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Immunohistochemical study on the antigenicity of each organ structure of *Clonorchis sinensis*

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Abstract: An immunohistochemical study was performed to demonstrate comparative antigenicity of each body structure of the liver fluke, *Clonorchis sinensis*, such as the digestive tract, reproductive organs, excretory system, tegument and suckers. Indirect immunoperoxidase technique was applied, using formalin-fixed and paraffin-embedded sections of *C. sinensis* as the antigen. Pooled cat sera obtained 10 weeks after an experimental infection with *C. sinensis* and peroxidase-conjugated goat anti-cat IgG were used as the primary and secondary antibodies, respectively.

The intensity of immunohistochemical stain was very sensitive upon the titers of the primary and secondary antibodies, and their optimum dilutions were found to be 1:1,000~1:2,000 and 1:1,000, respectively. The intestinal epithelial cells, intestinal content and excretory bladder showed strong positive coloring reactions even at lower titer (1:2,000) of the primary antibody, whereas the uterine wall and eggs, vitelline glands, and male reproductive organs showed only weak positive reactions despite an increase in the antibody titer (1:1,000). On the other hand, the suckers, tegument, subtegumental cells and other parenchyme portions did not reveal any positive immunoperoxidase reaction at the same antibody titers.

From the above results, it is highly suggested that the most potent antigenicity of *C. sinensis* occur from their excretory-secretory substances originated from the digestive and excretory organs.

Key words: *Clonorchis sinensis*, antigenicity, immunohistochemistry, immunoperoxidase stain, excretory-secretory antigen

INTRODUCTION

In *Clonorchis sinensis* infection, it is well known that there are significantly elevated antibody levels in the sera of infected patients (Lee *et al.*, 1981; Yang *et al.*, 1983; Hong, 1988) as well as infected animals (Wykoff, 1959). What kind of antigens, in terms not only of their physicochemical properties but of their production sites within the worm, are most responsible for the antibody response of the host, has been a popular subject of investigation for the past 30 years (Sadun *et al.*, 1959; Wykoff, 1959; Sun and Gibson, 1969a & b; Choi *et al.*, 1981; Lee *et al.*, 1988; Chu *et al.*, 1990). Unfortunately, however, the trials and efforts to purify chemically a potent and useful antigenic material from whole worm extracts have not yet been successful (Lee *et al.*, 1988).

On the other hand, several studies to figure

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out, or to visualize, important antigen-producing sites within the body of the worms have revealed some fruitful results, though the results are not uniformly agreed by different workers. Some immunofluorescent studies, for example, showed strong immunofluorescence from the tegument of *C. sinensis* (Cho and Soh, 1974; Im, 1974; Cho *et al.*, 1986), whereas other immunofluorescent studies or immunogold-labeling studies demonstrated that the intestinal content and intestinal epithelial layer, rather than the tegument, revealed stronger positive reactions (Sun and Gibson, 1969b; Sung *et al.*, 1988; Chu *et al.*, 1990). This discrepancy remains to be a question and should be investigated using various available techniques. In the present study, indirect immunoperoxidase technique was applied to visualize the antigenic sites of *C. sinensis* in worm sections.

MATERIALS AND METHODS

1. Collection of the metacercariae of *C. sinensis*

Pseudorasbora parva, the most well-know fish as the second intermediate host of *C. sinensis* in Korea, were collected from the Nakdong river, nearby Kimhae city. The metacercariae of *C. sinensis* were isolated under a stereomicroscope from the artificially digested material of the fish. The metacercariae were washed one or two times with 0.85% saline and stored in a 4°C refrigerator until use.

2. Preparation of worm sections and antibodies

Rabbits were fused or the preparation of worm sections in their livers. They were artificially fed the metacercariae of *C. sinensis*, 300 per each animal, through a gavage needle. They were sacrificed 2 or 4 weeks after the infection, and their livers were removed, from which many liver segments containing *C. sinensis* in the bile ducts were taken. After the segments were fixed with 10% neutral formalin, they were embedded in paraffin wax according to the conventional procedures, and serial sections of 4 μm thickness were made.

Five cats, 1.0∼1.5 kg in body weight, were used to prepare antisera to *C. sinensis*, *i.e.*, the primary antibody. The cats were infected with the metacercariae by the same method as in the case of rabbits. The cats revealed eggs in their feces after 4∼6 weeks. They were sacrificed 10 weeks after the infection, and the blood containing anti-*C. sinensis* antibody was drawn by cardiac puncture. The sera obtained from 5 cats were pooled.

Peroxidase-conjugated goat anti-cat IgG (Sigma) was purchased and used as the secondary antibody.

3. Immunohistochemical stain

Indirect immunoperoxidase staining technique (De Lellis, 1981) was applied. Briefly, the endogenous peroxidase was blocked by adding 0.02% hydrogen peroxide solution for 30 min. After the sections were washed with 0.01 M PBS (pH 7.2), normal goat serum (1:5 dilution) was applied for 30 min to block non-specific protein binding sites. Thereafter they were treated with pooled cat sera (primary antibody) diluted 1:125 to 1:16,000 by two-fold dilution method with PBS. After a wash with PBS, they were treated with peroxidase-conjugated goat anti-cat IgG (secondary antibody) for 30 min at room temperature. The dilution titers of the secondary antibody were in the range of 1:100∼1:1,000. After these procedures, 0.015% hydrogen peroxide and 0.25 mg/ml 3,3-diaminobenzidine-tetrachloride (DAB) was applied as the substrate for 10 min. These sections were counterstained with hematoxylin and observed under light microscopy. Yellowish-brown or dark brown color was regarded as positive reaction.

RESULTS

1. Optimum dilutions of antibodies

The optimum dilutions (titers) of the primary and secondary antibodies were determined, so as to discriminate real positive reactions from unwanted background stains. Positive peroxidase

stains were observed at 1:125~1:2,000 dilutions of the primary antibody and at 1:100~1:1,000 of the secondary antibody. The staining intensity was too strong when the primary antibody was diluted only to 1:125~1:500, so that the unwanted areas such as the hepatic cells and bile duct epithelia showed positive stains. Therefore, the optimum dilutions of the primary and secondary antibodies were set at 1:1,000~1:2,000 and 1:1,000, repectively.

2. Antigenicity of each organ structure of *C. sinensis*

The antigenicity of each organ structure of *C. sinensis* was compared by the lowest antibody titer at which positive reaction was recognized in each structure. When the dilution of the secondary antibody was set at 1:1,000, the staining results according to the serial dilutions of the primary antibody were as presented in Table 1.

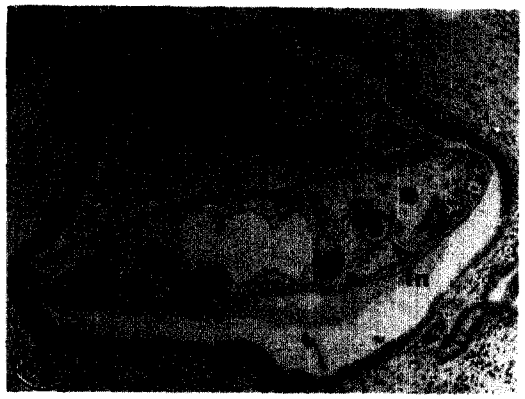
When the primary antibody was diluted more than 1:4,000, no positive stain was observed at any organ structures of *C. sinensis*. However, when the antibody dilution became 1:2,000 or 1:1,000, the intestinal epithelium, intestinal content, excretory bladder, uterine wall and eggs, vitelline glands and male reproductive

Table 1. Relative intensity of immunoperoxidase stain at various parts of sectioned *C. sinensis*

Organ structure	Primary antibody dilution*			
	1:500	1:1,000	1:2,000	1:4,000
Intestinal epithelium	+	+	+	−
Intestinal content	+	+	+	−
Excretory bladder	+	+	+	−
Uterine wall and eggs	+	+	−	−
Vitelline glands	+	+	−	−
Male reproductive organs	+	+	−	−
Suckers	+	−	−	−
Tegument	+	−	−	−
Parenchyme portions	+	−	−	−
Subtegumental cells	−	−	−	−

+ : positive stain, − : negative stain

* Optimum primary antibody dilution(1:1,000). The secondary antibody dilution was fixed at 1:1,000.



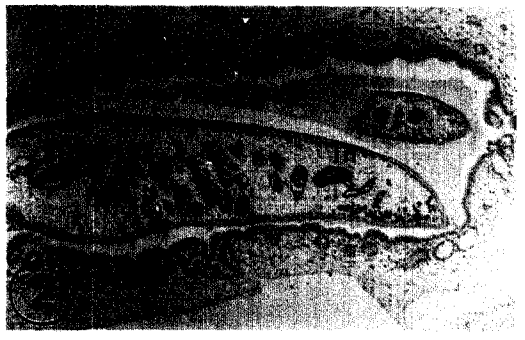


Fig. 1. Immunoperoxidase staining of sectioned *C. sinensis*. Note positive reactions in the intestine(In) (arrow heads), and excretory bladder(EB) (arrow heads). Primary antibody dilution 1:1,000. ×100.

Fig. 2. Another figure showing a strong positive reaction in the intestine(In) of the worm. Primary antibody dilution 1:500. ×100.

Fig. 3. Negative control for the immunoperoxidase staining of sectioned *C. sinensis*. The section was treated with all steps of immunoperoxidase procedure except using uninfected animal serum instead of infected one (primary antibody). Normal serum dilution 1:1,000. ×100.

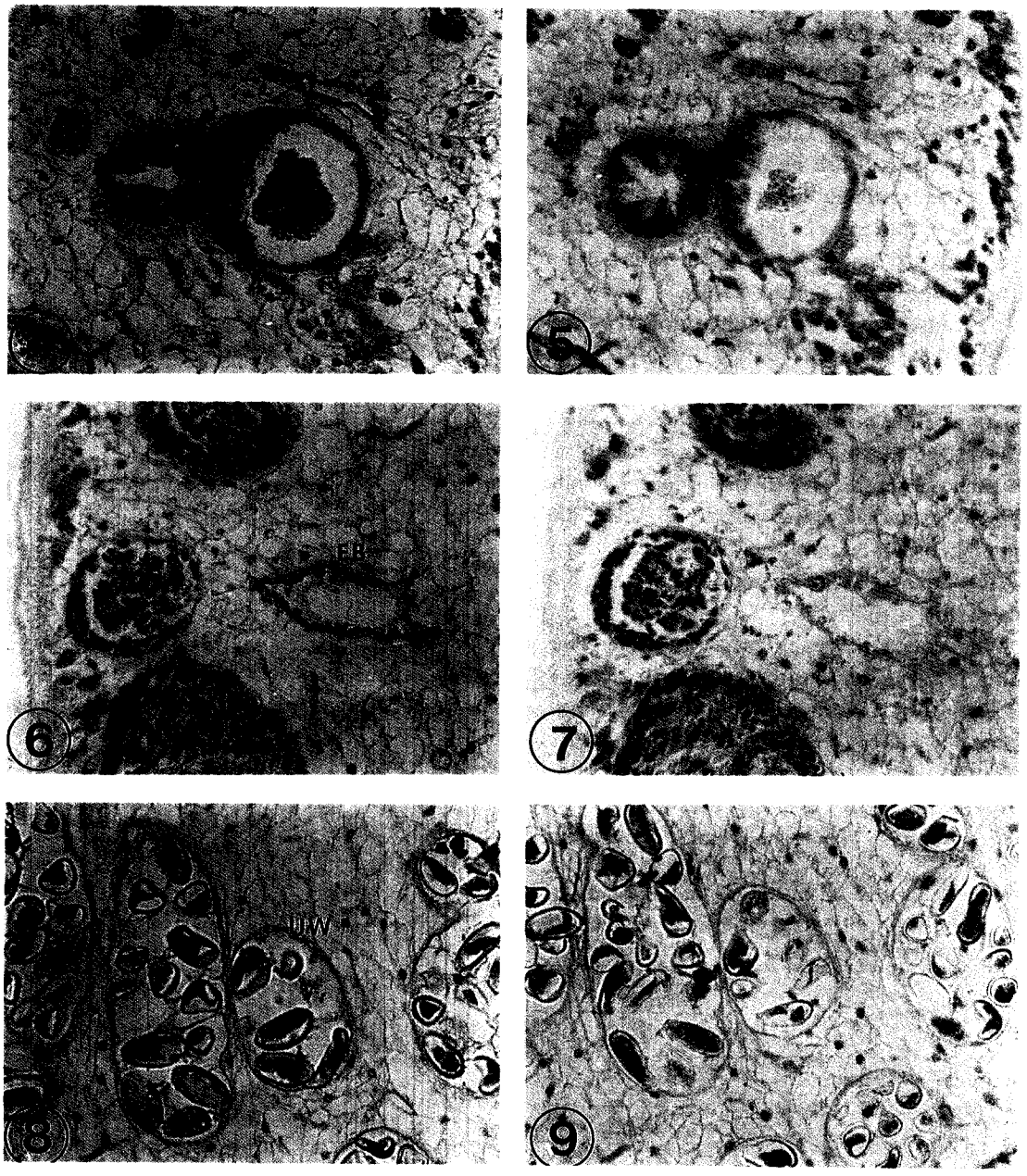


Fig. 4. Positive staining is observed in the intestinal epithelium(In) and intestinal content. Primary antibody dilution 1:1,000. ×400.

Fig. 5. Negative control of the same section of *C. sinensis*. Normal serum control group, dilution 1:1,000. ×400.

Fig. 6. Positive immunoperoxidase reaction is observed on the wall of the excretory bladder(EB). Primary antibody dilution 1:1,000. ×400.

Fig. 7. Negative control at the same sectional level. Normal serum control, dilution 1:1,000. ×400.

Fig. 8. Peroxidase reaction is observed on the uterine wall(UW) and internal substance of the eggs. Primary antibody dilution 1:500. ×400.

Fig. 9. Negative control of the same section. Normal serum control, dilution 1:500. ×400.

organs such as testes showed recognizable positive reactions (Figs. 1, 4 & 6). When the primary antibody was less diluted to 1:500, the staining intensity was increased at the intestine, excretory bladder, and other portions (Fig. 2).

Although the uterine wall and eggs, vitelline glands, and testes showed positive reactions at 1:1,000 dilution of the primary antibody, their staining intensity was weaker than that of the intestine or excretory bladder at the same dilution. The stain became evident and strong in those organs (Figs. 2 & 8) at 1:500 dilution. However, at this antibody titer, nearly all of other structures except subtegumental cells became weakly positive in the peroxidase staining (Table 1). Most of the positive reactions at 1:500, therefore, should be regarded as non-specific ones directed to non-specific sites.

Negative controls were employed to distinguish specific reactions from non-specific ones. The negative controls applied in this study were, 1) saline control; treated with PBS instead of the primary antibody, 2) normal serum control (Figs. 3, 5, 7 & 9); treated with normal cat serum instead of the primary antibody, 3) irrelevant antibody control; treated with cat anti-*Paragonimus westermani* antibody instead of the primary antibody, and 4) substrate control; all steps were followed, just omitting the primary and secondary antibodies. All of the above groups except the irrelevant antibody control did not show any positive reaction throughout the body compartments of *C. sinensis*. In the irrelevant antibody control, positive reactions were chiefly observed in vitelline glands and uterine eggs, however, the staining intensity was relatively weak.

DISCUSSION

In this study, paraffin sections of *C. sinensis* were stained by indirect immunoperoxidase technique for the purpose to demonstrate comparative antigenicity of each organ structure of the worm. It was observed that the strongest antigenicity was expressed at the intestinal epithelium, intestinal content and excretory bladder, and medium or weak antigenicity at the uterine wall and eggs, vitelline glands, and testes. The suckers, tegument and subtegumental cells revealed no antigenicity. This is in agreement with the previous authors (Sun and Gibson, 1969b; Sung *et al.*, 1988; Chu *et al.*, 1990), who reported that the intestinal wall and lumen showed strong fluorescence by indirect immunofluorescent and/or immunogold methods. The strong antigenicity of the excretory bladder in this study was also agreed to the latter two papers. However, the present observation that the tegument of the worms did not show any positive immunoperoxidase stain is comparable to the results obtained by Sun and Gibson (1969b), Im (1974) or Cho *et al.* (1986), who used immunofluorescent techniques.

The importance of excretory-secretory antigen in eliciting antibody response of the host to *C. sinensis* has been well documented since the works of Sun and Gibson (1969a, b & c), who observed strong antigenicity of metabolic products which are originated largely from the digestive and excretory organs of the worm. According to Sun and Gibson (1969a), the reason for the antigenicity of excretory-secretory substance of *C. sinensis* was suggested as follows. Inhabiting in the host's bile duct, the adult worms can give mechanical/chemical injuries to the biliary epithelial layer, and can obstruct the bile duct to cause stagnation and resorption of bile juice, thus making the metabolite of worms to come into the host tissues. Also in the case of *P. westermani*, the lung fluke, which inhabits within the lung tissue of definitive hosts, their excretory-secretory substance was reported to have strong antigenicity (Yogore *et al.*, 1965). Kim *et al.* (1983 & 1986) and Lee *et al.* (1986) and Lee *et al.* (1989) further emphasized antigenic potency of the intestinal content or metabolic product of *P. westermani*. Similar results were reported in the case of nematodes such as *Trichinella*

spiralis (Jackson, 1959) and *Nippostrongylus muris* (Jackson, 1960).

The nature and origin of excretory-secretory substance of *C. sinensis* or *P. westermani* has not yet been sufficiently understood and needs many studies. In one simple aspect, some of them should be of host origin. In the case of *C. sinensis*, for example, they may be tissue fragments uptaken by the worm or even blood drawn from the host (Chu *et al.*, 1982). These food materials will be degraded within the intestine of the worms and eventually constitute their intestinal content. Some of the content may be regurgitated through the oral sucker, and some may be absorbed through the intestinal wall of the worms. A part of the absorbed material will be excreted via the excretory bladder. It is speculated that the degraded or deformed host material might give strong antigenicity if they are resorbed by the host. In another aspect, on the origin of excretory-secretory substance, there should be some kinds of such material which are of parasite origin. Enzymes and metabolites can be the examples. Not many studies, however, have been given interests on this point.

Studies related to the tegumental antigenicity of *C. sinensis* have been reported several times. Choi (1981) suggested that some tegumental debris exfoliated into the culture supernatant might be responsible for the strong antigenicity of 24-hour culture fluid of this trematode. Sun and Gibson (1969b) observed specific immunofluorescence from the tegument of *C. sinensis* by immunofluorescent methods. Im (1974) and Cho *et al.* (1986) agreed to the antigenicity of the worm tegument. In this study, however, antigenicity of the tegument of *C. sinensis* appeared very weak or negligible. There could be several reasons for this discrepancy. First, difference in experimental settings such as the kinds of experimental animal, age of infection of the antisera, and dilution titer of anti-*C. sinensis* antibody might have been concerned. Second, whereas fresh frozen sections were used in the immunofluorescent studies by Sun and Gibson (1969b) and Cho *et al.* (1986), formalin-fixed paraffin sections were used in the present immunoperoxidase study. It should be considered that during the process of formalin fixation and paraffin embedding some loss of worm antigenicity especially of the tegument might occur. In order to answer to this question, it is necessary to do indirect immunoperoxidase staining using frozen-sectioned *C. sinensis*.

The eggs of *C. sinensis* have been studied for their antigenicity using various immunological methods (Sun, 1969; Sun and Gibson, 1969a & 1969b), and they were reported to have no antigenicity. By indirect fluorescent antibody technique, for example, specific fluorescence was not demonstrated in the eggs (Sun and Gibson, 1969b). The results of a circum-oval precipitin test (Sun, 1969) and a gel-diffusion test (Sun and Gibson, 1969a) also indicated that the eggs of *C. sinensis* were not an effective antigen. However, different results were reported in the case of other parasite eggs. Immunofluorescent studies have demonstrated that the eggs of *Schistosoma mansoni* have strong antigenicity (Andrade *et al.*, 1961) and immunoperoxidase studies on *P. westermani* have demonstrated positive antigenicity of their eggs (Lee *et al.*, 1989). The reason why the eggs of different kinds of parasites express different kinds of parasites express different antigenicity seems to be variable, and should be dependent upon many host-parasite factors such as the habitat of the parasite within the host. Sun (1969) insisted that the different antigenicity of *C. sinensis* and *S. mansoni* eggs should be due to, aside from a difference in their habitat, a difference in the ultrastructure of their shells and thus in the permeability of the eggs. In other words, unlike *S. mansoni*, the eggs of *C. sinensis* were believed not releasing metabolic products or secretions into the host body (Sun, 1969).

Contrary to this, weak antigenicity of some uterine eggs of *C. sinensis* was observed in this study. Kwon *et al.* (1984) also observed positive immunofluorescence from the eggs of *C. sinensis*.

These might have been non-specific reactions, however, it has to be cleared whether the suggestions given by Sun (1969), as above, are only partially true.

In the immunodiagnosis of clonorchiasis, antigens of *C. sinensis* chemically extracted by various methods have been applied. However, since the body of helminths such as *C. sinensis* is composed of very much complex substances, trials to purify potent antigens which have both high specificity and high sensitivity have been in most cases unsuccessful. Recently, whole worm extracts of adults and larvae of *C. sinensis* were analyzed by SDS-polyacrylamide gel electrophoresis, whereby 4∼5 immunoreactive antigen bands were obtained (Lee *et al.*, 1988). In spite of high immunoreactivity in ELISA there were cross reactions between these antigens and antisera obtained from other trematode infections such as paragonimiasis (*P. westermani*), so that further studies to purify specific antigens were needed (Lee *et al.*, 1988). A promising result was recently shown by Yong (1990) for the serodiagnosis of clonorchiasis, applying ELISA-inhibition test using a monoclonal antibody to *C. sinensis*.

In this study, sera of non-infected control group did not show false positive reactions to sectioned *C. sinensis* but sera of *P. westermani* infected controls revealed cross reactions with sectioned *C. sinensis*. This is in full agreement with previous authors such as Hunter *et al.* (1958), Kim and Blackwell (1963), and Sadun *et al.* (1959), who mentioned that cross reactions between *C. sinensis* and *P. westermani* are very frequent. In this study, vitelline glands and uterine eggs showed stronger cross reactions than other organs, which suggested that common antigens between *C. sinensis* and *P. westermani* might be located in these organs.

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=국문초록=

간흡충 충체의 부위별 항원성에 대한 면역조직화학적 연구

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간흡충의 충체 부위별 항원성을 알아보고자 면역조직화학적 방법을 이용하여 간흡충의 소화기관, 생식기관, 배설기관, 흡반, 표피 등의 염색강도를 비교 관찰하였다. 간흡충 실험 감염 후 2~8주 된 토끼에서 담관 내 충체를 포함하는 간 조직을 얻어 중성 포르말린 용액에 고정하고 파라핀으로 포매한 다음 4 μm 두께로 잘라 항원으로 이용하였다. 항-간흡충 항체(1차 항체)는 실험 감염 10주 된 고양이 혈청을 희석하여 사용하였고 peroxidase-conjugated goat anti-cat IgG를 2차 항체로 하여 간접 면역효소 염색을 시행하였다. 가장 적합한 1차 항체의 희석 농도는 1 : 1,000~1 : 2,000, 2차 항체의 희석 농도는 1 : 1,000이었다.

충체의 장 상피세포, 장 내용물 및 배설낭은 1차 항체 희석 농도 1 : 1,000~1 : 2,000에서 강한 양성 반응을 보인 반면, 자궁 및 일부 자궁 내 충란, 난황선, 웅성 생식기관 등은 1차 항체 희석 농도 1 : 1,000에서 미약한 양성 반응을 나타내었다. 한편, 흡반, 표피, 표피하 세포, 충체 실질 등은 1차 항체 희석 농도 1 : 1,000에서도 음성 반응을 나타내었다. 이상의 결과를 종합하면, 간흡충 감염시 숙주의 항체 반응은 충체의 소화-분비 기관에서 유래된 이른바 분비-배설 항원에 의해 가장 강력하게 유발되는 것으로 추측된다.

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