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# Modulation of Lung Granuloma Formation with a Monoclonal Antibody Producing Circumoval Precipitin Reactions with *Schistosoma japonicum* Eggs

FUMIE KOBAYASHI, TSUTOMU MORII, TOSHIHIRO MATSUI, TAKASHI FUJINO AND MORIYASU TSUJI

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## Abstract

A monoclonal IgM antibody producing the circumoval precipitin (COP) reaction with *Schistosoma japonicum* eggs was examined for its ability to modulate egg granuloma formation in lungs of egg-sensitized mice. The COP reaction-positive monoclonal antibody 3D10 suppressed lung granuloma formation in egg-sensitized mice to some extent. In the indirect immunofluorescent antibody test, the strongest fluorescence was observed in the space between miracidium and eggshell. Western blot analysis indicated that the IgM antibody recognized epitopes on molecules of 145 Kd of egg antigens. The antibody also recognized antigen(s) expressed on the surface membranes of mechanically transformed schistosomula. However, passive transfer of the antibody into mice resulted in no reduction in the recovery of adult worms.

**Key words:** *Schistosoma japonicum,* Soluble egg antigens, Monoclonal antibody, COP reaction

## Introduction

The circumoval precipitin (COP) reaction is a serological reaction occurring after incubation of schistosome eggs with sera from patients with schistosomiasis (Oliver-González, 1954). Because of its considerable sensitivity and specificity, the COP test has been used for immunodiagnosis of schistosomiasis japonica (Garcia, 1976; Tanaka, 1976; Yogore *et al.*, 1976). Garcia *et al.* (1985) reported that patients' sera forming large segmented precipitates in the COP test were effective in reducing granuloma formation in egg-sensitized mice. We previously produced and characterized monoclonal antibodies that produced COP reaction with eggs of *Schistosoma japonicum* (Kobayashi, 1986). In the present study, we examined the modulating effect of the COP reaction-positive IgM monoclonal antibody on lung granuloma formation in egg-sensitized mice. The effects of passive transfer of the antibody on *S. japonicum* infection were also examined.

*Department of Parasitology, Kyorin University School of Medicine, Mitaka, Tokyo 181, Japan.*
小林富美恵　森井　勤　松井利博　藤野隆志
辻　守康（杏林大学医学部寄生虫学教室）

## Materials and Methods

### *Mice*

Female C57BL/6 and BALB/c mice were purchased from Charles River Japan Inc., Atsugi, Japan. Female ddY mice were obtained from Shizuoka Laboratory Animal Center, Hamamatsu, Japan.

### *Parasites and antigens*

The Japanese strain of *S. japonicum* (from Yamanashi Prefecture) has been maintained in our laboratory using *Oncomelania hupensis nosophora* snails and ddY mice. Uninfected snails were collected from the Kofu Basin, Yamanashi Prefecture, Japan. Eggs were isolated from the intestines of ddY mice infected with *S. japonicum* for 8–9 weeks and soluble egg antigens were prepared by the method described previously (Kobayashi *et al.*, 1985a). Mechanically

transformed schistosomula were prepared by vortex mixing followed by passage of the cercarial suspension through a 22-gauge needle to allow complete detachment of the tails. They were then incubated in RPMI 1640, pH7.2, containing 20 mM Hepes, 100 U/ml penicillin, and 100 $\mu$g/ml streptomycin for 3 hrs at 37°C in 5% $CO_2$. Adult worms were obtained from the portal system of infected mice by perfusion and used to prepare cryostat sections.

*Infected and normal mouse sera*

Six- to eight-week-old female C57BL/6 mice were infected percutaneously with 30 to 50 cercariae. Sera of mice infected with *S. japonicum* were collected after 9 weeks and 16 weeks of infection. Normal sera were obtained from age- and sex-matched C57BL/6 mice.

*Monoclonal antibody*

The production of the monoclonal antibody used in this study has already been described (Kobayashi, 1986). Monoclonal antibody 3D10 is an IgM that produces circumoval precipitin (COP) reactions with eggs of *S. japonicum*. The subclass of antibody was determined by double immunodiffusion for mouse immunoglobulin types (Kobayashi *et al.*, 1985b). Ascites fluid or culture supernatant derived from the hybridoma was used as the source of monoclonal antibody. For some experiments, monoclonal antibody 3D10 was partially purified from ascites fluid by the twice precipitation in 50% saturated ammonium sulphate solution at pH 7.2. Partially purified 3D10 was dialyzed against 200 vol of PBS, pH 7.2, three times for 2 days. The monoclonal antibody was next concentrated to 5 mg protein/ml by Collodion Bags (Sartorius, Göttingen) and filtered. The protein concentration was determined by the method of Bradford (1976) using bovine serum albumin as a standard. Control ascites and supernatants were derived from non-fused NS-1 myeloma cells.

*Indirect immunofluorescence*

For immunofluorescence, sporocysts obtained from infected snails and mechanically produced schistosomula were fixed with 1% acetic acid in ethanol. Infected mouse livers and adult worms were obtained just before freezing. The frozen materials were placed into a pre-cooled cryostat (–20°C) and sections (5 $\mu$m) were cut. The fixed parasites and frozen sections were transferred to microscope slides and dried.

The indirect immunofluorescence was performed on the microscope slides at 25°C in a humidified box. Parasites were incubated for 30 min in undiluted hybridoma culture supernatants or sera from infected mice for 16 weeks as positive controls (diluted 1:40). After 3 washes in phosphate-buffered saline (PBS), pH 7.2, the parasites were incubated for 45 min in fluorescein-conjugated rabbit anti-mouse Ig (Miles Laboratories Ltd.) at a dilution of 1:100. After 3 further washes the parasites were mounted with coverslips in 10% glycerol in PBS and examined by using a Nikon fluorescence microscope. The degree of fluorescence ranged from negative (–) to very bright (+ + +).

*Lung granuloma assay and passive transfer*

Six- to eight-week-old female C57BL/6 mice were sensitized subcutaneously with 3,000 *S. japonicum* viable eggs. Two weeks later, the mice were injected intravenously with 0.5 ml of sera from infected mice or monoclonal antibody 3D10 (partially purified from ascites, 2.5 mg/mouse). Controls received normal mouse sera or control ascites. Twenty to 21 hours later, the mice were challenged intravenously with 3,000 viable eggs. One week later, they were killed and their lungs were perfused intratracheally with 10% phosphate-buffered formalin, removed, and processed for histologic examination. Sections from each lung, 5-$\mu$m-thick, were stained with hematoxylin and eosin. The diameter of the granuloma around single deposited eggs in the lung was measured in the tissue sections. At least 25 granuloma diameters per lung were measured, each diameter being the average of two diameters of a single egg granuloma measured at right angles to each other. Statistical analyses for the differences between control and experimental groups were performed using Student's $t$ test. Results yielding a $p$ value of $<0.05$ were considered to be significant.

*Adult worm assay and passive transfer*

Six- to eight-week-old female BALB/c mice received an intraperitoneal injection of 1 ml each of hybridoma ascites or control ascites (diluted 1:3) 21 hrs and 3 hrs before a challenge infection with 190 cercariae of *S. japonicum*. Mice were killed 4 wk after the challenge infection, and adult worms were recovered by portal perfusion.

*Western blot analysis*

Western blot analysis was performed as previously described (Kobayashi, 1986). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out using 10% gels according to Laemmli (1970). Samples for blotting were prepared using SDS sample buffer without 2-mercaptoethanol or boiling in an effort to preserve the epitope recognized by 3D10. At the completion of SDS-PAGE, the proteins were transferred from the gels to nitrocellulose membrane as described by Towbin *et al.* (1979). The nitrocellulose strips were then incubated with monoclonal antibody 3D10 (undiluted culture supernatant) or normal mouse sera (diluted 1:100). Diaminobenzidine was used as the substrate for peroxidase-conjugated antibodies.

## Results

*Stage specificity assay with a monoclonal antibody*

The reactivity of monoclonal antibody 3D10 with different developmental stages of *S. japonicum* is shown in Table 1. In the indirect immunofluorescence test, 3D10 reacted strongly with eggs in the frozen sections. The strongest fluorescence was observed in the space between miracidium and eggshell. The fluorescence was also observed around eggshell in the liver sections. In the miracidium, the antigen recognized by 3D10 was localized in the fore part of the parasite. Moreover, 3D10 bound to the surface membranes of sporocysts and mechanically transformed schistosomula. Although 3D10 reacted with gut of adult worms, there was no reaction of 3D10 with their tegument and stroma.

*Modulation of granulomas*

To examine the modulating effects of monoclonal antibody 3D10 on granuloma formation, the lung granuloma assay was performed. Egg-sensitized C57BL/6 mice were given either monoclonal antibody or serum, and challenged with eggs. Table 2 shows the modulating effects of various antibodies on lung

Table 1 Stage specificity of monoclonal antibody 3D10 assessed by immunofluorescence

| Test organism | Antibody | |
|---|---|---|
| | 3D10* | IMS† |
| Egg‡: miracidium | + + | + + |
| between miracidium and eggshell | + + + | + + + |
| around eggshell | + + | + + |
| Sporocyst | + | + + |
| Schistosomula | + | + + |
| Adult§: tegument | – | + + + |
| stroma | – | + + + |
| gut | + | + + + |

*Undiluted hybridoma culture supernatant of 3D10 was used as monoclonal antibody in these experiments.
†Pooled sera from C57BL/6 mice infected with *Schistosoma japonicum* for 16 weeks (diluted 1:40).
‡In frozen sections of infected mouse liver.
§Cryostat sections.

Table 2 Effect of passive transfer of monoclonal antibody 3D10 and sera from mice infected with *Schistosoma japonicum* on granuloma formation in the lungs of egg-sensitized mice*

| Substance passively transferred | No. of mice | Mean granuloma diameter ± SD ($\mu$m) | $p$ |
|---|---|---|---|
| Normal mouse serum | 5 | 227 ± 16 | – |
| 9-wk infected serum | 5 | 208 ± 23 | N.S.† |
| 16-wk infected serum | 5 | 197 ± 19 | $<0.05$ |
| Monoclonal antibody 3D10 | 6 | 139 ± 23 | $<0.001$ |
| Control ascites | 6 | 224 ± 22 | N.S. |

*C57BL/6 mice were given either 0.5 ml of serum or monoclonal antibody (partially purified from ascites, 2.5 mg) 20–21 hr before challenge. All mice were sacrificed one week after challenge and granuloma diameters were measured.
†Not significant.

granuloma formation, which were determined at 7 days after egg-challenge. Animals given 0.5 ml of serum from 16-week-infected mice showed a significant reduction in granuloma size ($p<0.05$). Injection of 0.5 ml of partially purified monoclonal antibody 3D10 (2.5 mg) also significantly reduced granuloma size in the lungs ($p<0.001$), while no effect was observed upon passive transfer of normal mouse serum or control ascites. Treatment of mice with 0.5 ml of serum from 9-week-infected animals tended to diminish granuloma size, but not significantly.

Fig. 1 shows the histological examination of the lesion around eggs injected into the lungs. The egg granulomas contained mainly neutrophils and histiocytes. Small numbers of lymphocyte-like

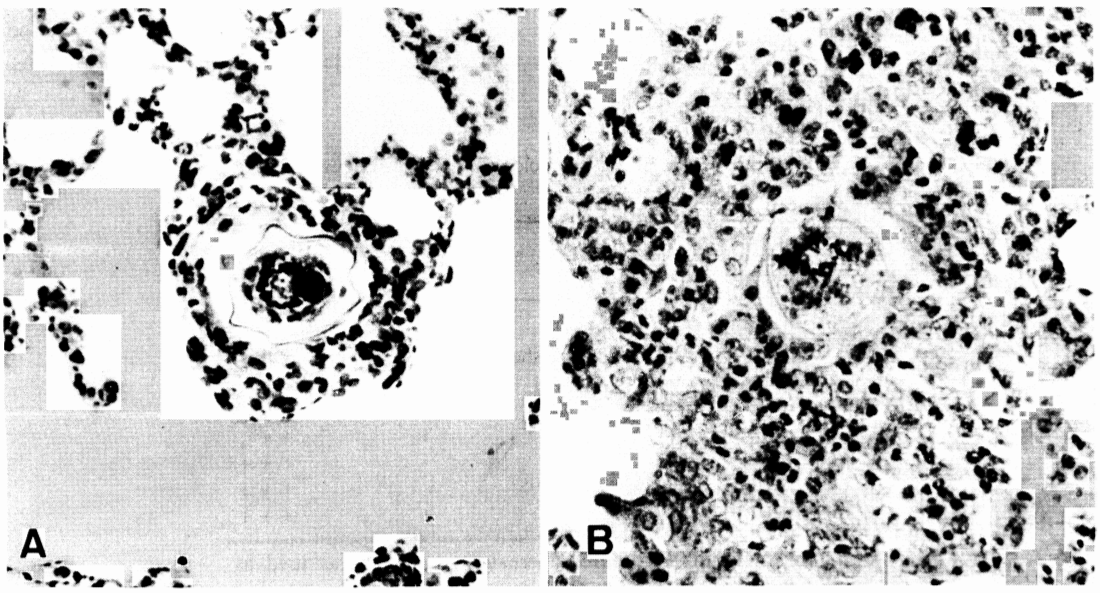


Fig. 1 Representative granulomas around *Schistosoma japonicum* eggs one week after their intravenous injection into the lungs of egg-sensitized C57BL/6 mice. Monoclonal antibody 3D10 (A) or normal mouse serum (B) was given intravenously 20–21 hr before egg challenge. (Hematoxylin and eosin, ×270)

Table 3 Effect of passive transfer of monoclonal antibody 3D10 on *Schistosoma japonicum* infection*

| Experiment | No. of mice | No. of adult worms recovered |
|---|---|---|
| Control ascites | 16 | 107.6 ± 23.7 |
| Monoclonal antibody 3D10 | 11 | 97.6 ± 21.6 |

*Ascites (diluted 1:3, 1 ml each) were given by intraperitoneal injection 21 hr and 3 hr before cercarial challenge. BALB/c mice were challenged with 190 cercariae by belly penetration and perfused 4 weeks thereafter.

cells were also observed in the granuloma periphery. The cellular composition of the granulomatous lesions was similar in both 3D10 treated and untreated mice.

*Effects of passive transfer on recovery of adult worms*

As the antigen recognized by 3D10 was shown to be present on the surface membranes of schistosomula, the experiments were performed to test the effects of this monoclonal antibody on *S. japonicum* infection in mice. Groups of BALB/c mice were injected intraperitoneally with 3D10 hybridoma ascites and challenged with *S. japonicum* cercariae. As shown in Table 3, administration of 3D10 21 hrs and 3 hrs before cercarial challenge resulted in no significant reduction in recovery of adult worms when compared with control ascites.

*Molecular weight of egg antigen detected by monoclonal antibody 3D10*

Anti-egg monoclonal antibody 3D10, which modulated the granuloma formation in the lungs, was used in Western blotting to determine molecular weights of the target antigens. As shown in Fig. 2, only one band was exhibited by 3D10 binding to soluble egg antigens. The antigen detected on eggs by the monoclonal 3D10 had an approximate molecular weight of 145 Kd.

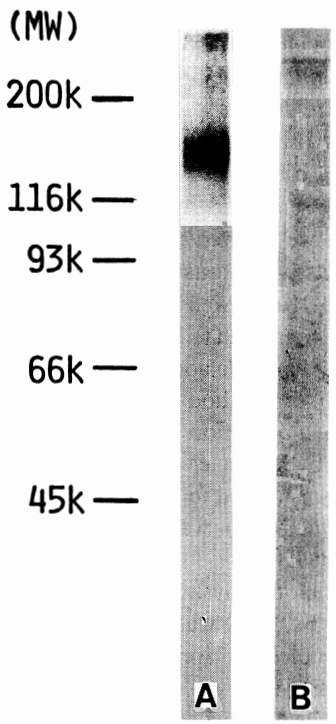


Fig. 2 Western blot analysis of *Schistosoma japonicum* egg antigens recognized by monoclonal antibody 3D10. Soluble egg antigens were subjected to SDS-PAGE under nonreducing conditions. Nitrocellulose membranes were incubated with monoclonal antibody 3D10 undiluted culture supernatant (A) or normal mouse serum (diluted 1:100) (B).

## Discussion

Recent studies have shown that T lymphocytes (Cheever *et al.*, 1985, 1989) and cytokines such as interleukin-2 (Kresina, 1991) and tumour necrosis factor $\alpha$ (Amiri *et al.*, 1992) may play an important role in the formation and modulation of egg granulomas in murine schistosomiasis.

However, the mechanisms of granuloma formation and modulation are considered to differ between infections with *S. japonicum* and *S. mansoni*. In the *S. japonicum* infection, subcutaneous sensitization with eggs is more effective than intraperitoneal sensitization, the latter route being more effective to elicit granuloma formation in *S. mansoni* infections (Warren *et al.*, 1975). Moreover, serum factors, but not lymphoid cells in animals chronically infected with *S. japonicum*, are involved in the modulation of granulomas around eggs. Olds *et al.* (1982) demonstrated that the $IgG_1$ fraction of serum from chronically infected mice decreased lung granuloma formation in egg-sensitized mice. Furthermore, Sidner *et al.* (1987) reported $IgG_1$ and $IgG_3$ monoclonal antibodies having modulating effects against pulmonary granulomas in egg-sensitized mice. In the present experiments, we showed that the COP reaction-positive, passively transferred monoclonal IgM antibody 3D10 as well as sera from 16-week-infected mice modulated granuloma formation. These results provide additional evidence for the importance of antibody in *S. japonicum* egg granuloma modulation and support the demonstration by Sidner *et al.* (1987) that the $IgG_1$ dependency in regard to serum immunoglobulins (Olds *et al.*, 1982) was not absolute at the monoclonal level.

Immunoblot analysis showed that the epitope recognized by 3D10 was present on a molecule having a molecular weight of 145 Kd (145-k antigen). Earlier, we showed that a 145-k molecule gave a strong reaction for carbohydrate with the periodic acid-Schiff (PAS) reagent, demonstrating the glycoprotein nature of the major antigen in schistosome eggs (Kobayashi, 1986). Furthermore, this circumoval precipitinogen may be a secretory antigen released from miracidium in the mature egg since the strongest fluorescence was observed in the space between the miracidium and eggshell. Tracy and Mahmoud (1982) isolated three egg antigens which sensitized mice to lung granuloma formation. One of the antigens, gp-1, was a glycoprotein having a molecular weight of 138 Kd. The molecular weight and strong PAS-reactivity of our 145-k antigen are closely similar to those of gp-1, suggesting that they may be identical. If so, the 145-k antigen may have two important roles in granuloma formation; viz., in the early phase of infection, this antigen molecule is secreted from mature eggs, trapped in host tissues, and sensitizes the host for granuloma formation; in the late phase of infection, the antibodies raised against this antigen regulate the cellular response and modulate the granuloma formation.

Monoclonal antibodies giving COP reactions with *S. japonicum* eggs have also been produced by Cruise *et al.* (1981). However, their COP-positive IgM monoclonal antibody did not modulate *S. japonicum* egg granuloma formation (Mitchell *et al.*, 1982). Garcia *et al.* (1985) considered that the failure of their colleagues to modulate egg-induced granulomas with their anti-egg IgM monoclonal antibody might reflect the use of lyophilized rather than viable eggs. In the present experiment, we used viable eggs for sensitization and challenge. Therefore, secretory antigens from live eggs may have a key role for granuloma formation and its modulation.

Harn *et al.* (1984) obtained an anti-egg monoclonal antibody, E.1, that protected against cercarial challenge in *S. mansoni* infection. Although 3D10 recognized antigenic determinants on the surface membranes of schistosomula, it did not have protective activity to a challenge infection. These results show that the cross-reactive egg antigens with the schistosomula membrane antigens are not always involved in the protective immunity in the challenge infection. The epitope(s) on the schistosomula recognized by 3D10 may disappear from membranes before the effective protective immunity in the host develops.

Further studies on the egg antigens responsible for COP reactions may facilitate progress towards the development of vaccines against granulomatous diseases, as well as immunodiagnosis for schistosomiasis japonica.

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