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The Effects of *Schistosoma japonicum* Infection on the Splenectomized SJL Mice

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Abstract

We investigated the effects of the *Schistosoma japonicum* infection on the splenectomized SJL/N mice. Splenectomy induced an increase of the white blood cell (W.B.C.) count including slight eosinophilia and the packed cell volume (P.C.V.).

In SJL/N mice the *S. japonicum* infection induced hepatosplenomegaly, enlargement of mesenteric lymph nodes, loss of body weight and thymic atrophy as well as in other tested mice. In a hematological examination, leucocytosis with eosinophilia was significant after 7 weeks post-infection (PI) and the red blood cell (R.B.C.) count was maintained, but the P.C.V. decreased significantly between 8 and 9 weeks PI (hypochromic anemia).

The infection on the splenectomized mice strengthened the leucocytosis and eosinophilia in the late stage and induced a significant decrease of R.B.C. count and P.C.V. between 8 and 9 weeks PI (normochromic anemia). Splenectomy, also, stressed hepatomegaly including the enhancement of granuloma size and IgE antibody production to soluble egg antigen in mice infected with *S. japonicum*.

Key words: Anemia, eosinophilia, hepatomegaly, IgE, leucocytosis, *Schistosoma japonicum*, schistosomiasis japonica, SJL/N mouse, splenectomy

Introduction

Since Warren & DeWitt (1958) reported that mice could be used as a good model to study the pathophysiology of human schistosomiasis mansoni and japonica, the parasitic factors responsible for the pathogenesis of schistosomiasis have been mainly decided by the use of these experimental models. In schistosomiasis, the host granulomatous response around eggs plays a crucial role in the pathogenesis of hepatosplenic disease. As Warren (1971) pointed out, however, the immunopathology of schistosomiasis japonica differed greatly from that of schistosomiasis mansoni. We tried to elucidate a responsible parasite factor for the hepatosplenomegaly in schistosomiasis japonica by observing murine models infected with *S. japonicum*.

It is well known that, in murine schistosomiasis, different inbred strains of mice show different responses in immunopathology (Mitchell *et al.*, 1981; Colley & Freeman, 1983). We previously reported the effects of splenectomy on pathophysiology of outbred ddY mice with *S. japonicum* infection (Amano & Oshima, 1984b, 1987). One characteristic effect of splenectomy was a distinct induction of eosinophilia in the early course of PI. Sugane and Oshima (1984) reported that eosinophilia in peripheral blood of SJL mice was most significant among 8 inbred strains infected with *Toxocara canis*. So we subsequently studied the effects of splenectomy on the pathophysiology including eosinophilia and the granulomatous reactions in SJL/N mice infected with *S. japonicum*.

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Materials and Methods

Animals and Splenectomy

SJL/N mice were originally purchased from the Central Institute for Experimental Animals (Tokyo) and bred in our laboratory. Five-week-old female ddY mice and 6-week-old male Sprague-Dawley rats were obtained from the Shizuoka Laboratory Animal Center (Hamamatsu).

In the first experiment, mice were divided into 4 groups: 1) a sham treated control group, 2) a splenectomized and non-infected group, 3) a sham treated group, infected with 20 cercariae and 4) a splenectomized infected with 20 cercariae group. In the second experiment, mice were divided into 3 groups, without a sham treated control group. Six-week-old female SJL/N mice were carefully splenectomized without leaving accessory spleens or sham treated under pentobarbital anesthesia by the previously described method (Amano & Oshima, 1984a, b). None of the mice developed any manifestations of bleeding or infection. When the mice were killed, the mice were carefully inspected for spleen remnants and accessory spleens in the peritoneal cavity, but no evidence of such was found.

Strain of Parasite and Method of Infection

The Japanese strain of *S. japonicum* was obtained from Dr. Kamo, E., Yamanashi Medical Institute and maintained in our laboratory with mice and *Oncomelania hupensis nosophora*. In order to secure infection in the experimental animals, at least ten of the infected snails were crushed in water droplets and the emerged cercariae were pooled in a glass tube. The active cercariae swimming near the water surface were used for infection. At one week after splenectomy or sham treatment, each of the anesthetized SJL/N mice was exposed percutaneously with 20 cercariae of *S. japonicum* by the cover slip method on the shaved abdominal wall (Amano & Oshima, 1984a, b).

Collection of *S. japonicum* Eggs and Preparation of Soluble Egg Antigen (SEA)

Schistosome eggs were collected from the livers and intestines of female ddY mice exposed with 40 cercariae of *S. japonicum* at 8 weeks post-infection (PI) by a modified Coker & Lichtenberg's method (1956). The livers and intestines of the infected mice were removed, washed with and incubated overnight in 0.2 M NaCl solution, and homogenized with a Waring blender twice for 20 seconds at a time. The homogenate was strained through several wire meshes, successively from rough to fine size and schistosome eggs were finally trapped on the size 45 μm mesh. The schistosome eggs suspension was centrifuged many times until the supernatant became clear and the final sedimentations were used for the preparation of SEA by a modified Boros & Warren's method (1970) as follows: eggs in Dulbecco's phosphate buffered saline were homogenized in a glass tissue homogenizer at 4°C for 30 minutes. The homogenate was stored at 4°C overnight and centrifuged at 100,000 g for 2 hours at 4°C. The supernatant was sterilized by passing through a 0.45 μm filter membrane (Millipore Corp., Bedford, MA, USA) and stored as SEA at −80°C. The protein content was measured by Bradford's method (1976).

Measurement of Peripheral Blood Cells

Blood samples were taken from the lateral tail vein weekly and the number of white blood cells (W.B.C.) and eosinophils were counted by a previously described ordinal method (Amano & Oshima, 1984a).

In the first experiment, mice were killed at 9 weeks PI and in the second at 8 weeks PI. Mice were sacrificed under ether anesthesia and the blood samples were collected by the heart puncture method. The number of red blood cells (R.B.C.) and packed cell volume (P.C.V.) were measured by the ordinal method and the rest of the blood samples were pooled to collect sera.

Number of Schistosome Eggs in the Liver

The liver of each mouse was removed and weighed immediately on an electric reading balance. A part of the liver, about 20% of the total weight, was fixed in 10% neutral formalin for the preparation of tissue sections.

The residual liver was weighed to decide the ratio to a total weight and incubated in 50 ml of 5% W/V KOH solution for 6 to 8 hours in a 37°C warm bath to make it easy to count the number of schistosome eggs (Cheever & Anderson, 1971). The number of schistosome eggs was counted from three 0.15 ml samples under a microscope and the number of the eggs in each liver was correlated (Amano & Oshima, 1984a).

Measurement of Granuloma Size

Though *S. mansoni* eggs are found to be scattered in tissue, *S. japonicum* eggs are often found to be congregated in tissue. Therefore, it is not easy to measure the exact size of *S. japonicum* egg granuloma in the liver. The liver sections were stained with hematoxylin and eosin. We tried to find the granuloma produced by a single schistosome egg in the liver sections. Then we measured the size of granulomas automatically by Digigkammer (Mutoh Industries, Tokyo) by projecting the figure of granuloma on a screen. At least 30 areas of single egg granuloma in a liver were measured.

Anti-SEA IgG titer

The enzyme-linked immunosorbent assay (ELISA) technique used to assess specific anti-SEA IgG involved the following procedures. SEA were diluted to a concentration of 10 μg/ml in phosphate-buffered saline (PBS). 50 μl of this solution was incubated in each well of a micro immuno plate II (Nunc, Denmark) and incubated overnight at 4°C. Unbound material was flicked out and washed three times with PBS, containing 0.05% Triton 100. The sera were first diluted to 80 times and then repeated in two-fold dilutions. 50 μl of diluted antisera or control serum was added to each well and incubated for one hour at room temperature. Following incubation, the plate was again washed three times with PBS-Triton buffer. 50 μl of the peroxidase-conjugated rabbit antimouse IgG preparation (Cappel, USA: diluted 1:500 with PBS) was applied and incubated for one hour at 37°C, then washed three times as described. The substrate for the assay was prepared by dissolving 15 mg of ABTS (Sigma, USA) in 100 ml of citrate-phosphate buffer, to which 50 μl of 30% H_2O_2 was added. The substrate was added to each well. The plate was then incubated for 20 minutes at room temperature in total darkness. The plate was inserted into the microelisa reader (Corona, Tokyo) and then read at wavelength 410 nm. The positive range was expressed by the lowest limit, which was higher than 2 standard deviations above the mean of the control sera.

Passive Cutaneous Anaphylaxis (PCA) Reaction

Titration of IgE antibody to SEA of *S. japonicum* was performed by PCA reaction on the Sprague-Dawley rats' skins as follows. Serially diluted 100 μl of pooled sera from the *S. japonicum* infected mice were intradermally injected into the shaved back of an anesthetized rat and, 4 hours later, 1 ml of 0.3% Evans blue solution including 500 μg SEA was intravenously injected into the rat. After another 30 minutes, the diameters of the blue spots formed on the back were measured. Blue spots larger than 5 mm in diameter were defined as positive reactions and the PCA titers of the reaction were expressed as the highest dilutions of the sera that showed positive reactions. All the positive sera in PCA were heat treated at 56°C for 30 minutes and PCA reactions were tested again using these sera (Sugane & Oshima, 1984).

The significant deviation in the data among experimental groups were tested by the parametric Student's *t*-test.

Results

All the infected mice, especially those splenectomized, gradually decreased in weight and ruffled coats after 6 weeks PI. We had to stop the first experiment at 9 weeks PI and the second one at 8 weeks PI.

Body and Organ Weights

As shown in Table 1, splenectomy had no influence on weight of body and organs (thymus, mesenteric lymph nodes) in the non-infected control mice. In both experiments, the infec-

Table 1 Changes of body and organ weight in the splenectomized SJL/N mice with *Schistosoma japonicum* infection

No. of groups	No. of mice	Body (g)	Liver (mg)	Spleen (mg)	Thymus (mg)	Mesenteric lymph nodes (mg)
Experiment 1						
1	6	21.8±1.0	1,097± 39	139±25	42±2	38± 3
2	6	21.9±0.5	1,142± 26	——	39±5	42± 3
3	5	17.4±0.4*	1,719±124*	537±50*	14±8*	239±16*
4	5	18.4±0.4*	2,150±128*†	——	15±7*	323±42*††
Experiment 2						
2	5	20.5±0.3	1,011± 25	——	51±2	46± 3
3	5	17.8±0.4*	1,724± 58*	553±32*	8±1*	238±16*
4	6	18.7±0.5*	2,285±104*†	——	10±4*	295±28*††

The mice were sacrificed at 9 weeks PI in experiment 1 and at 8 weeks PI in experiment 2. The number of groups shows the following things: 1, sham treated control mice group; 2, splenectomized control mice group; 3, sham treated and infected mice group; 4, splenectomized and infected mice group.
significantly different group 3 vs. group 4 (†: $P < 0.001$, ††: $P < 0.01$)
**: significantly different group 1 vs. group 3 and group 2 vs. group 4 ($P < 0.001$).*
Each data shows mean ± standard error (compared with the parametric Student's t-test).

tion of *S. japonicum* induced hepatosplenomegaly, enlargement of mesenteric lymph nodes, thymic atrophy and body weight loss. However, among mice infected with *S. japonicum*, splenectomy resulted in significant hepatomegaly and hypertrophy of the mesenteric lymph nodes. Hepatomegaly in the infected mice was much more prominent in the splenectomized mice than in the non-splenectomized mice ($P < 0.001$).

White Blood Cell Count (W.B.C.)

Splenectomy induced an increase of the W.B.C. count in peripheral blood including slight increase of eosinophils. From 2 to 9 weeks after treatment, an arithmetic mean and standard error of the W.B.C. count were 18,500 ± 1,800/mm^3 in the sham operated mice group and 25,700 ± 3,000/mm^3 in the splenectomized mice group. As shown in Fig. 1, the infection of *S. japonicum* induced the first low peak of leucocytosis at the 2nd week of infection in the splenectomized mice and at the 3rd week in the non-splenectomized mice. The levels of leucocytosis were much higher in the splenectomized mice. After 5 weeks PI, the splenectomized mice showed significant leucocytosis and the W.B.C. count reached 73,200 ± 5,100/mm^3 at 9 weeks PI. In the sham treated mice, however, the leucocytosis began from 7 weeks PI and the level was lower than that of the splenectomized mice, 46,600 ± 8,900/mm^3 at 9 weeks PI.

Eosinophil

Splenectomy seemed to have very little effect on the eosinophil count among control mice (control; 390 ± 80: splenectomized; 508 ± 60). As shown in Fig. 2, eosinophilia in SJL/N mice with schistosomiasis japonica appeared earlier in the splenectomized mice, but in the sham treated mice it appeared after 7 weeks PI. In the splenectomized mice, a weak peak of eosinophilia was investigated at 2 weeks PI. However, the levels of eosinophils were almost the same in both groups at 9 weeks PI.

The ratio of eosinophils to the W.B.C. count was almost constant through the course of infection.

Red Blood Cell Count (R.B.C.) and Packed Cell Volume (P.C.V.)

As shown in Table 2, splenectomy had no influence on R.B.C. count, but induced an in-

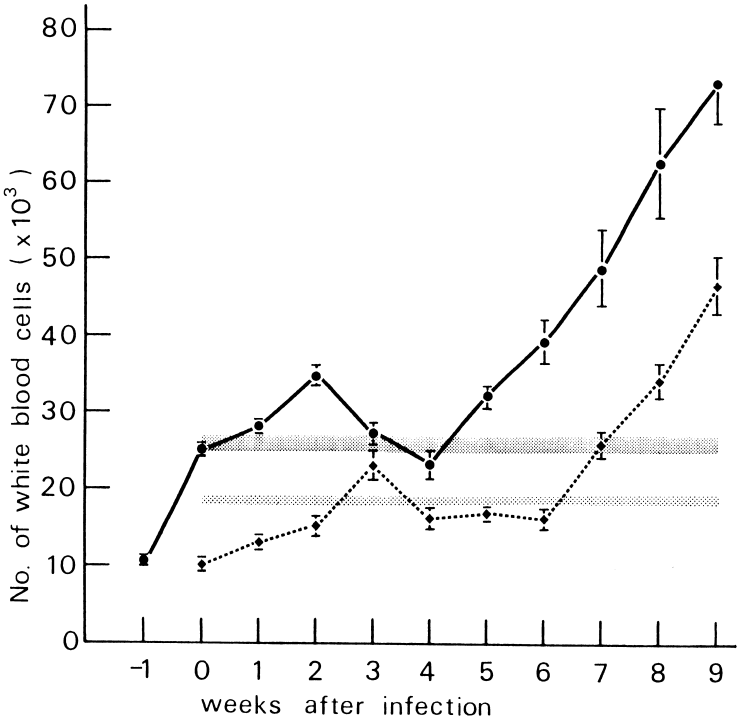


Fig. 1 The changes of the number of white blood cells in peripheral blood of sham operated and splenectomized SJL/N mice infected with 20 cercariae of *Schistosoma japonicum*.
Each point represents the arithmetic mean of determinations from splenectomized mice (●) and sham operated mice (♦). The brackets show the standard errors of the means.
(▒) shows the mean ±S.E. of splenectomized mice. (▒) shows the mean ±S.E. of sham treated mice.

crease of P.C.V. in the control mice group. Among the sham treated mice infected with *S. japonicum*, the R.B.C. count did not change, but P.C.V. decreased. On the other hand, in the infected and splenectomized mice, the R.B.C. count and P.C.V. decreased significantly. As a result, the mean corpuscular volume (M.C.V.) decreased to $32.5 \pm 1.0\ \mu m^3$ among the sham treated mice infected with *S. japonicum* (hypochromic anemia), but M.C.V. did not change in the infected and splenectomized mice (normochromic anemia).

Number of Schistosome Eggs in the Liver

As shown in Table 3, splenectomy had no influence on the accumulation of schistosome eggs in the liver. In the second experiment, the number of schistosome eggs was more than in the first one. This was suspected to be due to a faster rate of infection in the second experiment, though we did not examine the number of adult worms.

Size of Granuloma around Schistosome Eggs

We measured only the granuloma around single eggs with developed miracidium in the stained liver sections. More than 30 granulomas in each liver were measured by Digigkammer. As shown in Table 4, a significant increase in granuloma size was observed in the livers of the splenectomized mice. The cellular compositions of the enlarged granuloma, however, did not differ from that of the sham-treated group. Eosinophils, plasma cells, epitheloid cells and

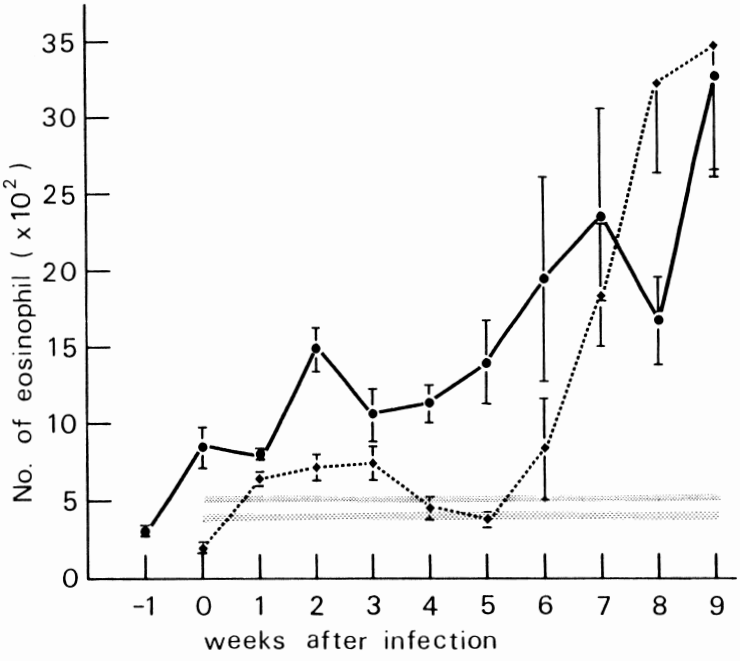


Fig. 2 The changes in the eosinophil number in peripheral blood of sham operated and splenectomized SJL/N mice infected with 20 cercariae of *Schistosoma japonicum*.
Each point, bracket and band mean the same things in Fig. 1.

Table 2 Changes of peripheral blood in the splenectomized SJL/N mice with *Schistosoma japonicum* infection

No. of groups	No. of mice	P. C. V. (%)	R. B. C. ($\times 10^4$/mm^3)	M. C. V.
Expeaiment 1				
1	6	42.4±1.8	971±30	42.7±1.4
2	6	45.2±0.9	966±44	47.1±1.4
3	5	31.6±0.6*	976±28	32.5±1.0*
4	5	22.9±1.2*†	503±34*†	45.8±1.3
Experiment 2				
2	5	39.7±1.7	839±24	47.3±1.7
3	5	26.8±1.6*	856±19	31.3±1.8*
4	6	18.2±1.0*††	426±22*†	42.9±2.0

The number of experiments is explained in the foot-notes of Table 1.
significantly different group 3 vs. group 4 (†: $P < 0.001$, ††: $P < 0.01$)
**: significantly different group 1 vs. group 3 and group 2 vs. group 4 ($P < 0.001$)*
Each data shows mean ± standard error (compared with the parametric Student's t-test).

macrophages comprised the bulk of the lesions (Photos 1 and 2).

Table 3 The effect of splenectomy on the schistosome eggs production in the liver of SJL/N mice

Treatment of mice	No. of eggs/liver*
Experiment 1†	
sham operated	32,200±6,900
splenectomized	40,700±9,900
Experiment 2†	
sham operated	58,700±6,400
splenectomized	48,700±2,200

*: Each data shows mean ± standard error.
†: The mice were sacrificed at 9 weeks PI in experiment 1 and at 8 weeks PI in experiment 2.

Anti-SEA IgG Antibody Titers

Sera from each group were pooled to determine IgG antibody titers to SEA. Optical densities greater than 0.384 were decided as positive. All sera of infected mice groups showed the same titer (× 40,960). No difference of IgG antibody was investigated between splenectomized and non-splenectomized mice.

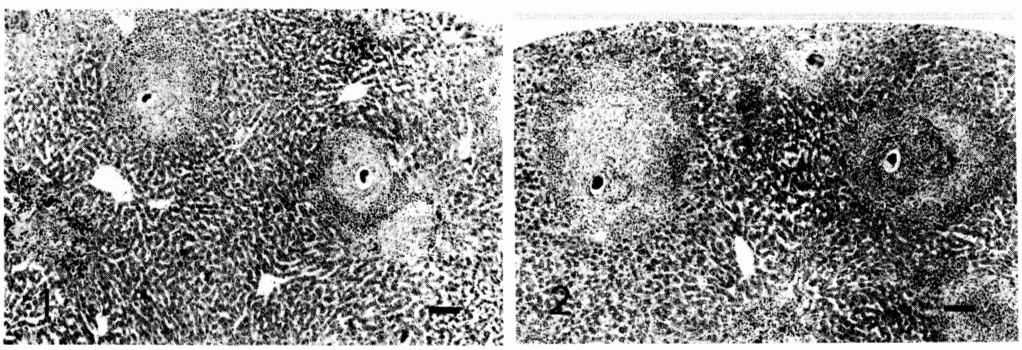
IgE Antibody to SEA in the S. japonicum Infected Mice

Sera from each group were pooled to determine IgE antibody titers to SEA. PCA reaction was performed in three rats. As shown in Table 5, the PCA titers of both sera at 8 and 9 weeks PI were higher in the splenectomized mice than in the sham treated mice. After heat treatment, those sera lost the ability to react to SEA on

Table 4 The effect of splenectomy on the granuloma size around one schistosome egg in the liver of SJL/N mice*

Treatment of mice	No. of examined granuloma	Size of granuloma ($\times 10^{-4}/mm^2$)
Experiment 1		
sham operated	231	970.0±62.5
splenectomized	209	1,774.4±79.4†
Experiment 2		
sham operated	117	985.6±79.5
splenectomized	163	1,631.5±28.0†

*: The mice were sacrificed at the 9th week of infection in experiment 1 and at the 8th week of infection in experiment 2.
Each data shows mean ± standard error and is compared with the parametric Student's *t*-test (†: $P < 0.001$).



Photos 1 and 2. The granuloma around schistosome eggs in the liver of sham operated (1: left) and splenectomized mice (2: right) at 8 weeks PI.

Table 5 IgE antibody titers to SEA of *S. japonicum* in SJL/N mice

Treatment of mice	anti-SEA IgE titer*	
	8 weeks	9 weeks
sham-treated	1 : 40	1 : 160
splenectomized	1 : 320	1 : 640

*: IgE titers were measured by PCA reactions using pooled sera at 8 or 9 weeks PI.

the back of the rat.

Discussion

Mice are the most common experimental models for studying the pathophysiology of human schistosomiasis mansoni and japonica, because mice and humans show relatively similar symptoms in the course of schistosomiasis. It is also well known that among inbred strains of mice, hosts respond differently to schistosome infection, varying in rate of infection, mortality and grade of resistance to reinfection (Class & Deelder, 1979; Mitchell *et al.*, 1981; Colley & Freeman, 1983; Kaji *et al.*, 1983).

We reported previously that splenectomy in ddY mice induced eosinophilia in the early course of the *S. japonicum* infection. Sugane & Oshima (1984) observed that SJL mice showed most significant eosinophilia among 8 inbred strains infected with *T. canis*. In this study, we used SJL/N mice to examine the effects of splenectomy on *S. japonicum* infection.

SJL mice are, also, known to develop a high incidence of Hodgkin's-like reticulum cell sarcoma (Dunn & Deringer, 1968; Siegler & Rich, 1968). Immunologically, SJL mice have non-specific suppressor T-cells on IgE production (Watanabe *et al.*, 1976), actively suppress immunoglobulin allotype synthesis (Jacobson & Herzenberg, 1972) and have other defects of T-cell and B-cell in response to synthetic polypeptide (Mozes *et al.*, 1975). When SJL mice were mildly irradiated, high titers of IgE antibody appeared during the course of parasitic infection (Watanabe, *et al.*, 1976). Warren (1969) first observed the suppression of granuloma formation around *S. mansoni* eggs in aged SJL mice which exhibited a Hodgkin's-like disease. Many researchers have used SJL mice with other strains to analyze susceptibility of parasites (Mitchell *et al.*, 1981; Colley & Freeman, 1983; Sugane & Oshima, 1984).

In this study, *S. japonicum* infection in SJL mice induced loss of body weight, thymic atrophy, hepatosplenomegaly and enlargement of the mesenteric lymph nodes. The mean weight of the mesenteric lymph nodes increased about 7 times that of the control mice. These changes were observed in ddY mice, which included the difference of degree (Amano & Oshima, 1983, 1984a).

Physiologically, *S. japonicum* infection induced leucocytosis with eosinophilia. After 7 weeks PI, leucocytosis and eosinophilia became progressively evident. The eosinophilia in SJL/N mice was more significant than that in C3H/He mice and in other inbred strains (Amano & Oshima, 1985, unpublished data). This result was the same as in inbred strains infected with *T. canis* (Sugane & Oshima, 1984), but the early response of leucocytosis to infection was very weak.

Splenectomy showed various effects on the *S. japonicum* infected mice. In a previous study (Amano & Oshima, 1984b), we pointed out that in ddY mice splenectomy effected significant eosinophilia in the early course of PI, but this reaction was weak in the splenectomized SJL mice. On the other hand, eosinophilia late in the course was not effected in SJL mice. The mechanisms of eosinophilia may be different between early and late course of infection. Leucocytosis appeared earlier and more significantly in splenectomized mice than in sham treated mice. Normochromic anemia was investigated in SJL mice in the same way as in ddY mice.

We recognized that the splenectomy of SJL mice before infection induced a significant increase of hepatomegaly, including enlargement of granuloma size around eggs, compared with sham treated and infected mice during the

course of *S. japonicum* infection. The effect of splenectomy on hepatomegaly was different from that of the ddY mice with *S. japonicum* infection (Amano & Oshima, 1984b) and CF1 mice with *S. mansoni* infection (Hood & Boros, 1980). In our previous study (Amano & Oshima 1984b, 1987), spleen removal before infection had no effect on hepatomegaly and splenectomy after infection, especially at the granulomatous stage, diminished the degree of hepatomegaly. Contrary to this, Hood & Boros (1980) found that splenectomy, carried out at the 8th week of infection, enhanced the granulomatous reaction in the liver of CF1 mice infected with *S. mansoni*. In the chronically *S. mansoni* infected mice, the granulomas around schistosome eggs were modulated with T suppressor lymphocytes (Colley, 1975). Hood & Boros (1980) suggested that the removal of T suppressor lymphocytes by splenectomy allowed an oversecretion of lymphokine, which resulted in augmented granulomatous responses of CF1 mice infected with *S. mansoni*. In the mice infected with *S. japonicum*, the size of circumoval granuloma decreased between 7 and 10 weeks of infection (Warren *et al.*, 1978; Cheever *et al.*, 1984). But in schistosomiasis japonica, the mechanisms of modulation of granuloma formation have not been made clear yet.

Aged SJL/N mice with moderate and advanced Hodgkin's-like disease showed the significant decrease of granuloma size around *S. mansoni* eggs (Warren, 1969). It can be assumed that these SJL/N mice have the effect of modulating the granuloma formation from a young age and splenectomy reduces the effect to modulate the granuloma formation around schistosome eggs. As a result, granuloma size around eggs might increase in the splenectomized SJL mice, but in our study the cellular compositions of granuloma showed no difference between splenectomized and non-splenectomized SJL/N mice.

Another interesting effect of splenectomy was the increase of IgE antibody titer to SEA, but no effect on the production of IgG to SEA. Little *et al.* (1982) reported that anti SEA-IgE antibody measured by PCA appeared 6 weeks PI and reached a peak at 9 weeks PI in CD1 mice infected with *S. japonicum*. They also reported that specific IgG, especially IgG1 antibody increased dramatically to a peak at 8 weeks PI and continued to rise thereafter until 20 weeks PI.

Watanabe *et al.* (1976) reported that SJL mice had non-specific T suppressor cells on the IgE production, which were sensitive to radiation. Watanabe & Ovary (1977) also noticed that the irradiated SJL mice produced high titers of IgE antibody and again suppressed the IgE production by the transfer of spleen cells from normal SJL mice. From these experiments, we can assume that splenectomy induces the decrease of non-specific T suppressor cells on the IgE production, which results in enhancement of IgE antibody to SEA and stimulates the granuloma formation in SJL/N mice infected with *S. japonicum*.

Warren *et al.* (1975) pointed out that the constitutions of granulomas around *S. japonicum* differed from those around *S. mansoni*. They suggested that the type of hypersensitivity reaction was different between these two schistosome infections. Ishii *et al.* (1982) pointed out that immediate hypersensitivity reaction was stronger to SEA than to adult worm antigen in experimental animals with *S. japonicum* infection. From the fact that IgE antibody titers are enhanced and enlarged granuloma is observed in the splenectomized SJL mice with *S. japonicum* infection, it might be suggested that an immediate type allergic reaction plays an important role in granuloma formation in schistosomiasis japonica, but more experimentation will be necessary to elucidate the relationship between granuloma size and IgE antibody titer to SEA.

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