# Characteristics of eosinophilic inclusions within *Schistosoma japonicum* eggs

M. HIRATA[1]*, T. NAKASHIMA[2] *and* T. FUKUMA[1]

[1] *Department of Parasitology, Kurume University School of Medicine, Kurume 830, Japan*
[2] *Kurume Laboratory, CRC Inc, Kurume, Japan*

(*Received 1 July 1998; revised 28 September 1998; accepted 29 September 1998*)

SUMMARY

Although eosinophilic bar- or droplet-like inclusions are frequently detectable inside eggs deposited in the livers of *Schistosoma japonicum*-infected animals, little is known of their exact nature. In the livers of mice implanted with freshly laid eggs, inclusion-positive eggs were found in 28·7 and 46·2% of deposited eggs at 2 and 4 weeks, respectively, after implantation, but in 4·3% at 5 weeks when most of the eggs had already degenerated. When the extent of granuloma formation was investigated, granulomas around inclusion-positive eggs were smaller than those around negative eggs. Host factors associated with the formation of inclusion were sought using *in vivo* and *in vitro* studies. Following the administration of anti-egg antigen serum into egg-implanted mice, no increase in occurrence of inclusion-positive eggs was seen. In a co-culture of mature eggs with infected rabbit or mouse serum, inclusions were rarely found. In contrast, they were found in 17·9% of eggs in the presence of splenic cells. The present study is the first to show that there is decreased granuloma formation in the presence of eosinophilic inclusions inside eggs and our *in vitro* study suggests that host cell–egg interaction is responsible for the formation of inclusions.

Key words: *Schistosoma japonicum*, egg, eosinophilic inclusion, granuloma formation.

## INTRODUCTION

In tissues of *Schistosoma japonicum*-infected animals, bar- or droplet-like inclusions are frequently detectable inside deposited eggs. They are present between the vitelline membrane and the egg-shell and can be stained with eosin in formalin-fixed, paraffin-embedded tissues. In their comparative study of schistosome infection in the hamster, Lichtenberg, Erickson & Sadun (1973) observed the presence of inclusions inside *S. japonicum* eggs and hypothesized that this was a manifestation of intra-ovular immune precipitates or a reverse Hoeppli phenomenon. Since such findings were not described for *S. mansoni*- or *S. haematobium*-infected animals in their study, it is probable that the appearance of inclusions is unique or else occurs at a much higher frequency in infections with this species. The functional significance or nature of these inclusions has not been reported, nor has it been ascertained whether they are derived from schistosome eggs, a host component or their complexes. We have shown that host immunoglobulins penetrate eggs in the livers of *S. japonicum*-infected mice and the appearance of inclusions was assumed to be associated with this phenomenon (Hirata, Hieda & Tsutsumi, 1986*a*). Since it is difficult to clarify matters using infected animals, the present study was carried out using eggs which were implanted into the livers of mice or cultured *in vitro* with host substances, serum or splenic cells.

* Corresponding author: Department of Parasitology, Kurume University School of Medicine, Kurume 830, Japan. Tel: +81 942 31 7550. Fax: +81 942 31 0344. E-mail; hiramizu@med.kurume-u.ac.jp

## MATERIALS AND METHODS

### *Animals and parasites*

CBA/J mice and BALB/c nude mice were purchased from Shizuoka Laboratory Animal Center, Japan, and rabbits (New Zealand White) were obtained from KBT Oriental Co. Ltd, Japan. Wistar rats were given to use by the Animal Center of Kurume University School of Medicine. A Japanese strain of *Schistosoma japonicum* has been maintained in our laboratory by passage through *Oncomelania hupensis nosophora* and rabbits for 25 years. Mice, rats and rabbits were exposed by subcutaneous injection to about 30, 100 and 700 cercariae/animal, respectively. Groups of 2 CBA/J mice were killed 5, 6, 8 and 10 weeks after infection. Three nude mice and 3 rats were killed at 7 and 6 weeks, respectively. One rabbit was killed at 7 and another at 10 weeks. Livers from the animals were fixed in 10% neutral buffered formalin and stained with haematoxylin and eosin. Serum was collected from the infected animals and stored at −80 °C. Throughout the experiment, animals were housed under conventional conditions with controlled temperature, a diurnal light cycle and free access to food and water.

 

*Immune serum and transfer*

Eggs were isolated by pronase (0·01 %) and collagenase (0·01 %) digestion of the intestines of infected rabbits. Extraction of soluble egg antigen (SEA) was carried out according to our previous report (Hirata *et al*. 1986*b*). Immune serum against SEA was produced in rabbits. A mixture (1 ml) of equal volumes of SEA (100 $\mu$g/ml) and Freund's adjuvant was injected at several sites along the back. These injections were repeated 4–6 times biweekly and the rabbits were further boosted by 1 or 2 intraperitoneal injections of SEA. Serum was collected 10 days after the final injection.

A 0·5 ml volume of immune rabbit serum or normal rabbit serum was injected i.p. into mice previously implanted with freshly laid eggs at 3, 5, 9 and 11 days after implantation.

*Hepatic granuloma formation*

In order to estimate the occurrence of inclusion-positive eggs during the life-time of deposited eggs, freshly laid eggs were implanted into the livers of mice. Eggs were obtained by the culture for 2 days, in serum-free RPMI-1640, of mated worms taken from infected rabbits. In total, 1500 eggs were implanted into the livers of CBA/J mice through the caecal vein, as previously described (Hirata *et al*. 1991). The use of freshly laid eggs has been previously confirmed to produce granulomas analogous to those seen in infected animals with regard to the cellularity and magnitude of tissue lesions. Groups of 2–4 mice were killed at defined periods after implantation. For histological examination, livers were removed, fixed in 10 % neutral-buffered formalin, and then embedded in paraffin wax. Serial sections were made 50 $\mu$m apart and stained with haematoxylin and eosin. To evaluate the development of the granulomas around the eggs, diameters (the mean width and length of the lesion) were measured using a micrometer. For an estimation of the occurrence of inclusion-positive eggs, more than 20 eggs per mouse liver, excluding eggs exhibiting only shells, were examined.

*Fluorescent antibody staining*

The technique has previously been described (Hirata *et al*. 1986*a*). Briefly, livers taken from serum-injected mice were fixed in cold 95 % alcohol, dehydrated and then embedded in paraffin wax. Sections were stained with FITC-labelled anti-mouse IgG or anti-rabbit IgG (1 : 100 dilution) that had been treated with rabbit IgG or mouse IgG, respectively, to prevent cross-reaction.

In vitro *culture*

Freshly laid eggs were obtained in a similar manner to that described above, except for the culture of mated worms in the presence of 10 % foetal bovine serum, and the eggs were then left for 10 days until fully mature miracidia had formed within the eggs. In order to investigate the participation of antibody or host cells in the occurrence of inclusions, 150 mature eggs were plated on 24-well plates and then further incubated for 4 days in medium containing 25 % infected rabbit or mouse serum, or splenic cells ($4 \times 10^6$ cells/well) from infected mice. Eggs (600/specimen) were collected, centrifuged and mixed with 50 % starch that had been previously dissolved by heating, and subsequently the mixture was poured into the removed mouse caecum. After being fixed in 10 % neutral formalin, tissues were processed as described above. The number of eggs examined exceeded 70 in each specimen.

*Statistical analysis*

Student's unpaired *t*-test was used for the difference in granuloma size between inclusion-positive eggs and negative eggs (Fig. 2). In cases where the mouse groups comprised 3 or more animals (Fig. 4), the mean granuloma size in each mouse liver was evaluated using ANOVA (Bonferroni's adjustment). Values of $P < 0{\cdot}05$ were considered to indicate significant differences.

## RESULTS

*General features*

Inclusion-positive eggs (Fig. 1A) were detected in the livers of infected mice, rats and rabbits (not shown), thus their appearance seems to be common in *S. japonicum*-infected animals. Interestingly, they were also present in the livers of infected BALB/c nude mice (Fig. 1B). The number and size of inclusions varied greatly with each egg, ranging from 1 to 6 in number, and from small dots of 50 $\mu$m in length. It is a characteristic feature that they were always localized between the egg-shell and the vitelline membrane, well demarcated from their surroundings.

*Occurrence of inclusion-positive eggs*

The occurrence varied considerably with each mouse, ranging from 16·7 % to 36 % at 2 weeks of implantation. Summarizing the data, each group consisting of 2–3 mice, had an occurrence of 28·7 and 46·2 % at 2 and 4 weeks, respectively, but 4·3 % at 5 weeks when most of the eggs had already degenerated. When mice infected for 5 weeks were examined, no inclusions were found. These results indicate that inclusions appear in mature live eggs, but not in immature eggs or eggs that have degenerated. Although eosinophilic fringes around the eggs, called Hoeppli phenomenon, were sought, we could not find any in the egg-implanted tissues.

https://doi.org/10.1017/S0031182098003837 Published online by Cambridge University Press

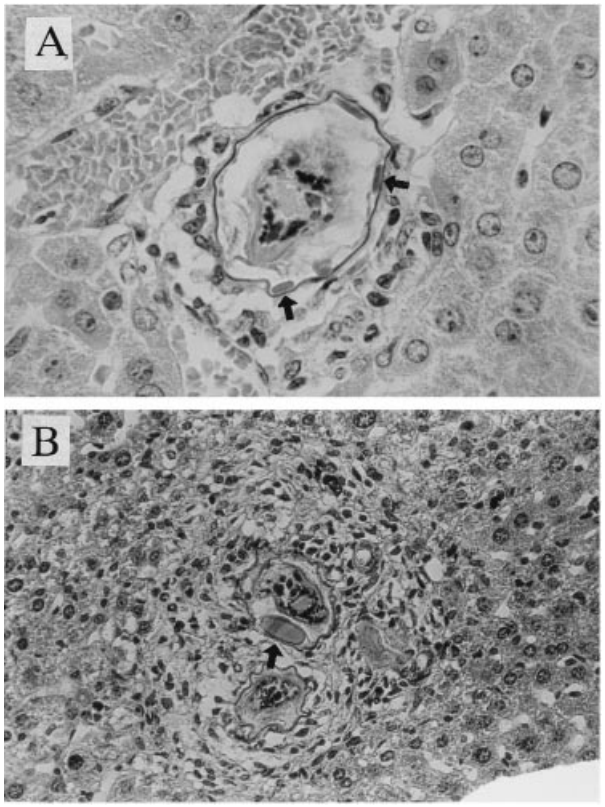


Fig. 1. Appearance of inclusions inside eggs in the liver of an egg-implanted mouse (A) and an infected BALB/c nude mouse (B). Arrows indicate inclusion. Note the extent of cellular response around inclusion-positive eggs is only slight.

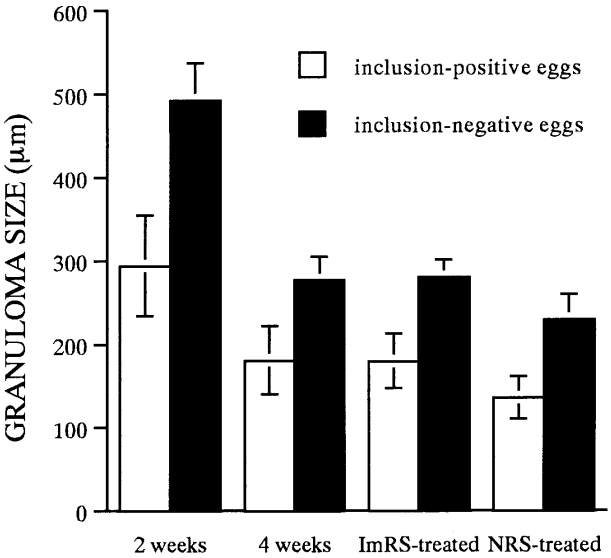


Fig. 2. Comparison of granuloma sizes around inclusion-positive and negative eggs in different groups of egg-implanted mice. See Fig. 4 for ImRS- or NRS-treated mice. Bar indicates S.E.

### *Relationship with granuloma formation*

When the extent of granuloma formation around inclusion-positive and negative eggs was studied comparatively in egg-implanted mice, granulomas

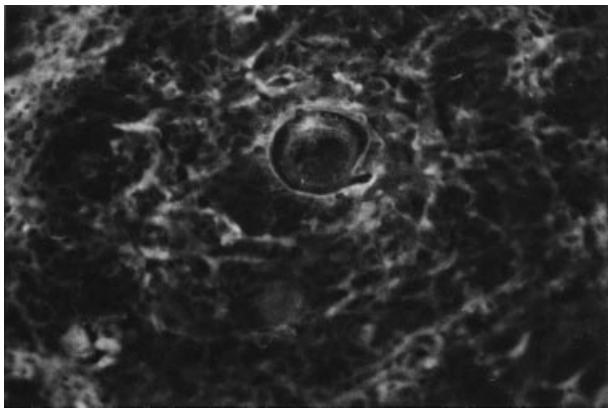

Fig. 3. Fluorescent antibody staining in the liver of immune rabbit serum (ImRS)-injected, egg-implanted mice, showing that rabbit IgG is distributed over the granulomatous area or inside the eggs.

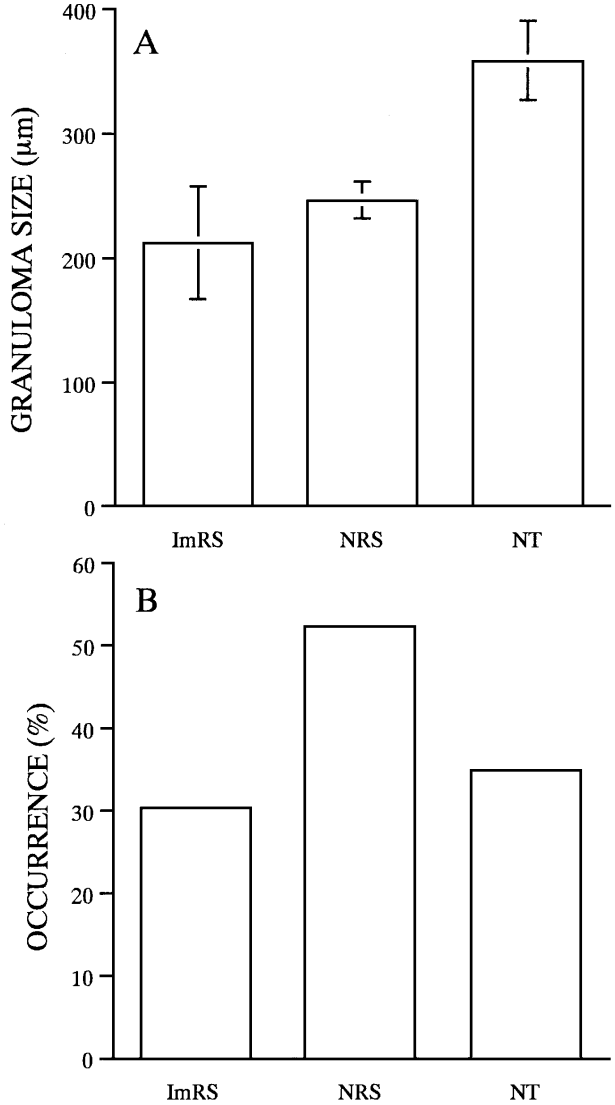


Fig. 4. Effect of immune rabbit serum (ImRS) or normal rabbit serum (NRS) injection on granuloma formation (A) or the occurrence of inclusion-positive eggs (B) in mice with 2 weeks of egg implantation. NT, untreated. Each mouse group consisted of 3–4 animals. Significant difference between NT and NRS-, or ImRS-treated group. Bar indicates S.D.

https://doi.org/10.1017/S0031182098003837 Published online by Cambridge University Press

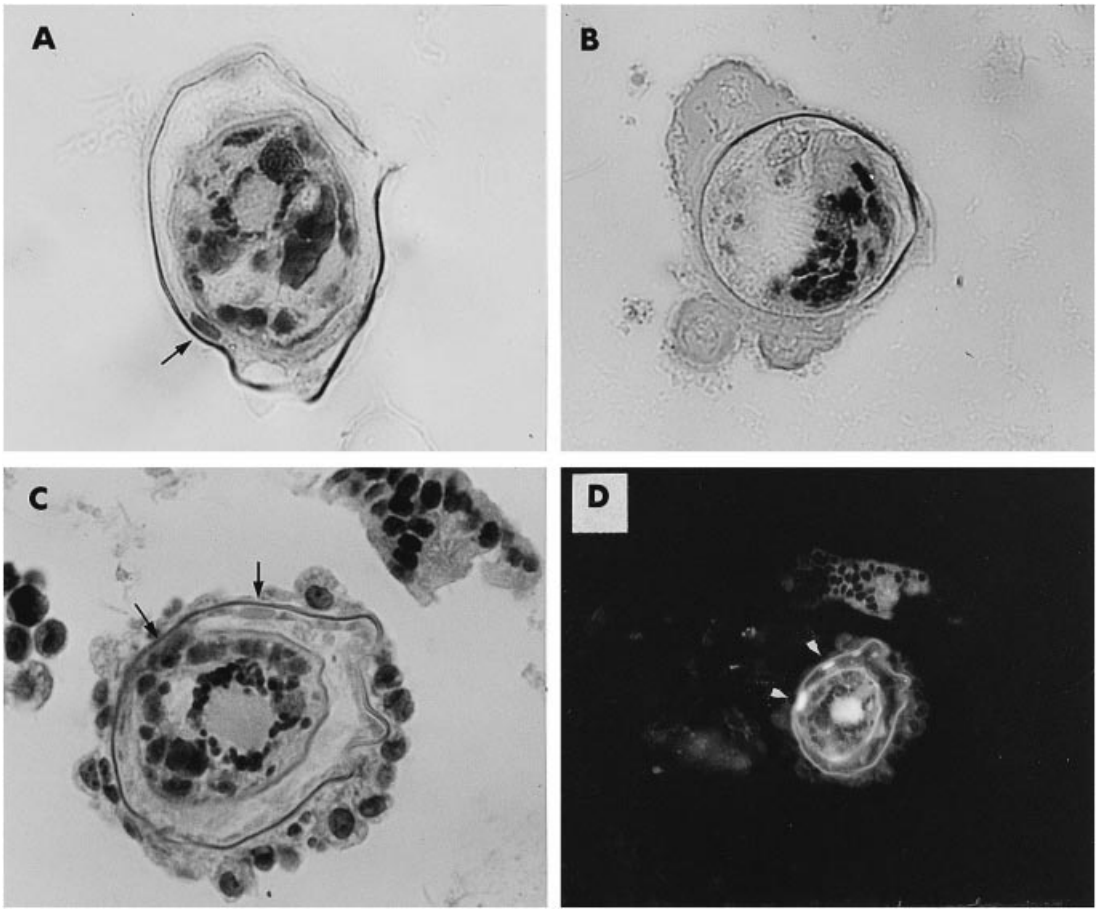


Fig. 5. Mature eggs co-cultured with medium only (A), serum (B) or splenic cells (C). (D) Shows autofluorescence of haematoxylin and eosin-stained egg (the same as C). An ambiguous substance is visible in (A) (arrow). Arrows in (C) and (D) indicate inclusion.

around positive eggs were smaller than those around negative eggs at 2 and 4 weeks after implantation (Fig. 2). Although there were no statistical differences between the sizes of granulomas, a similar tendency was consistently found in all of the 6 different groups of mice examined (data either below or not shown). The distribution of occurrence of inclusion-positive eggs according to granuloma size, which was calculated from 121 eggs deposited in the livers of mice implanted for 2 weeks, indicated that there was a negative correlation between these two factors. It was also noted that there was a drastic decrease in the occurrence for large granulomas, those measuring 300 $\mu$m or more in diameter. In these granulomas, positive eggs were rarely found.

*Effect of immune serum on occurrence of inclusion-positive eggs*

When the fluorescent antibody technique was performed 2 weeks after implantation on the livers of egg-implanted mice which had earlier received injections of immune rabbit serum against SEA, exogenous rabbit IgG was found to be distributed throughout the granulomatous area, sometimes even inside the eggs (Fig. 3). In mice that had not been treated with rabbit serum, no reaction was seen. A similar pattern of distribution, however, was seen in the tissues of normal rabbit serum-injected mice and also in the staining for mouse IgG. The size of the granulomas, measured in the presence or absence of eggs, shows an apparent decrease in both immune serum- and normal serum-treated mice, compared to untreated mice (Fig. 4A). We did not find any increase in the occurrence in immune serum-treated mice, compared to normal serum-injected mice or untreated mice (Fig. 4B). In these serum-treated mice, the granulomas around the inclusion-positive eggs were again smaller than those around the negative eggs (Fig. 2).

In vitro *study on occurrence of inclusion-positive eggs*

In order to ascertain the origin of inclusions, mature eggs were incubated with infected rabbit or mouse serum, or splenic cells for 4 days, and then the eggs were processed in a similar manner to tissues. In the absence of cells or serum, eggs contained ambiguous substances with a morphology similar to that of inclusions (Fig. 5A) except that they were not eosinophilic. In the presence of serum, although circumoval precipitates were stained with eosin, inclusion-positive eggs were rarely found; 0% for infected rabbit serum (Fig. 5B) and 1·8% for

https://doi.org/10.1017/S0031182098003837 Published online by Cambridge University Press

infected mouse serum. In contrast, in the presence of splenic cells, eosinophilic inclusions appeared at a rate of 17·9% and associated cells around the eggs were mostly macrophages (Fig. 5C). When haematoxylin and eosin-stained eggs were observed on a fluorescent microscope fitted with filters for FITC, eosinophilic inclusions were clearly visible because eosin emitted autofluorescence (Fig. 5D).

## DISCUSSION

The presence of bar- or droplet-like inclusions inside *S. japonicum* eggs has been described by Lichtenberg *et al*. (1973). However, as far as we know, no study has been performed to elucidate their occurrence, pathological significance or origin. The present study employed artificial granuloma formation induced by the implantation of freshly laid eggs into the livers of mice. This enabled us to examine the various characteristics of inclusions. In particular, the results show that there is decreased granuloma formation when there is presence of inclusions, inside the eggs. Furthermore, the results of an *in vitro* culture of eggs strongly suggest that inclusions are formed during interaction with the host cells and are not spontaneously produced.

Lichtenberg *et al*. (1973) have hypothesized that the appearance of inclusions is a manifestation of intraovular immune precipitates. We previously detected host immunoglobulin inside eggs in tissues of *S. japonicum*-infected mice (Hirata *et al*. 1986*a*) as was confirmed by administering rabbit hyperimmune serum in the present study. In an immunohistochemical election microscopy study on *S. mansoni*, no IgG was found inside eggs incubated with serum from infected patients (Demaree & Hillyer, 1981). Nevertheless, our results did not support the hypothesis of Lichtenberg *et al*. (1973). (1) In immune serum-administered animals, there was no increase in the occurrence of inclusion-positive eggs. (2) The addition of infected rabbit or mouse serum to the culture of mature eggs did not seem to influence the occurrence. (3) Inclusion-positive eggs were detected in nude mice where antibody production is poor. These observations seem to refute the participation of antibody in the appearance of inclusions. In addition, it has been reported that antibody and immune complexes are independent of the acute phase of granuloma formation in the case of B cell-depleted mice (Cheever *et al*. 1985). The present results of the co-culture of eggs with splenic cells from infected mice suggest that inclusions are formed during interaction with the host cells.

As factors decreasing granuloma formation, eosinophils may act as candidate host cells, because several studies with *S. mansoni* have indicated that eosinophils mediate egg destruction, eventually reducing granuloma formation (Hsu *et al*. 1980; Olds & Mahmoud, 1980; James & Colley, 1978; de Brito, Kazura & Mahmoud, 1984; Kazura *et al*. 1985). Furthermore, based on the immunohistology of tissues of *S. mansoni*-infected patients, Kephart, Andrade & Gleich (1988) demonstrated the presence of an eosinophil-derived major basic protein (MBP) in close proximity to the eggs, suggesting that the eosinophilic fringes formed around eggs, referred to as Splendore-Hoeppli phenomenon in their study, can be accounted for in part by the deposition of eosinophil granule MBP. In our study, the kinds of cells present within the vicinity of positive eggs varied with each granuloma according to whether the predominant cells comprised neutrophils, eosinophils, or epithelioid cells or whether the granuloma was almost lacking in any inflammatory cells. Neutrophils have been reported to participate in the damage of *S. mansoni* eggs (de Brito *et al*. 1984; Kazura *et al*. 1985). However, the fact that the acute phase of large granulomas, which mostly consisted of confluent neutrophils (seen 2 weeks after the implantation of eggs), rarely exhibited inclusion-positive eggs seems to suggest that neutrophils are independent of the occurrence of inclusions. The existence of toxic substances such as MBP inside the eggs remains to be studied in detail. The present results of *in vitro* culture suggest that macrophages are among the candidate cells.

The results showing that serum transfer decreased granuloma formation differ from those of our previous study (Hirata *et al*. 1997). It is possible that the effect may be caused by the larger volume (0·5 ml) of serum and greater number (5) of injections in the present study, compared to the previous study (0·3 ml volume and 3–4 times). In fact, no decrease was seen with 3 injections of the same volume of serum in our preliminary experiment. Regarding the relationship between granuloma formation and the occurrence of inclusion-positive eggs, it should be pointed out that decreased granuloma formation is not necessarily implicated in the increase in occurrence of inclusion-positive eggs because the associated suppressive mechanisms may differ with each model. In our preliminary experiments, we observed some increased occurrence (66·1%) in mice with SEA-suppressed granuloma formation (Hirata *et al*. 1997), suggesting that the protocol is an effective method for inducing inclusion-associated factors.

Finally, the present study reveals several characteristics of eosinophilic bar- or droplet-like inclusions inside eggs. (1) Inclusion-positive eggs are commonly seen in tissues of *S. japonicum*- infected animals and even in T cell-deficient mice, and inclusions appear at the mature stage of eggs when intense cellular response occurs. (2) There is decreased granuloma formation around inclusion-positive eggs, although it is not known whether inclusions are primarily involved in the suppression

of granuloma formation. (3) Inclusions seem neither to be formed by antigen-antibody interaction, nor to be spontaneously produced by the eggs themselves. (4) The results of *in vitro* co-culture suggest that the inclusions are formed during interaction with host haematopoietic cells.

We wish to thank Manami Ohba for her excellent technical assistance. The English used in this manuscript was revised by K. Miller (Royal English Language Centre, Fukuoka, Japan). This work was supported in part by a Grant-in-Aid (10670245) from the Ministry of Education, Science and Culture, Japan.

## REFERENCES

DE BRITO, P. A., KAZURA, J. W. & MAHMOUD, A. A. F. (1984). Host granulomatous response in schistosomiasis mansoni: antibody and cell-mediated damage of parasite eggs *in vitro*. *Journal of Clinical Investigation* **74**, 1715–1723.

CHEEVER, A. W., BYRAM, J. E., HIENY, S., LICHTENBERG, F. VON, LUNDE, M. N. & SHER, A. (1985). Immunopathology of *Schistosoma japonicum* and *S. mansoni* infection in B cell depleted mice. *Parasite Immunology* **7**, 399–413.

DEMAREE, R. S. JR. & HILLYER, G. V. (1981). Immunoperoxidase localization by electron microscopy of soluble egg antigen and human IgG in circumoval precipitin reactions around *Schistosoma mansoni* eggs. *American Journal of Tropical Medicine and Hygiene* **30**, 402–405.

HIRATA, M., HIEDA, T. & TSUTSUMI, H. (1986*a*). Immunoglobulins inside *Schistosoma japonicum* eggs from the livers of mice. *Journal of Parasitology* **72**, 578–582.

HIRATA, M., HIEDA, T., TSUTSUMI, H., SHIMADA, M., SATO, K., KOECH, D. K. & WAMBAYI, E. (1986*b*). Immunodiagnosis of schistosomiasis haematobia using eggs collected from urine as antigen. *Japanese Journal of Parasitology* **35**, 437–443.

HIRATA, M., TAKUSHIMA, M., KAGE, M. & FUKUMA, T. (1991). Induction of experimental murine granuloma formation against *Schistosoma japonicum* eggs produced by *in vitro* ova deposition, *in vitro* tissue extraction, or lyophilization. *Parasitology Research* **77**, 315–319.

HIRATA, M., KAGE, M., HARA, T., NAKAO, M. & FUKUMA, T. (1997). Inhibitory effect of circulating egg antigens on *Schistosoma japonicum* egg-induced granuloma formation. *Journal of Parasitology* **83**, 842–847.

HSU, S. Y., HSU, H. F., MITROS, F. A., HELMS, C. M. & SOLOMON, R. I. (1980). Eosinophil as effector cells in the destruction of *Schistosoma mansoni* eggs in granulomas. *Annals of Tropical Medicine and Parasitology* **74**, 179–183.

JAMES, S. L. & COLLEY, D. G. (1978). Eosinophil-mediated destruction of *Schistosoma mansoni* eggs. *Journal of the Reticuloendothelial Society* **20**, 359–374.

KAZURA, J. W., DE BRITO, P. A., RABBEGE, J. & AIKAWA, M. (1985). Role of granulocyte oxygen products in damage of *Schistosoma mansoni* eggs *in vitro*. *Journal of Clinical Investigation* **75**, 1297–1307.

KEPHART, G. M., ANDRADE, Z. A. & GLEICH, G. J. (1988). Localization of eosinophil major basic protein onto eggs of *Schistosoma mansoni* in human pathologic tissue. *American Journal of Pathology* **133**, 389–396.

LICHTENBERG, F. VON, ERICKSON, D. G. & SADUN, E. H. (1973). Comparative histopathology of schistosome granulomas in the hamster. *American Journal of Pathology* **72**, 149–178.

OLDS, G. R. & MAHMOUD, A. A. F. (1980). Role of host granulomatous response in murine schistosomiasis mansoni. Eosinophil-mediated destruction of eggs. *Journal of Clinical Investigation* **66**, 1191–1199.