THE BASIC IMMUNOLOGICAL STUDIES ON EXPERIMENTAL SCHISTOSOMIASIS JAPONICA

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INTRODUCTION

In order to explain the immunological reaction on the course of liver disease, a definite method for the detection should be needed. The antigen antibody reaction, which is accompanied with morphological reaction in the course of liver disease, is extremely variable. Accordingly, meanwhile, the change in relationship of antigen antibody reaction and the functional and structural change in the reaction are important. A part of these problems are treated by the authors in this paper.

In the experimental studies on Schistosomiasis japonica in rabbits, a part of these problems was studied by the authors. The localization of the antigen antibody reaction in adult worm, ova, miracidia and granuloma with ova in the organs after infection of cercariae were observed by the direct and indirect fluorescent antibody technique. At the same time, rise and fall of the antibody titer was serologically observed in the precipitin test and the tannic acid hemagglutination test. Liver function tests were performed to study the process of destruction of the liver cell.

Furthermore, after infection, the transformation of the serum fraction by the immunoelectrophoresis was studied and finally the immunological reaction of Schistosomiasis japonica was investigated.

MATERIALS AND METHODS

Experimental animal

Eighty male rabbits weighing approximate 2.0-3.0 kg which were infected with *S. japonicum* cercariae (about 500) of *Oncomelania nosophora* snails and 20 untreated rabbits were used as a control. Three normal goats and an infected goat were sensitized with normal rabbit serum, normal gamma-globulin serum, and serum of rabbits infected with *Schistosoma japonicum*.

Gathering method of infected rabbit serum and organs

The serum of infected rabbits was collected every two weeks from 2 weeks

to 20 weeks after infection. The serum of untreated rabbits was collected as a normal. These sera were treated as follow : the tannic acid hemmagglutination test, the precipitin test, the fluorescent antibody technique, and the immuno-electrophoresis. The above sera was also subjected to liver function tests.

Infected rabbits were sacrificed every two weeks and thymus, lung, heart, liver, kidney, adrenal gland, intestine and lymph nodes were removed immediatly. Untreated rabbit's organs were applied as a control.

Tannic acid hemmagglutination test

A modified Boyden's tannic acid hemmagglutination test (Tsuchiya's method) was used. 1 : 10,000 tannic acid solution was used.

1) Antigen

Male and female adult worms collected from the portal vein of infected rabbits were fully washed with physiological saline solution. After that, they were lyophilized and kept in a deeper freezer. These lyophilized worms were powdered and an extract (1 : 3,000) of phosphate buffered saline was made. This antigen was placed in a water bath at 30°C for 10 minutes for sensitization. After that, the high speed refrigerated centrifuge was used to obtain a clear supernatant fluid.

2) Antiserum

The sera collected from the infected rabbits and untreated rabbits were used. The serum was inactivated in a water bath at 56°C for 30 minutes.

Following the addition of 0.05% bovine serum albumin, the serum was diluted in P.B.S. at pH 6.4, to make 1 : 10, 1 : 20, 1 : 50, 1 : 100,, 1 : 100,000, and 1 : 200,000 dilutions.

Precipitin test

1) Antigen

The same lyophilized adult worms as an antigen for the hemmagglutination test were used.

2) Antiserum

The serum which was collected by the above-mentioned method was used as the antiserum. The test serum was inactivated in a water bath at 56°C for 30 minutes.

Fluorescent antibody technique

1) Antigen

a) Schistosoma japonicum

Cercaria, adult worm, and miracidium were used as an antigen.

b) Rabbit organs

Organs of rabbits infected with *S. japonicum* cercariae and untreated rabbits were used as the antigen. The organs were cut into small pieces of tissue (1.0×1.0×0.2 cm).

A piece of fresh tissue was fixed in a mixture of 95% ethyl alcohol and acetone and inbedded in paraffin.

[illegible]

TABLE 2
*Results of the hemagglutination test in serum of
 infected rabbit using adult worm as an antigen*

Serum Antigen	Normal	2 W	4 W	6 W	8 W	9 W	12W	14W	20W
10×	±	+	+	+	+	+	+	+	+
20×	—	+	+	+	+	+	+	+	+
50×	±	+	+	+	+	+	+	+	+
100×	±	+	+	+	+	+	+	+	+
200×	±	±	±	+	+	+	+	+	+
500×	—	—	—	±	+	+	+	+	+
1,000×	—	—	—	—	+	+	+	+	+
2,000×	—	—	—	—	+	+	+	+	+
5,000×	—	—	—	—	+	+	+	+	+
10,000×	—	—	—	—	+	+	+	+	+
20,000×	—	—	—	—	+	+	+	+	+
50,000×	—	—	—	—	+	+	+	+	+
100,000×	—	—	—	—	±	±	±	±	±
200,000×	—	—	—	—	—	—	—	—	—
Control	—	—	—	—	—	—	—	—	—

TABLE 3
*The amount of protein and F/P mol ratio of the fluorescent antibody
 solutions which were used in the fluorescent antibody technique*

F. A. solution		Protein (mg/dl)	F/P mol ratio
untreated	1 st fraction	0.4752	0.6599
	2 nd fraction	0.7265	1.0220
6 W	1 st fraction	0.3083	0.5134
	2 nd fraction	0.2331	0.4840
8 W	1 st fraction	0.2800	0.3906
	2 nd fraction	0.1134	0.9946
10 W	1 st fraction	0.0618	0.3542
	2 nd fraction	0.2200	0.6736
12 W	1 st fraction	0.5226	0.7347
	2 nd fraction	0.4496	1.3671
20 W	1 st fraction	0.5388	0.3657
	2 nd fraction	0.1548	1.6534
Anti-normal rabbit γ -gl. goat γ -gl.	1 st fraction	0.1658	0.9344
	2 nd fraction	0.3462	1.5275

*After twice absorption by the liver powder of mice, 1 st fraction was
 used in this experiment.*

after infection, increased to 300 fold 4 weeks after infection and further increased to 2,400 fold between 6 and 10 weeks (TABLE 1).

2) Tannic acid hemmagglutination test

An antigen titer of antiserum of infected rabbit was 100 fold by 4 weeks after infection, 200 fold in 6 weeks and then increased to 50,000 fold between 8 and 20 weeks. Uninfected rabbit's serum as a control revealed only 200 fold in antigen titer (TABLE 2).

3) Liver function tests

a) Changes in an electrophoretic pattern of serum proteins were shown in Fig. 1, 2, 3, 4, 5, 6. Albumin decreased remarkably 8 weeks; α_1 -globulin decreased gradually from 8 weeks; α_2 -globulin increased slightly after 8 weeks. Beta-globulin increased extremely in 8 weeks and decreased slightly at 12 and 20 weeks. Gamma-globulin began to increase 8 weeks, and tended to rise more up to 20 weeks (Fig. 1, 2, 3, 4, 5).

Fig. 1. Transformation of albumin fraction in serum of infected rabbit.

Fig. 2. Transformation of α_1 -globulin fraction in serum of infected rabbit.

Fig. 3. Transformation of α_2 -globulin fraction in serum of infected rabbit.

Fig. 4. Transformation of β -globulin fraction in serum of infected rabbit.

Fig. 5. Transformation of γ -globulin fraction in serum of infected rabbit.

Fig. 6. Transformation of transaminase in serum of infected rabbit.

b) Transaminases

Both s-GOT and s-GPT increased 4 weeks abnormally and then decreased thereafter (Fig. 6).

4) Immunoelectrophoresis

Immunoelectrophoretic patterns of infected rabbit's sera, using croat anti-serum against normal rabbit serum, were shown in Fig. 19. Reinforcement of precipitate line in beta-globulin fraction was observed in those 2, 4, 6, 8, 10, 12, 14, and 20 weeks after infection (Fig. 19).

5) Fluorescent antibody technique

a) Indirect technique

Schistosoma japonicum :

First, serum of normal and infected rabbits 20 weeks post infection was used. The degree of dilution of F.A. solution and serum dilution were decided by the box titration test. In the same condition, cercaria, adult worm and miracidium were treated by the indirect technique to the serum of normal and infected rabbits 2-20 weeks after infection.

cercaria :

In F.A. dilution $4\times\sim 16\times$, serum dilution $10\times\sim 100\times$, no fluorescence was seen in normal rabbits serum but seen slightly between 2 weeks and 20 weeks after infection.

adult worm :

In F.A. dilution $8\times\sim 32\times$, serum dilution $10\times\sim 100\times$, no fluorescence was seen in them until 4 weeks after infection but seen slightly from 8 weeks.

miracidium :

In F.A. dilution $4\times\sim 16\times$, serum dilution $10\times\sim 100\times$, no fluorescence was seen in them until 6 weeks after infection but seen between 8 weeks and 20 weeks (TABLE 4, 5).

Liver

H-E staining findings :

Lymphocytes, plasma cells, phagocytes and giant cells were seen in the surroundings of the nodules due to ova. Furthermore, the circumference was enclosed with collagen fibers of poor cellularity. Remarkable degenerative necrosis of liver cells was rarely seen. Hyperplasia of bile duct was not remarkable (Fig. 13).

TABLE 4

Representative titration in the fluorescent antibody technique using miracidium as an antigen for optimal dilutions of serum and fluorescent antibody solution

Serum specimen	F. A. dilutions Serum dilutions	1 ×	2 ×	4 ×	8 ×	16 ×	32 ×	64 ×
20 W	1 : 1	++	++	+	+	+	±	—
	1 : 10	+	+	+	+	±	—	—
	1 : 100	+	+	+	+	±	—	—
	1 : 200	+	+	±	±	—	—	—
	1 : 400	+	+	±	±	—	—	—
Normal	1 : 1	+	+	±	—	—	—	—
	1 : 10	+	±	—	—	—	—	—
	1 : 100	±	±	—	—	—	—	—
	1 : 200	±	—	—	—	—	—	—
	1 : 400	±	—	—	—	—	—	—
Control	0.85% Saline	—	—	—	—	—	—	—

TABLE 5

Results obtained in the indirect fluorescent antibody technique for serum of rabbit infected with Schistosoma japonicum

Week	F. A. dilutions	4 ×		8 ×		16 ×	
		Serum dilutions		Serum dilutions		Serum dilutions	
		1 : 10	1 : 100	1 : 10	1 : 100	1 : 10	1 : 100
Normal		—	—	—	—	—	—
2 W		—	—	—	—	—	—
4 W		—	—	—	—	—	—
6 W		—	—	—	—	—	—
8 W		±	±	±	—	—	—
12 W		±	±	±	±	±	—
16 W		+	±	±	±	—	—
20 W		+	+	±	±	±	±
Control (0.85% Saline)		—	—	—	—	—	—

PAS staining findings :

Positive reaction was seen in the shell of the ovum, and collagen fibers of the granulomas surrounding it.

Fluorescent antibody technique :

Fluorescence which glittered greenly at the part of collagen fibers surrounding the ova, was seen in each serum in 6, 8, 12 and 20 weeks. Slight fluorescence was seen in the above-mentioned part in normal rabbits serum, and, very slight fluorescence was also seen at the same part in a control (0.85% saline). Fluorescence shining a gold color, which looked non-specific, was seen in the shell of the ovum. Yet these findings were recognized in the liver progressed 6, 8, 12 and 20 weeks after infection but not recognized at all in the liver of the normal rabbit (Fig. 15).

Intestine

H-E staining results :

Staining results were similar to the liver. Lymphocytes, plasma cells, phagocytes, and giant cells were found surrounding the ova and collagen fibers in which there was not an abundance of cells (Fig. 14).

PAS staining :

Positive reaction was seen in the shell of the ovum and collagen fiber of the granuloma surrounding the ova.

Fluorescent antibody technique :

The findings were quite similar to the liver. Fluorescence was recognized in the part of the collagen fiber of the granuloma surrounding the ova. As compared with the part of the nodules due to ova, slight fluorescence was recognized. The above-mentioned reaction was recognized in each serum progressed 6, 8, 12, and 20 weeks after infection and in serum of normal rabbits; a slight fluorescence was recognized in some of the controls (0.85% saline) very slight fluorescence was also seen in the same part. Though the above-mentioned findings were not seen at all in normal intestine, the same findings were also recognized in the intestine progressed 6, 8, 12, and 20 weeks after infection (Fig. 16).

Spleen

In fluorescent antibody technique, fluorescence shining apple-green was recognized firmly in the red pulp. This fluorescence was also recognized in each week's serum and the same findings were also recognized in the spleen progressed 6, 8, 12, and 20 weeks after infection. Furthermore, a slight fluorescence was recognized in some of the controls (0.85% saline) (Fig. 17).

b) Direct technique

Gamma-globulin progressed 6, 8, 12, and 20 weeks after infection, and the normal was labelled with F.I.T.C. and used.

Schistosoma japonicum

Cercariae, adult worms, and miracidia revealed very slight fluorescence in untreated rabbit gamma-globulin. In rabbit gamma-globulin progressed 6, 8, 12,

and 20 weeks after infection, three groups had almost the same fluorescence in about the same degree of dilution and the gradual difference was not recognized among three groups (Fig. 7, 8, 9, 10, 11,).

Liver, Intestine

These findings were remarkably different from the indirect technique findings. Namely, in gamma-globulin progressed 6, 8, 12, and 20 weeks after infection, fluorescence brightening apple-green was recognized in the ova, but in granuloma, which was found by the indirect technique, only slight was recognized. Yet as compared with the above-mentioned findings, in normal gamma-globulin, decrease of the fluorescent reaction was seen. The above-mentioned findings were seen in the organs progressed 6, 8, 12, and 20 weeks after infection but not in normal organs (Fig. 12, 18).

Spleen

In this organ, fluorescent reaction which was recognized in the red pulp in the indirect technique decreased markedly.

Thus a very slight fluorescence was recognized and nothing more.

DISCUSSIONS AND CONCLUSION

The authors presented the investigations in which the antibody production of rabbit infected with cercaria was measured by ring test and tannic acid hemagglutination test by using the adult worm as an antigen. In the results, it was shown that the antibody level began to rise 4 weeks after infection and continued to increase up to 20 weeks.

According to the lowering of the A/G ratio, the increase of beta-globulin, the increase of gamma-globulin and especially the increase of transaminases with the increase of the antibody appearance ratio, it seemed that liver function and morphological changes found, were related with destruction of the liver.

However, histologically, the findings of piecemeal like necrosis in the liver were not observed but considerable exudative inflammation of the nodules due to ova with granuloma and relative destruction of the limiting membrane of the liver was found.

It was considered that the increase of ratio of the antibody appearance, which was measured by the above mentioned methods, was mainly caused by the direct infection of cercariae.

The indirect approach of fluorescent antibody technique was employed as the method of serological diagnosis and serum antibody certification in schistosomiasis japonica. Sadun et al. (1962) employed *Schistosoma mansoni* cercaria as an antigen. Sato (1965) compared and investigated the difference of the speciality in miracidium and cercaria.

The indirect fluorescent reaction between the experimental rabbit serum and the miracidium antigen were employed. As the results of this test, fluorescent

reaction of the serum from 2 weeks to 6 weeks after infection was not clear but remarkable in the serum from 8 weeks to 20 weeks. Thus, it was considered that the infected serum contained some specific reaction factor for miracidium. The specific fluorescent reaction was seen in cercaria until the end of 20 weeks after infection.

Positive fluorescent reaction was clearly seen in male and female adult worms, however, according to anatomical difference, the fluorescent reaction revealed marked variance of intensity. For instance, the nonspecific fluorescent reaction was marked in the esophagus, intestine and immature ova in the uterus. Therefore, it was considered that the antigens of each sex were found to differ. It was difficult to compare sexually the antigens of the adult worm because of big, thick worm body.

In these adult worm and cercaria, it was hard to deny that the specific antigen was included and common antigen also existed in them as miracidium.

F/P ratio of each fluorescein-labelled rabbit gamma-globulin which was used in the direct and indirect technique revealed as TABLE 3.

In the direct fluorescent antibody technique, first, marked fluorescent reaction, which was more remarkable than in the indirect technique, was seen in the direct technique between cercaria, adult worm and miracidium and the fluorescein-labelled rabbit gamma-globulin 6, 8, 12, 20 weeks after infection.

Popper et al. (1961) employed the fluorescent antibody technique for the organs in experimental schistosomiasis mansoni, and immunological reaction of the spleen and nodules due to ova in schistosomiasis mansoni was revealed by the indirect technique.

The reaction on the liver, intestine, spleen, lymph nodes, lung and heart etc. in the direct technique and indirect technique were made. The infected rabbit gamma-globulin revealed more remarkable specific fluorescent reaction in the ovum of the liver and intestinal submucous tissue. It was considered that the infected rabbit gamma-globulin had the similar significance as cercaria, adult worm and miracidium as mentioned above.

In the case of 6 weeks after infection, the fluorescent reaction, which differed from the reaction of the direct technique, was clearly revealed in the part enclosing the ovum and collagen fiber of granuloma of relatively poor cellularity, in the indirect technique. It was considered as the specific reaction. In the direct technique, no remarkable reaction to the gamma-globulin of each weeks 6, 8, 12, 20 weeks was observed in the above-mentioned part but remarkable in the ovum. It was considered that the antigen of cercaria, adult worm and miracidium had the specific and common reactive factor. In fact, it was hard to deny that different antigen production to granuloma around the ovum existed in *Schistosomiasis japonica*. If the existence of the above-mentioned different antibody production has a possibility, it is hard to deny that the different antibody provokes the interstitial tissue reaction which is a part of

the autoimmunoreaction in the liver and may be one of the factors of chronic liver disease, in addition to the antigen antibody reaction with cercaria, adult worm and miracidium. It was unclear whether the fluorescent reaction to the granuloma of the liver and intestine depended upon the same factor of the antigen antibody reaction or not. It may be considered that the antibody is produced in the same body also contains the autoantibody of the organs which is produced in the organs with granuloma of schistosomiasis japonica.

The success of solving the important immunological problems the field of schistosomiasis japonica are expected.

SUMMARY

The rabbits were infected with about 500 cercariae of *Schistosoma japonica*. The localization, specificity, and change of the antigen antibody reaction in adult worm, ovum, and miracidium and granuloma with ova in the organs after infection of cercariae were observed by the direct and indirect fluorescent antibody technique. At the same time, rise and fall of the antibody titer was serologically observed in the precipitin test and the tannic acid hemagglutination test. The liver function tests were performed to study of destruction of the liver. Furthermore, after infection, the transformation of the rabbit serum fraction by the immunoelectrophoresis was studied. The results of the above experiments were as follow.

1) In the precipitin test and the tannic acid hemagglutination test using adult worm as antigen, antibody titer rose in 6-8 weeks after infection and it continued until 20 weeks.

2) It was observed that the serum protein fraction and the transaminase changed remarkably. Albumin fell and beta-, gamma-globulin increased in 8 weeks after infection. Gamma-globulin tended to increase more in 20 weeks after infection. Both s-GOT and s-GPT increased remarkably in 4 weeks after infection and decreased remarkably in 8 weeks, it increased and decreased until 20 weeks at the line of the normal rate.

3) Specific strong precipitate line in beta-globulin region was observed in those 2, 4, 6, 8, 10, 12, 14, and 20 weeks after infection by immunoelectrophoresis.

4) The direct fluorescent antibody technique was applied to cercaria, adult worm and miracidium and the fluorescein-labelled infected rabbit gamma-globulin with *Schistosoma japonica*. The specific fluorescence was seen in each. It was hard to deny that common antigen existed in each. The fluorescent reaction of cercaria, adult worm, and miracidium of *Schistosoma japonica* in each period (6-, 8-, 12-, and 20 weeks after infection) was not found to differ. Meanwhile, slight fluorescence was also observed in the control which was normal rabbit gamma-globulin.

5) In the indirect technique, fluorescent reaction of cercaria, adult worm, and miracidium was observed in untreated rabbit and infected rabbit from 2 weeks to 20 weeks after infection.

In cercaria, slight fluorescent reaction was observed from 2 weeks to 20 weeks after infection. (F.A. dilution $4\times\sim 16\times$ dilutions, serum dilution $10\times\sim 100\times$ dilutions)

In adult worm, no fluorescence was seen in them until 4 weeks after infection but seen slightly from 8 weeks to 20 weeks. (F.A. dilution $8\times\sim 32\times$, serum dilution $10\times\sim 100\times$)

In miracidium, no fluorescence was seen in them until 6 weeks after infection but seen between 8 weeks and 20 weeks. (F.A. dilution $4\times\sim 16\times$, serum dilution $10\times\sim 100\times$).

6) Markedly different findings were seen in the fluorescent reaction to labelled gamma-globulin in the infected rabbit organs.

Bright apple-green like specific fluorescence to labelled gamma-globulin 6, 8, 12, and 20 weeks after infection was observed in the ovum of the liver and intestine in the direct technique. However, very slight fluorescence was seen in the granuloma.

Meanwhile, in the indirect technique, bright apple-green fluorescence was seen in the ovum, the granuloma around the ovum of the liver, intestine, and the red pulp of the spleen. However, this reaction was partially seen in the control.

7) No fluorescence was seen in the normal organs.

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EXPLANATION OF FIGURES

- Fig. 7.* Cercaria has been treated with fluorescein-labelled rabbit gamma-globulin of 8 weeks after infection (Direct technique).
- Fig. 8.* Miracidium has been treated with fluorescein-labelled rabbit gamma-globulin of 12 weeks after infection (Direct technique).
- Fig. 9.* Adult worm (male and female) has been treated with fluorescein-labelled rabbit gamma-globulin of 20 weeks after infection (Direct technique).
- Fig. 10.* Head of adult worm (male) has been treated with fluorescein-labelled rabbit gamma-globulin of 20 weeks after infection. Non specific fluorescein was observed in testis (Direct technique).
- Fig. 11.* Adult worm (female) has been treated with fluorescein-labelled rabbit gamma-globulin of 8 weeks after infection (Direct technique).
- Fig. 12.* Liver of a rabbit 8 weeks after infection with *Schistosoma japonicum*. The section has been treated with fluorescein-labelled rabbit gamma-globulin 6 weeks after infection. Specific fluorescein is observed in the ovum. The shell of the ovum shows golden-yellow fluorescence (Direct technique).
- Fig. 13.* A schistosomal granuloma in a rabbit with infection of 8 weeks standing. The section of liver has been stained with hematoxylin-eosin.
- Fig. 14.* Rectum of a rabbit 8 weeks after infection with *Schistosoma japonicum*. The section has been stained with hematoxylin-eosin.
- Fig. 15.* Liver of a rabbit 20 weeks after infection with *Schistosoma japonicum*. The section has been treated with fluoresceinated anti rabbit gamma-globulin. Fluorescence is observed within the granuloma and ovum and at its periphery. The shell of the ovum shows bright yellow fluorescence (Indirect technique).
- Fig. 16.* Rectum of a rabbit 8 weeks after infection with *Schistosoma japonicum*. The section has been treated with fluorescein labelled anti rabbit gamma-globulin. The fluorescence is observed within the granuloma and ovum (Indirect technique).
- Fig. 17.* Spleen of a rabbit 8 weeks after infection with *Schistosoma japonicum*. The section has been treated with fluoresceinated anti normal rabbit gamma-globulin. Fluorescence is observed in the red pulp (Indirect technique).
- Fig. 18.* Rectum of a rabbit 8 weeks after infection with *Schistosoma japonicum*. The section has been treated with fluorescein-labelled rabbit gamma-globulin of 8 weeks after infection. Fluorescein is observed within the ovum. The shell of the ovum shows golden-yellow autofluorescein (Direct technique).

Fig. 7

Fig. 8

Fig. 9

Fig. 10

Fig. 11

Fig. 12

Fig. 13 *Fig. 14* *Fig. 15* *Fig. 16* *Fig. 17* *Fig. 18*

Fig. 19. Transformation of serum protein fraction in infected rabbit by the immunoelectrophoresis.