[Jpn. J. Parasitol., Vol. 36, No. 2, 63–67, April, 1987]

Presence of IgG Inside of Eggs Isolated from or Trapped in the Liver of *Schistosoma japonicum*-Infected Rabbits

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(Received for publication; September 18, 1986)

Abstract

Using the immunoperoxidase technique with protein A (PA) as the primary "antibody", PA-reactive material was detected inside of eggs trapped in the livers of *Schistosoma japonicum*- infected rabbits. The material, determined by blocking methods to be primarily IgG, was also localized inside of eggs isolated from liver tissue. Intraovular IgG occurred in focal and diffuse patterns, with 20–60% of eggs staining positively between 7 and 35 weeks post-infection. The incidences in immature and degenerated eggs were lower than those in fresh and mature eggs. The present study indicates that immunoglobulins penetrate into schistosome eggs *in situ* in *S. japonicum*-infected liver tissues resulting in intraovular precipitin reactions.

Key words: *Schistosoma japonicum*, eggs, IgG, rabbit, immunoperoxidase technique, protein A

Introduction

Marked deposits of immunoglobulins (Igs) in egg granulomas and in perivascular locations are consistently observed in immunohistologic studies of the liver in schistosome-infected animals. These locally distributed deposits of Igs are reported to be associated with the sequestration of egg antigens (Von Lichtenberg, 1964) as well as with the regulatory processes of the periovular granulomatous reaction (Warren *et al.*, 1975). In *Schistosoma japonicum* infection, modulation of egg granuloma size in the chronic stages of infection has been transferred successfully by serum Ig (IgG1) but not by T-cells, in contrast with the case of the *S. mansoni* infection (Olds *et al.*, 1982). Although the details of this mechanism have not been elucidated, Garcia and Mitchell (1982) hypothesized that granuloma modulation in the *S. japonicum* infection may be caused by the effects of antibodies on the maturation or longevity of miracidia. We are currently carrying out a series of immunohistologic studies to elucidate the roles of antibody distributed at the egg granuloma site. Using the fluorescent antibody technique, we previously have shown a spatial relationship between Igs and eggs in the livers of *S. japonicum*-infected mice (Hirata *et al.*, in press). Here, using the immunoperoxidase technique, we report the detection of intraovular IgG in the livers of *S. japonicum*-infected rabbits.

Materials and Methods

Specimens

Fifteen rabbits, each weighing 2.5 kg, were infected by intraperitoneal injection of 200–500 Japanese strain *S. japonicum* cercariae. The rabbits were necropsied 6–35 weeks after infection. Pieces of liver tissue (approx. 10 x 10 x 5 mm) were excised and fixed in cold 95% ethanol for overnight using a magnetic stirrer. Some additional tissues were also fixed in Bouin's fixative (saturated picric acid: formalin:acetic acid, 20:5:1). Tissues were embedded in paraffin and serial sections were cut at 4 μm for staining with immunoenzyme

This study was financially supported by the Japanese Ministry of Education, Science and Culture (No. 60570183).

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and haematoxylin-eosin techniques. Eggs, isolated from the liver of a 12-week infected rabbit, using 0.1% trypsin digestion, were also used for the immunohistologic study. These eggs were fixed in 95% ethanol and then washed, in a cold room, with three successive changes of 0.01M phosphate-buffered saline, pH 7.4, containing 10%, 15%, and, finally, 20% sucrose. The eggs were then embedded in OCT compound (Miles Laboratories), quick frozen in dry ice-acetone, and sectioned in the cryostat at 6 μm.

Immunoperoxidase technique

Rabbit IgG was localized in the infected liver tissues or in isolated eggs using the protein A (PA) – peroxidase-antiperoxidase (PAP) technique essentially following the method of Notani *et al.* (1979). Deparaffinated tissue sections or frozen sections of eggs were treated with 0.5% H_2O_2 in methanol to block the endogenous peroxidase activity found in inflammatory cells. After washing in 0.05M tris-HCl, pH 7.6, the sections were layered first with either normal rabbit serum (diluted 1:10) or 1% bovine serum albumin for 30 min to reduce non-specific staining, followed by PA (1:100 dilution) (Pharmacia Fine Chemicals), and then by rabbit PAP (1:50 dilution) (DAKO Chemicals). The peroxidase reaction was developed with 0.03% diaminobenzidine (DAB) and 0.025% H_2O_2. Controls included buffer or normal rabbit serum used in place of PA, PAP, or DAB. PA mixed with the rabbit IgG fraction which had been prepared through DEAE-cellulose chromatography of an ammonium sulfate-precipitated fraction of normal rabbit serum (Levy and Sober, 1960) was used for the blocking reactions.

Results

The results of staining in an alcohol-fixed liver section obtained from a 9 week-infected rabbit using the PA-PAP method are shown in Fig. 1. The reaction products were found inside of eggs, as well being distributed in the granulomas and perivascular areas and within the cytoplasm of the inflammatory cells. Staining intensity associated with the inflammation infiltrates decreased considerably in Bouin's fluid-fixed preparations in comparison to alcohol-fixed preparations; however, intraovular staining was not affected. Thus, in Bouin's-fixed tissues, the reaction inside of eggs was strikingly apparent in comparison to the other stained areas (Fig. 2).

In control sections, when the various reagents had been replaced with buffer or normal rabbit serum, no specific staining was present in the eggs nor in inflammatory tissues. Although staining was seen within the cytoplasm of inflammatory cells, this was determined to be the result of endogenous peroxidase activity by staining non-specifically with substrate only. Blocking tests using the rabbit IgG fraction completely eliminated the specific staining indicating that IgG was mainly responsible for the reactions (Fig. 3).

Reactions indicating the presence of intraovular IgG were not seen in all eggs. The rates of positive staining ranged between 20% and 60% from 7 weeks post-infection through the last studied samples at 35 weeks post-infection. Tissues from two 6 week-infected rabbits showed no intraovular staining. The rate of reaction was characteristic for the cluster of eggs in each different composite granuloma. When the eggs in the granuloma were fresh and mature, positive staining occurred at a high rate. When stained with haematoxylin-eosin, these intraovular IgG positive eggs were deeply stained with eosin (Fig. 4). Eggs of other stages of development or integrity were stained with much lower frequencies. One type of less reactive egg was weakly eosinophilic, contained granular constituents, and had distorted egg shells. These were considered to be newly laid or immature eggs (Fig. 2, upper egg). Another type of eggs which were weakly eosinophilic and had round egg shells, contained miracidia which were clearly damaged or only miracidial remnants. Eggs with earlier signs of degeneration were occasionally positively stained (Fig. 5, upper egg), but eggs with advanced degeneration were less reactive or negative (Fig. 5, lower egg).

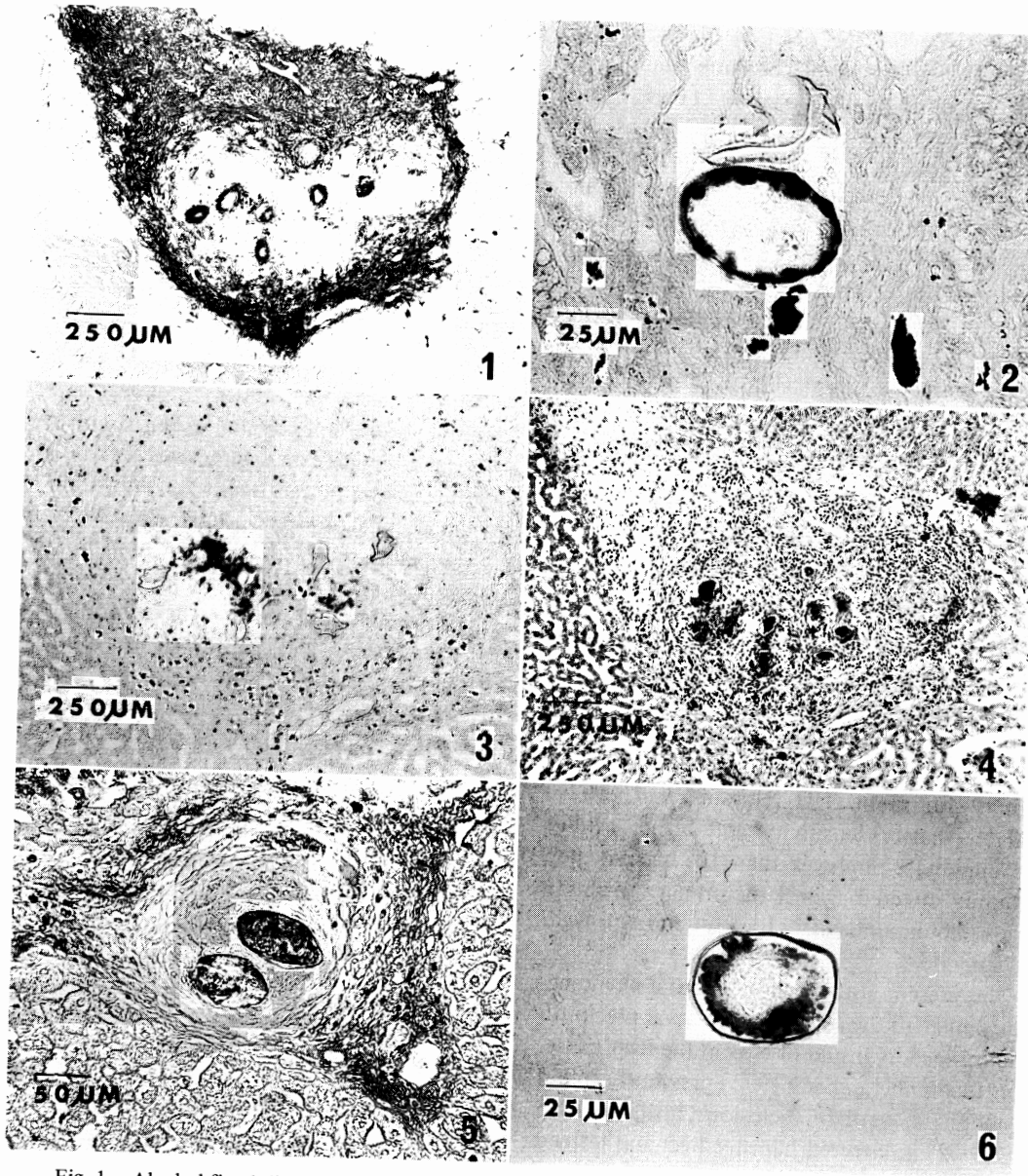

Fig. 1 Alcohol-fixed liver tissue from a 9 week-infected rabbit. Reaction products are seen inside eggs.

Fig. 2 Bouin's fixative-fixed liver tissue from a 25 week-infected rabbit. A rather degenerated egg (center egg) shows focal pattern of staining. Note no reaction in an egg with distorted egg shell (upper egg). Worm pigments are seen outside eggs.

Fig. 3 The same granuloma as Fig. 1. The result of blocking test. No reaction is seen excepting for inflammatory cells.

Fig. 4 The same granuloma as Fig. 1. H & E stain. Intraovular IgG-positive eggs (Fig. 1) are deeply stained with eosin.

Fig. 5 Alcohol-fixed liver tissue from a 34 week infected rabbit. A diffuse pattern of staining is seen in degenerated egg (upper egg). An egg with advanced degeneration (lower egg) slightly reacted.

Fig. 6 An egg isolated from an infected rabbit. Cryostat section. Reaction products are seen along body surface of a larva.

Localization of the reaction products to a restricted area between the miracidium and egg shell was frequently observed (Figs. 1 and 2). This type of staining, referred to as the "focal pattern", was seen in degenerating eggs as well as in fresh eggs, irrespective of the period of infection. Staining over the whole miracidial body, also seen in both types of egg, was termed the "diffuse pattern" (Fig. 5).

Intraovular IgG was also localized in cryostat sections of eggs which had been isolated from a 12 week-infected rabbit (Fig. 6). As with the eggs studied *in situ*, specific staining was not seen in control preparations of isolated eggs.

Discussion

Using PA as a bridge between rabbit IgG and PAP, intraovular IgG was found in the liver of *S. japonicum*-infected rabbits. The reactivity and specificity of PA for Igs of several mammalian species has been established (Goding, 1978), and is confirmed in the present study. It has also been shown that PA has a high affinity for rabbit IgG (Notani *et al.*, 1979) and has better sensitivity and higher avidity for immune complexes than the second step antibody directed against the primary antibody in a radioimmunoassay (Jonsson and Kronvall, 1974).

The present study confirms that a phenomenon similar to the *in vitro* circumoval precipitin reaction occurs inside of eggs in the liver tissue. Von Lichtenberg *et al.* (1973) previously found eosinophilic, bar-like inclusions inside eggs in *S. japonicum*-infected hamster liver and termed them "intraovular reverse precipitation". Eggs considered to be newly laid or immature were less reactive or negative. It is likely that the antigenic material associated with the egg is present in insufficient quantities to develop precipitin reactions both outside and inside eggs in the tissues. Immature eggs which were obtained from cultured adult worms did not show any reaction on incubation with infected rabbit serum (unpublished data). Degenerated eggs occasionally showed intraovular IgG, but eggs in advanced states of degeneration were mostly negative. These eggs are also known to be less reactive in the *in vitro* circumoval precipitation reaction. Their occasional positive reaction probably reflects a residue of intraovular precipitates occurred at the secretory phase of egg development. Thus, the intraovular precipitin reactions seen in host tissue seem to result when reactive eggs are exposed to a sufficient level of antibody.

In our previous study (Hirata *et al.*, in press), IgG fluorescence-positive substance localized in the restricted area between the miracidium and egg shell was shown to appear as eosinophilic amorphous material in haematoxylin-eosin stained tissues. Similar observations were made in the present study, as shown in Figs. 1 and 4. Since concentrations of antibody in the cytoplasm of plasma cells (Russell bodies) are eosinophilic, the deep staining of precipitate with eosin could indicate the deposition of Igs.

The occurrence of Igs inside of eggs has been described by several authors (Kawasaki *et al.*, 1968; Nakano, 1969; Von Lichtenberg *et al.*, 1973), though detailed studies have not been done. Together with our previous study, the present experiments confirm and extend the observations of these authors by demonstrating IgG in isolated eggs. The question now to consider is the role of the intraovular IgG. Interestingly, Garcia and Mitchell (1982) speculated that the reduced granuloma sizes in the later stage of *S. japonicum* infection may be the result of antibody on embryonation or longevity of eggs. Cheever *et al.* (1985), however, could not find any difference in the morphology of eggs between B-cell-depleted *S. japonicum*-infected mice and immune-intact controls. Information about the immunopathology of *S. japonicum* infection is still relatively scarce in comparison to that about *S. mansoni* infection. Further studies will be required to clarify the role of antibody at the granuloma site.

Acknowledgements

We would like to express thanks to Dr. J. E. Byram, Department of Pathology, Brigham and Women's Hospital, U.S.A., for reviewing the manuscript.

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