathol.*, **51**, 307–321 (1967).
12) Haese, W. H., Smith, D. L. and Bueding, E. Hycanthone-induced hepatic changes in mice infected with *Schistosoma mansoni. J. Pharmacol. Exp. Ther.*, **186**, 430–440 (1973).
13) Haese, W. H. and Bueding, E. Long-term hepatocellular effects of hycanthone and of two other antischistosomal drugs in mice infected with *Schistosoma mansoni. J. Pharmacol. Exp. Ther.*, **197**, 703–713 (1976).
14) Miyasato, M. Experimental study of the influence of *Schistosoma japonicum* infection on carcinogenesis of mice liver treated with N-2-fluorenylacetamide (2-FAA). *Jpn. J. Parasitol.*, **33**, 41–48 (1984).
15) Cheever, A. W. Parasitic disease and hapatic cancer. *In* "Primary Hepatoma," ed. W. J.

Burdette, pp. 98–99 (1965). Univ. of Utah Press, Utah.
16) Kusama, S. Ein fall vom primaeren parenchymatosen Leberkrebs bei der Schistosomiasis hepatis. *Gann*, **1**, 670–716 (1907).
17) Iuchi, M., Nakayama, Y., Ishiwa, M., Yamada, H., Chiba, K., Iio, M. and Kameda, H. Studies on primary carcinoma of liver associated with schistosomiasis japonica. *Naika*, **27**, 761–766 (1971) (in Japanese).
18) Iuchi, M., Nomura, M., Nozawa, K., Muto, J., Aida, K., Sakato, M. and Kitani, K. Hepatocellular carcinoma with schistosomiasis japonica. *J. Yamanashi Med. Assoc.*, **12**, 21–24 (1984) (in Japanese).
19) Iwamura, K. Hepatic involvement of schistosomiasis japonica. *Tokai J. Exp. Clin. Med.*, **2**, 143–152 (1977).
20) Kamo, E. and Ebato, T. A clinical analysis of primary carcinoma of the liver in relation with schistosomiasis japonica. *J. Yamanashi Med. Assoc.*, **9**, 23–37 (1982).
21) Higginson, J. The geographical pathology of primary liver cancer. *Cancer Res.*, **23**, 1624–1633 (1963).
22) Lyra, L. G., Reboucas, G. and Andrade, Z. A. Hepatitis B surface antigen carrier state in hepatosplenic schistosomiasis. *Gastroenterology*, **71**, 641–645 (1976).
23) Tsuda, H., Sarma, D. S. R., Rajalakshmi, S., Zubroff, J., Farber, E., Batzinger, R. P., Cha, Y. and Bueding, E. Induction of hepatic neoplastic lesions in mice with a single dose of hycanthone methanesulfonate after partial hepatectomy. *Cancer Res.*, **39**, 4491–4496 (1979).
24) Cheever, A. W. *Schistosoma japonicum*: the pathology of experimental infection. *Exp. Parasitol.*, **59**, 1–11 (1985).
25) Warren, K. S. and DeWitt, W. B. Production of portal hypertension and esophageal varices in the mouse. *Proc. Soc. Exp. Biol. Med.*, **98**, 99–101 (1958).
26) Becker, F. F. Morphological classification of mouse liver tumors based on biological characteristics. *Cancer Res.*, **42**, 3918–3923 (1982).
27) Goldfarb, S., Pugh, T. D. and Koen, H. Classification of mouse hepatocellular neoplasms. *Cancer Res.*, **44**, 869–870 (1984).
28) Warren, K. S. The pathology of schistosome infections. *Helminthol. Abstr.*, **42**, 591–633 (1973).
29) Suzuki, S., Matsuoka, A., Niki, R. and Takagaki, Y. Pathological studies on spontaneous tumors in mice. *Exp. Anim.*, **30**, 407–420 (1981) (in Japanese).
30) Yamamoto, H., Imai, S., Tsutsui, K. and Kikumori, M. Spontaneous tumor formation in NIH-Swiss, ddY: SLC and ICR:SLC mice. *Exp. Anim. Tech.*, **18**, 57–65 (1983) (in Japanese).