[Jpn. J. Parasitol., Vol. 37, No. 1, 1–8, February, 1988]

Iron in the Schistosome Pigment, Egg and Adult Worm of *Schistosoma japonicum* Detected by Electron Probe X-ray Microanalysis

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(Received for publication; April 6, 1987)

Abstract

In relation to iron turnover and anemic state of schistosome infection, location and distribution of iron in the murine hepatic Kupffer cell, egg in the granuloma in the 6th week after infection, and in the alimentary tract and its adjacent area of *Schistosoma* japonicum (3 weeks old) were examined by electron probe X-ray microanalysis electron microscopy.

The pigment examined by X-ray microanalysis electron microscopy showed a peak of 6398 eV which indicated the presence of iron. It could be demonstrated directly that the schistosome pigment contained a large quantity of iron. The brownish granules in the intestinal lumen of schistosome worm also contained iron, but no iron could be detected in the worm body near the intestine and egg in the liver granuloma. It was considered that the schistosome pigment in the Kupffer cell was derived from host hemoglobin digested by the worms. Mass of iron deposited in the liver was measured by the method of nitroso-DMAP as a reagent. Iron concentration in infected mouse liver showed 3.3 mg iron/100g of liver 9,000xg sediment in the 9th week and 4.4 mg in the 15th week after infection. These values are 1.15 and 1.29 times higher than those in control mice, respectively.

Key words: *Schistosoma japonicum,* schistosome pigment, X-ray microanalysis, hematin

Introduction

Animals and human patients with schistosome infection show an accumulation of brownish pigment in the hepatic Kupffer cells. It was disputed whether this pigment is a hemoglobin derivative or melanin (Johnson *et al.,* 1954; Sawada *et al.,* 1956; Sano and Ishii, 1979).

However, by means of biochemical techniques, it was suggested that the pigment was an iron-containing derivative of hemoglobin, and probably formed by digestion of erythrocytes in the alimentary tract of worms (Ostrow and Warren, 1965). To get a direct evidence containing iron in the murine hepatic Kupffer cell, egg in the granuloma, and alimentary tract and its adjacent area in a body of *Schistosoma japonicum,* pigment granules in their sites were examined by electron probe X-ray microanalysis. X-ray microanalysis combined with an electron microscope and an energy-dispersed X-ray spectrometer has become a useful method for the analysis of elements and fine distribution of them in biological materials (Mizuhira 1971, Mizuhira 1976).

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Materials and Methods

Electron microscopy and X-ray microanalysis

Young male closed-colony mice of ddY strain (4 weeks old) obtained from Kitayama LABES Co., LTD. (Kyoto, Japan) were intraperitoneally infected with 60 cercaiae of *Schistosoma japonicum* (Kofu strain).

The liver of infected mice were examined in the 6th week and 25th week after infection. The liver was cut to pieces and fixed in cold Karnovsky's glutaraldehyde and paraformaldehyde mixture for 20 hr at 4℃. Post-fixation

was carried out with 2% OsO4 for 2 hr after washing away aldehyde with 0.1 M cacodylate buffer (pH 7.4) containing 4.5% sucrose to adjust osmotic pressure. The liver specimens were dehydrated and embedded in a mixture of Epon 812 and 815 resin. Pairs of *S. japonicum* at 3 weeks of age were treated with the same ways. The liver and *S. japonicum* specimens were sectioned to 0.2 μm-thick by a Portar-Blum Ultramicrotome for light microscopy, transmission electron microscopy and X-ray microanalysis. The sections were stained with 0.03% toluidine blue for an observation of egg granuloma in the liver by a light microscope. For X-ray microanalysis, at first, the thick unstained sections were observed under a transmission electron microscope (Hitachi HU-12A) and then examined under a scanning transmission electron microscope (HU-12A) equipped with energy-dispersed X-ray spectrometer (EDAX 711). The following conditions were used for the stationary spot analysis; accelerating voltage: 25 kV, spot size: 30-50 nm, electron beam current: $1X10^{-10}$ amperes, analyzing time: 100-200 sec.

Determination of iron contents in the liver

The liver of C3H mice (7 weeks old) infected with 40 cercariae of *S. japonicum* were examined for iron contents at the 9th and 15th week after infection. The liver of a mouse at each stage was perfused with 0.15 M KC1 and homogenized with three volumes of the same solution for 5 min. After centrifugation (9,000xg for 20 min), 0.5g of tissue sediment was dissolved in a mixture of 5 ml of concentrated nitric acid (S.G. 1.38) and 0.5 ml of 60% perchloric acid. Iron concentration in the mixture was determined in duplicate by reading the change in optical density at 750 nm of 2-nitroso-5-dimethylaminophenol (Toei *et al.*, 1975). The iron concentrations of the 9,000xg supernatant (S9) of 3 mice was measured at each stage by the direct nitroso-PSAP method (FeC Kit, Wako Pure Chem. Indust., Tokyo) and were expressed as mean values.

Results

Preliminary observation of the liver by light-microscopy

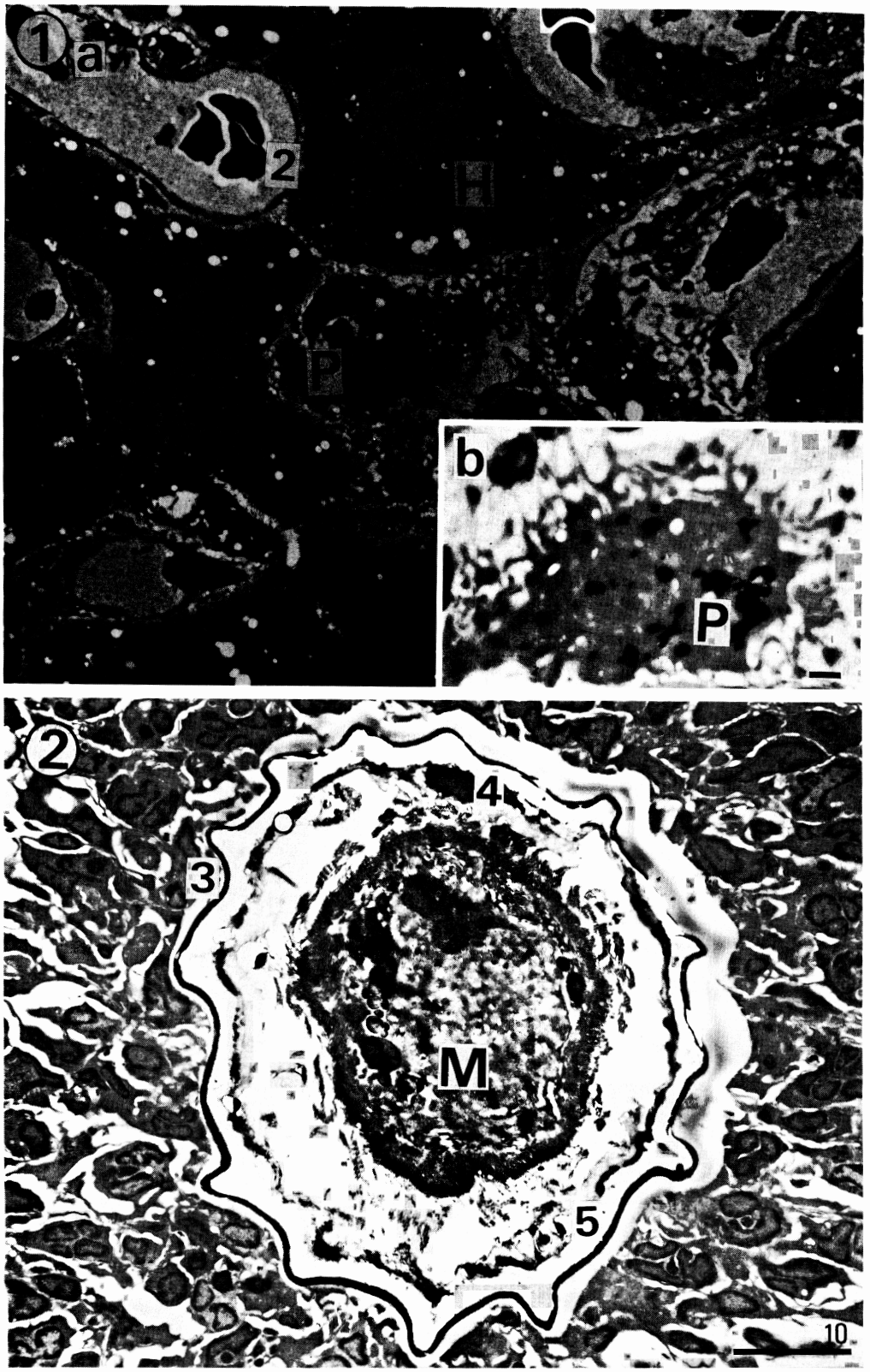
A granuloma with infiltration of various kinds of cells around the egg was seen in the liver of ddY mouse at 6 weeks after infection. Hepatic cells occupied the central region of the picture and disruption of hepatic cords was observed. The Kupffer cells in sinusoid contained a large amount of the schistosome pigment which looked to be brownish granules. Paired schistosome worms of 3 weeks after infection was sectioned transversely at the anterior part. The female worm was located inside of the gynecophoral canal of the male. Many brownish granules were seen in the intestinal lumen of male and female worms. In the body of a male worm, a number of granules looks like brownish pigment granules in the intestinal lumen were noticed near the intestine.

Preliminary observation by an electron microscope for the choice of sites to be determined by X-ray microanalysis

As shown in Figs. 1a and 1b, the schistosome pigment (1 and P) accumulates as osmophilic and electron-dense granules in the Kupffer cells. Red blood cell containing iron as hemoglobin was selected as reference (2 in Fig. 1a). The distribution of iron was examined in the egg shell (3 in Fig. 2) and electron-dense areas (4 and 5 in Fig. 2) around miracidium.

Fig. 1. Fig. 1a shows an electron microscopical view of the liver. The Kupffer cell with schistosome pigment (P) in the sinusoid, red blood cell (2) and normal hepatocyte (H) are seen. As shown in high-magnificated picture (Fig. 1b), the schistosome pigments (1 and P) are seen as osmophilic, irregular size granules in the Kupffer cell (bar indicates 1 um).

Fig. 2. This electron microscopical photograph shows an egg in egg granuloma of the liver. X-ray microanalysis was done at the sites of egg shell (3), electron-dense area (4) and electron-dense granule (5) around a miracidium (M).



(3)

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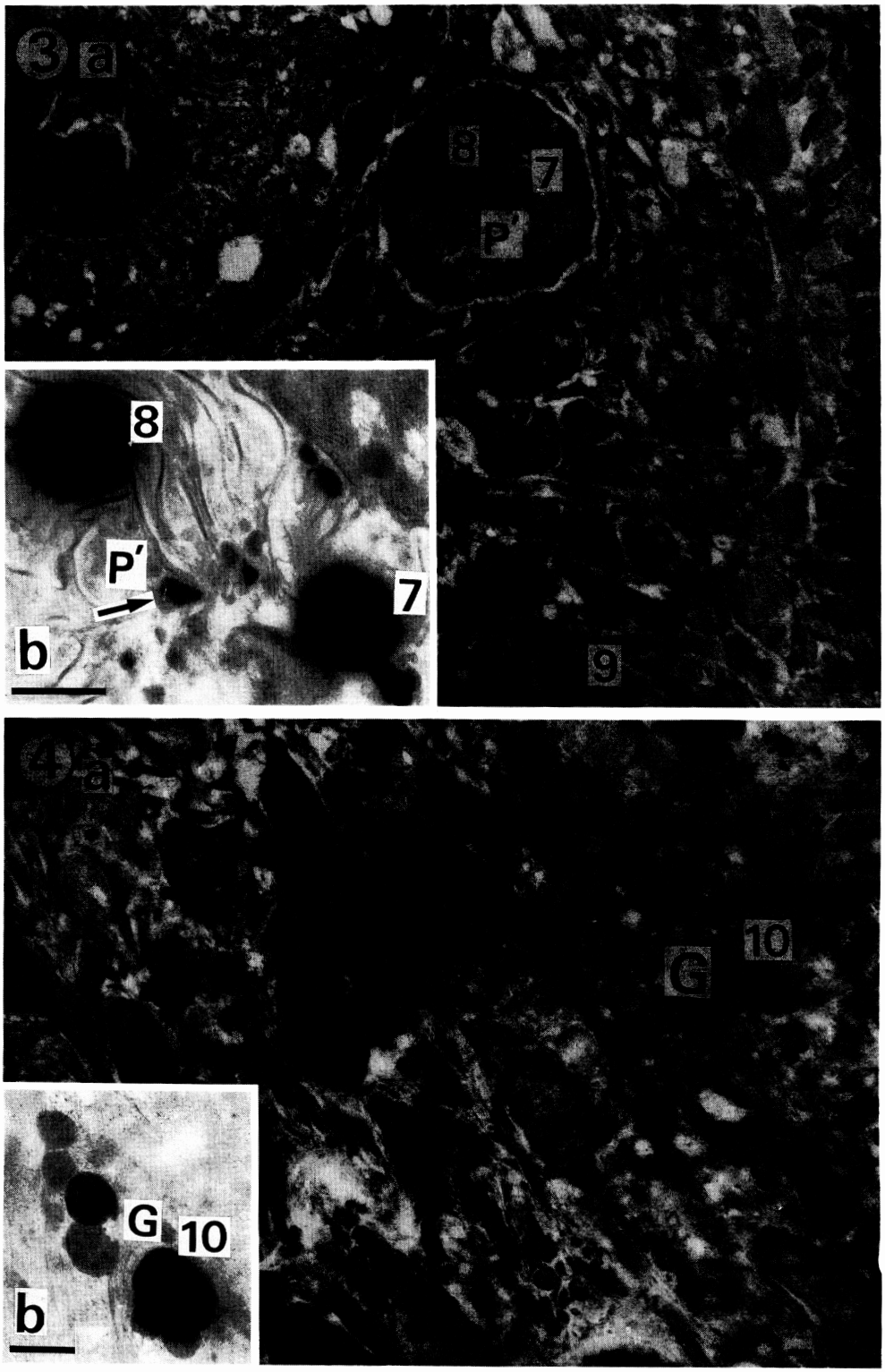

In the intestinal lumen of schistosome worms, many osmophilic and morphologically irregular granules (Figs. 3a and 3b) which be similar to the schistosome pigment in the Kupffer cell were observed. One of the granules (7 and P' in Fig. 3) as examined by X-ray spot microanalysis. Though the diameter of the beam spot in X-ray spot microanalysis was 30-50 nm, the burned spot mark on the specimen by the beam was about 1 μm in diameter. The granule-free area (8 in Fig. 3a) in the intestinal wall and the nucleus (9 in Fig. 3a) were selected as reference for spot analysis of iron. Osmophilic granules (10 and G in Figs. 4a and 4b) near the intestine of male worm were also examined by X-ray spot analysis. The granules are round in shape and regular in size of about 0.5 um. They are morphologically distinct from the schistosome pigment in the Kupffer cell.

X-ray microanalysis

The results by X-ray microanalysis in the liver (1-6 in Fig. 5) and the worms (7-10 in Fig. 6) are summarized in Figs. 5 and 6, respectively. Abscissa, expressed in KeV, shows the characteristic and specific X-ray energy spectrum dispersed from elements exposed to the electron beam. Ordinate shows the intensity of the X-ray signals of individual elements.

The schistosome pigment (1 in Fig. 5) in the Kupffer cell had a clear peak of iron at 6398 eV. Other peaks showed osmium at 1914 eV which was used as fixative solution, and copper at 8040 eV which reflected the copper-grid itself. In red blood cell (2 in Fig. 5), the peak of iron was not detected. Egg shell (3 in Fig. 5) did not reveal the peak of iron. However, a minor peak of calcium at 3690 eV was seen in addition to peaks of osmium and copper. The diffuse and electron-dense area (4 in Fig. 5) of

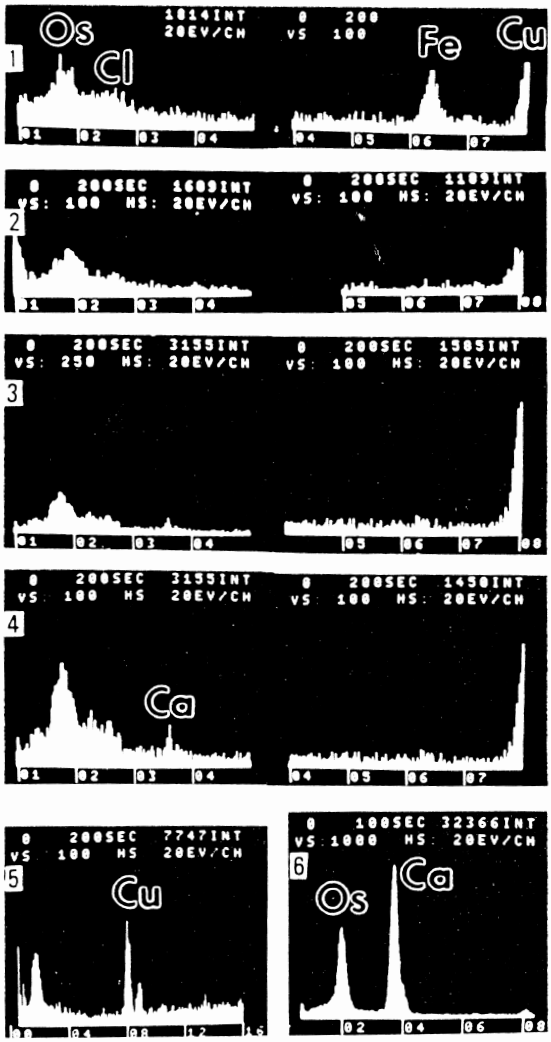

Fig. 5. Results of X-ray microanalysis in the liver are summarized in this figure. The numbers from 1 to 5 correspond with those in Fig. 1 and Fig. 2, respectively. The number of 6 shows a result with an egg in the liver at 25 weeks after infection.

Fig. 3. An electron microscpical view of the female worm's intestine (Fig. 3a). Granules (P', arrow) in the intestinal lumen (Fig. 3b) are osmophilic and looked like to the schistosome pigment morphologically. Each bar indicates 1 um.

Fig. 4. An electron microscopical view of the male worm at a site near the intestine. Numerous round granules (10 and G) are seen in the tissue (Fig. 4a). In high-magnificated picture (Fig. 4b), the granules (10 and G) are osmophilic but distinguished with its feature from the schistosome pigment or granules in the intestinal lumen. Each bar indicates 1 um.

the peri-miracidial space showed the same spectrum as the egg shell. The granule (5 in Fig. 5) in the peri-miracidial space did not show any peaks of iron and calcium. Two peaks in the center of the spectrum showed Kα (8040 eV) and Kβ (8904 eV) of copper. Egg shell (6 in Fig. 5) of 25 weeks after infection showed only two peaks of osmium and calcium. The peak of calcium was remarkably high. Iron could not be detected in neither eggs at 6 weeks nor those at 25 weeks after infection.

On the granules (7 in Fig. 6) in the intestinal lumen of female worm, the peak of iron could be detected clearly at 6398 eV. Other granule-free areas (8 in Fig. 6) in the intestine, nucleus (9 in Fig. 6) and osmophilic granules (10 in Fig. 6) near the intestine of the male worm did not show the peak of iron.

Iron content in the liver

As shown in Fig. 7, iron concentration in S9 preparation of the control mice did not show a prominent change and its values ranged from 318 to 434 μg/ml. However, in the experimental group, iron concentration decreased at around 13 weeks and reached 204 μg/ml at 15 weeks, the value of which was about a half of that in the control. Accumulation of iron in the tissue increased gradually in proportion to infection period. In the 9th week, the

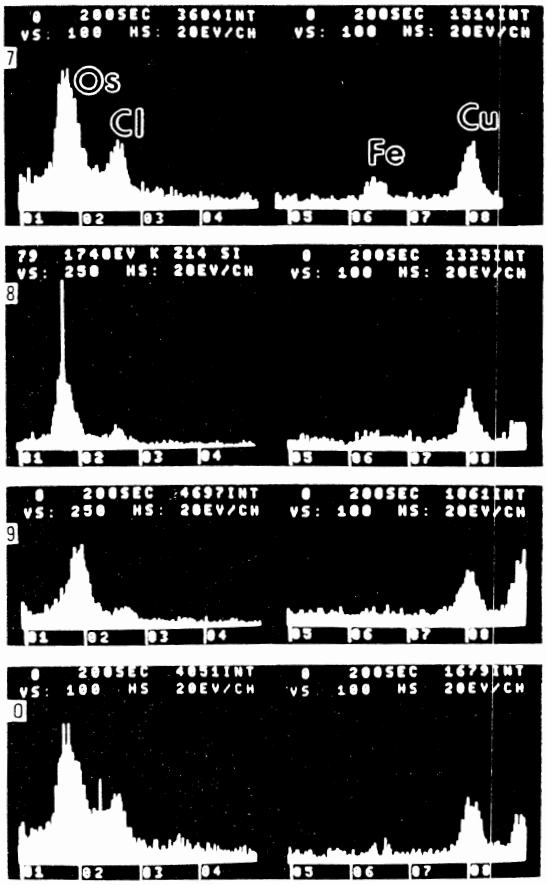

Fig. 6. Results of X-ray microanalysis in the worm are summarized in this figure. The numbers from 7 to 10 correspond with those in Fig. 3 and Fig. 4, respectively.

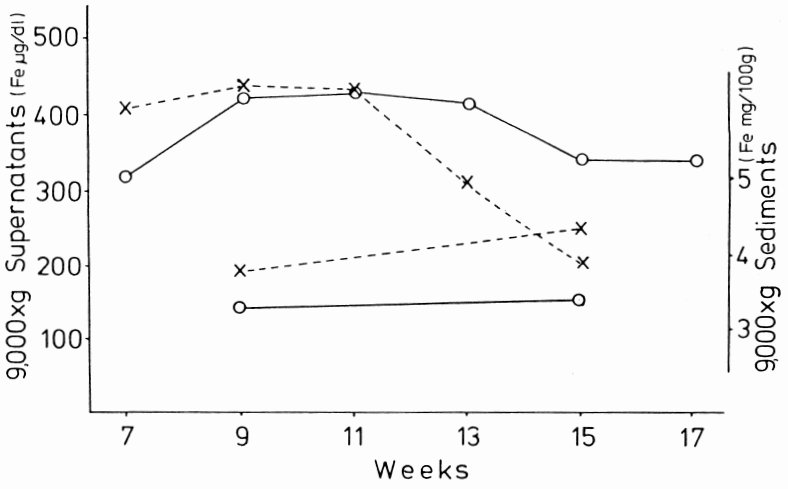

Fig. 7. Iron contents in 9,000×g sediments of the liver (lower lines) and its supernatant (S9 preparations; upper lines). (x------x: experiment, O------O: control)

concentration of the experimental group showed 3.8 mg/100g of 9,000xg liver sediment which was 1.15 times of the control. In the 15th week iron content reached 4.4mg which was 1.29 times on the control. A total amount of 0.6 mg iron/100g of liver sediment accumulated in the liver of infected mice during 6 weeks.

Discussion

Schistosome pigment accumulates gradually in the Kupffer cell throughout schistosomiasis. The nature of the pigment has been examined histochemically by a light microscope. Sawada *et al.* (1956) described that the pigment in the Kupffer cell showed a marked affinity to silver and the lack of any evidences for the presence of ferric, ferrous and masked iron. However, Ostrow and Warren (1965) reported that the pigment was benzidine-positive but Prussian-blue-negative, and had characteristic absorption spectra of acid and alkaline hematin. They suggested that the pigment was an iron-containing derivative of hemoglobin. Sano and Ishii (1979) examined both schistosome pigment and malarial pigment by elemental X-ray microanalysis. They concluded that both pigments contained iron.

On the other hand, the pigment itself appeared intensely osmophilic, and had ovoid or doughnut-shaped configurations (Stenger *et al.*, 1967). In the present study, osmophilic granules were also found in the intestinal lumen, somatic tissue near the intestine of schistosome worms and egg in the granuloma of the mouse liver. It is very useful to apply electron probe X-ray microanalysis for the examination of iron microlocalization in these granules. It is said that X-ray microanalysis can detect theoretically a minimum absolute concentration of 10^{-13} g of elements if all analytical conditions are suitable. Yamada and Ishikawa (1977) could detect a small amount of iron in fresh frozen-dried erythrocyte, but failed in washed erythrocyte with physiological saline or fixed erythrocyte with 3% glutaraldehyde. Consequently, it is very difficult to detect iron in a plastic embedded section of red blood cell after a conventional fixation (Mizuhira, 1976). As relative concentration of iron is 0.347% of hemoglobin, the absolute iron content in one red blood cell is estimated 1.1×10^{-9} g when the blood has 500×10^4 cells/mm^3 and 16g of hemoglobin/100ml. In this study iron could not be detected in the red blood cell used as reference under the embedded condition, but a clear peak of iron at 6398 eV was obtained from the schistosome pigment. This indicates that a large amount of iron is contained in the schistosome pigment and accumulates in the liver as non-utilized iron because the schistosome pigment is said to be water-insoluble. Iron could also be detected in the granules in the intestinal lumen. This result agreed with other report (Kloetzel and Lewert, 1966) which described the granules as aggregations of host cell debris containing iron derived from the hemoglobin of red blood cell. It has been shown that the schistosome pigment is a metabolite released by worms and then accumulated in the Kupffer cells or phagocytic cells in the liver.

Pigment formation by schistosome worm cultured *in vitro* was reported by Kloetzel and Lewert (1966). Daily pigment production, expressed in terms of hemin, was 1.19 μg/pair of *S. mansoni*. As iron content in hemin is 8.57% (Windholz, 1976), daily iron production amounts 10.2 ng/pair. Accumulation of iron in the liver was 0.6 mg/100g of liver sediment for 6 weeks in our experiment. Daily accumulation was calculated about 28.7 ng if we postulated 2 g of liver sediment could be obtained from one mouse. This value is equivalent to about 3 pairs of *S. mansoni*. In fact, the worm burden recovered from portal vein was 7-12 pairs throughout the experiment. Iron deposit was slightly lower than the value of Kloetzel and Lewert (1966). This difference may be due to the species difference and that of between *in vitro* and *in vivo* experiments. In *in vivo* experiment, a large amount of iron should be excreted out of the body for long infection period.

In this study micro-distribution of iron in

schistosomiasis were examined by electron probe X-ray microanalysis. A large amount of iron was detected in the Kupffer cells as non-utilized iron, but could not be detected in the egg and the worm body.

Acknowledgments

The advices and supports of Dr. H. Iwatsuki of Department of Anatomy, Kawasaki Medical School are gratefully acknowledged. The authors also wish to thank Mr. K. Uehira of Laboratory Center of Electron Microscope, Kawasaki Medical School for his excellent technical assistances.

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