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Differences in Larvicidal Activity of Complement and Chemiluminescent Response of Phagocytes in Carp (*Cyprinus carpio*), Crucian carp (*Carassius auratus*) and False Dace (*Pseudorasbora parva*) against Excysted Metacercariae of *Clonorchis sinensis*

Ki Hong Kim†, Yoon Jung Hwang, Se Ryun Kwon and Jae Bum Cho

Department of Aquatic Life Medicine, College of Fisheries Sciences, Pukyong National University Pusan 608-737, Korea

Differences in larvicidal activity of complement and chemiluminescent response of phagocytes in carp (*Cyprinus carpio*), crucian carp (*Carassius auratus*) and false dace (*Pseudorasbora parva*) against excysted metacercariae of *Clonorchis sinensis* were investigated. The prevalence of *C. sinensis* metacercariae of false daces caught at Nakdong river (NR) was 100%. However, no *C. sinensis* metacercariae were found in false daces, which were collected at a reservoir in Chinyoung (CY), crucian carps and carps. The sera of false dace, which were intensively infected with *C. sinensis* metacercariae, killed excysted metacercariae of *C. sinensis* more readily than that of carp. However, the serum obtained from *C. sinensis* metacercariae-free false dace showed the lowest larvicidal ability. The larvicidal ability of sera collected from each fish species completely disappeared when the complement was inactivated by heating. When supernatant of excysted metacercariae homogenate were added to phagocytes of each species, the chemiluminescent responses were significantly ($p<0.05$) diminished in false dace and carp. The inhibition ratio of chemiluminescent responses by the supernatant was 22.9% in false dace, 9.6% in crucian carp and 12.4% in carp.

Key words: *Clonorchis sinensis*, Excysted metacercariae, Freshwater fish, Complement, Chemiluminescent response

Introduction

Clonorchiasis is a representative freshwater fish-mediated human helminthiasis in Korea. *Clonorchis sinensis* shows a low degree of specificity for the fish host, and approximately 36 species of freshwater fish were found to serve as second intermediate hosts in Korea (Rim, 1990). Among them, false dace (*Pseudorasvora parva*) is known as the representative host for *C. sinensis*.

The host specificity of a parasite can be determined in part by the protecting ability of the host against invading parasites. Bower and Woo (1977) have shown that *Cryptobia catostomi* infecting an atypical host was lysed by stimulating host's complement cascade. A similar mechanism has been demonstrated in *Trypanosoma salmositica*, also (Wehnert and Woo, 1980). Fisher *et al.* (1982) reported that the chemiluminescent response of granulocytes against *Nematospiroide dubius* in Lewis rat (resistant to the infection of *N. dubius*) was considerably higher than that in Nmri rat (susceptible to the infection of *N. dubius*).

Although there are many reports concerning wormicidal effect of skin mucus on *C. sinensis* cercariae in various freshwater fish species (Chun, 1964;

†Corresponding author

Rhee, 1974; Rhee *et al.*, 1979, 1980a,b, 1982, 1983, 1984a,b), little is known about the reactions of complement and phagocytes of fish against *C. sinensis* metacercariae.

In the present study, therefore, differences in larvicidal activity of complement and chemilu- minescent response of phagocytes in carp (*Cyprinus carpio*), crucian carp (*Carassius auratus*) and false dace (*Pseudorasbora parva*) against excysted metacercariae of *Clonorchis sinensis* were investigated.

Materials and Methods

Fish

False daces and crucian carps were collected using a small trawl or bait traps from the lower reaches of Nakdong river (NR) and a reservoir in Chinyoung (CY), Kyoungsangnam-do in Korea. Carps were obtained from the fish farm in Pukyong National University of Korea. Collected fish were transported to the laboratory in live state, and randomly sampled 10 fish of each species were examined for the presence of *C. sinensis* metacercariae.

Collection and excystation of *C. sinensis* metacercariae

C. sinensis metacercariae were collected using digestion method with artificial gastric juice. The collected metacercariae were excysted by immersing in artificial intestinal juice, and the excysted metacercariae were washed three times in sterile 0.85% NaCl.

Homogenization of excysted metacercariae

Three-thousand excysted metacercariae in 3 m*l* Hank's balanced salt solution (HBSS) on ice were homogenized using ultrasonic processor. The homogenate was centrifuged at 500 g at 4°C for 30 min, and the supernatant was filter sterilised (0.4 μm).

larvicidal effects of serum complement

Fish were anaesthetized with benzocaine and bled from the caudal vein using a sterile 1 ml syringe. Blood was allowed to clot at 22°C, stored overnight at 4°C, then centrifuged at 1000 g for 5 min. To each well of a microtitre plate was added fresh serum of each fish species and 20 excysted metacercariae. The parasite mortality was assessed on the basis of responsiveness to stimulation.

The inactivation of serum complement activity was accomplished by heating at 47°C for 30 min, and larvicidal effect of this complement-inactivated serum of each fish species was assessed as above.

Chemiluminescent (CL) response of head kidney phagocytes

Isolation of head kidney phagocytes. Head kidneys were removed aseptically from each fish species and passed through a 100 μm nylon mesh using HBSS containing heparin (10 units/m*l*, Sigma), penicillin (100 μg/m*l* Sigma) and streptomycin (100 U/ m*l*, Sigma). The resulting cell suspension was placed on a 34/51% Percoll density gradient and centrifuged at 400 g for 30 min at 4°C. The interphase was collected and the cells were washed twice at 400 g for 5 min in HBSS containing heparin and antibiotics. The cell viability was examined with trypan blue exclusion and was evaluated to be greater than 95%. The phagocytes, including neutrophils and macrophages, were adjusted to 3.0×10^6 cells/ml HBSS in all fish species.

Opsonization of zymosan. To exclude influence of the species-specific complement factors in CL assay, serum of tilapia (*Oreochromis niloticus*) cultured in the fish farm of Pukyong National University was used as an opsonin of zymosan. Blood samples were collected with syringes from the caudal vein of the tilapia, and serum was separated by centrifugation. Zymosan (Sigma) was mixed with the fresh serum and incubated at 30°C for 30 min. Zymosan was separated from the serum by centrifugation, washed three times and suspended in HBSS.

CL assay. The ROIs(reactive oxygen intermediates) produced by stimulated phagocytes were quantified using an automatic photoluminometer (Bio-Orbit 1251, Finland). Each test cuvette (4 m*l*) con-

tained 0.7 m*l* luminol(Sigma) made according to the method of Scott and Klesius (1981), 0.2 m*l* cell suspension of each fish species, and 0.2 m*l* filtered supernatant of excysted metacercariae homogenate or 0.2 m*l* HBSS as a control. The mixture was incubated for 15 min at 22°C. Then, 0.3 m*l* opsonized zymosan was added just prior to measurement. The control blank cuvette contained only luminol, cells and HBSS. The measurements were made for 100 minutes in triplicate and the light emission was recorded as mV.

Statistical analysis

The CL data were analysed using the Student's *t*-test to determine differences among fish species.

Results

Prevalence of *C. sinensis* metacercariae in each fish species

The prevalence of *C. sinensis* metacercariae of false daces caught at Nakdong river (NR) was 100%. However, no *C. sinensis* metacercariae were found in false daces collected at a reservoir in Chinyoung (CY), crucian carps and carps.

larvicidal effects of serum complement

The time required for complete killing of all metacercariae by fresh serum was 5 min in false dace caught at NR, 6 min in crucian carp, and 50 min in carp (Fig. 1). However, the serum of false dace collected at CY(free from *C. sinensis* metacercariae) required considerably more time (60 min) for complete killing of all metacercariae than those of false dace collected at NY and the other fish species.

The larvicidal ability of sera collected from each fish species completely disappeared when the complement was inactivated by heating.

Chemiluminescent (CL) response of leucocytes

Head kidney leucocytes of carp and crucian carp produced markedly higher CL responses than those

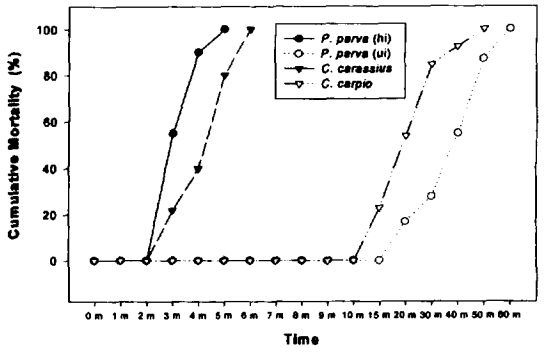

Fig. 1. Cumulative mortality (%) of excysted metacercaria of *Clonorchis sinensis* by serum of carp, crucian carp and false dace (hi, heavily infected; ui, uninfected).

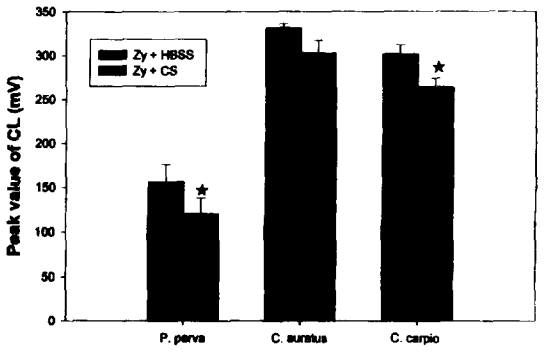

Fig. 2. Effect of the homogenate of *Clonorchis sinensis* metacercaria on the chemiluminescent response of kidney phagocytes of carp, crucian carp and false dace. Values are mean±SE. (★, indicate statistical significance at P<0.05).

of false dace in response to zymosan (Fig. 2). When supernatant of excysted metacercariae homogenate was added to phagocytes of each species, the CL responses were significantly ($p<0.05$) diminished in false dace and carp (Fig. 2). The inhibition ratio of CL responses by adding the supernatant was 22.9% in false dace, 9.6% in crucian carp and 12.4% in carp.

Discussion

The complement system of teleosts is activated through the classical and alternative pathway as in mammals (Iwama and Nakanishi, 1996). In the present study, the heat-inactivated serum of all fish

species had no larvicidal effect. This suggests that the wormicidal activity of serum might be mediated through activation of complement cascade. Although the larvicidal ability of serum from NR false dace infected with *C. sinensis* metacercariae was higher than carp serum, the serum obtained from *C. sinensis* metacercariae-free CY false dace showed the lowest larvicidal activity. This result suggests that false dace may produce antibody againt *C. sinensis*, and the serum complement collected from false dace infected with *C. sinensis* metacercariae might be activated not only alternative but also classical pathway. Relatively little information is available concerning specific immune responses of fish to metacercarial infections. Cottrell (1977) demonstrated that plaice (*Pleuronectes platessa*) produced specific antibody to metacercariae of *Cryptocotyle lingua*. Rainbow trout (*Salmo gairdneri*) could produce antibody against sonicated metacercariae of the eye fluke, *Diplostomum spathaceum*, injected intraperitoneally (Bortz *et al.*, 1984). Wood and Matthews (1987) found that grey mullet (*Chelon labrosus*) produced specific antibody in response to *C. lingua*, also.

Granulocytes and macrophages possess a phagocytic activity, which is the initial step in the immune response in fish, and is the major line of defence for all foreign material, including pathogenic agents (Olivier *et al.*, 1986). During phagocytosis, fish macrophages increase their oxygen consumption as well as the production of reactive oxygen intermediates (ROIs) (Chung and Secombes, 1988) such as the superoxide (O_2^-), hydrogen peroxide (H_2O_2), and the hydroxyl radical (OH^-). These ROIs play an important role in the anti-pathogenic activity of phagocytic cells (Allen *et al.*, 1972; Babior, 1984). On the other hand, parasites excrete various substances to evade the host's immune responses. For example, *Echino- coccus granulosus* in mouse excretes some substances, which inhibit the activation of macrophages (Robinson and Arme, 1986). Moreover, parasites excrete various enzymes including superoxide dismutase, catalase, glutathione peroxidase to prevent damages by ROIs and to neutralize ROIs (Callahan *et al.*, 1988). In the present study, the CL responses of all fish species were diminished when supernatant of *C. sinensis* excysted metacercariae homogenate was added in assays. From this fact, it can be conjectured that *C. sinensis* has some substances which can inhibit activation of fish leucocytes or can neutralize ROIs produced by respiratory burst of phagocytes. The diminished ratio of CL response by adding *C. sinensis* was greater in false dace than in carp and crucian carp, and this suggests that the coping ability of false dace leucocytes against invasion of *C. sinensis* may be lower than that of carp and crucian carp.

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References

Allen, R. C., Stjernholm, R. L. and Steele, R. H.: Evidence for generation of an electronic excitation state(s) in human polymorphonuclear leucocytes and its participation in bactericidal activity. Biochem. Biophy. Res. Comm., 47: 679-684, 1972.

Babior, B. M.: Oxidants from phagocytes agents of defense and destruction. Blood, 64: 959-966, 1984.

Bortz, B. M., Kenny, G. E., Pauley, G. B., Garcia-Ortigoza, E. and Anderson, D. P.: The immune response in immunised and naturally infected rainbow trout (*Salmo gairdneri*) to *Diplostomum spathaceum* as detected by enzyme-linked immunosorbant assay (ELISA). Dev. Comp. Immunol., 8: 813-822, 1984.

Bower, S. M. and Woo, P. T. K.: *Cryptobia catostomi*: incubation in plasma of susceptible and refractory fishes. Exp. Parasitol., 43: 63-68, 1977.

Callahan, H. L., Crouch, R. K. and James, E. R.: Helminth anti-oxidant enzymes: a protective mechanism against host oxidant? Parasitol. Today, 4: 218-225, 1988.

Chun, S. K.: Studies on the experimental mode of infections of *Clonorchis sinensis* III. Studies on the wormicidal effect of external mucous substance of some fresh water fish on the larva of *Clonorchis sinensis*.

Korean J. Parasit., 2: 148-158, 1964 (in Korean).

Chung, S. and Secombes, C. J.: Analysis of events occurring within teleost macrophage during the respiratory burst. Comp. Biochem. Physiol., 89B: 539-544, 1988.

Cottrell, B.: The immune response of plaice (*Pleuronectes platessa* L.) to the metacercariae of *Cryptocotyle lingua* and *Rhipidocotyle johnstonei*. Parasitol., 74: 93-107, 1977.

Fischer, H., Brinkmann, V., Hovestadt, I., Makimura, S., Mossmann, H., Possart-Schmitz, P., Schmitz, B. and Speth. V.: Chemiluminescence as a tool in parasite research. *In* Immune reactions to parasites, pp. 101-112, W. Frank (eds.). Gustav Fischer Verlag, Stuttgart, New York, 1982.

Iwama, G. and Nakanishi, T.: The fish immune system. Organism, pathogen, and environment, Academic Press, Inc., 380 p., 1996.

Olivier, G., Eaton, C. A. and Campbell, N.: Interaction between *Aeromonas salmonicida* and peritoneal macrophages of brook trout (*Salvelinus fontinalis*). Vet. Immunol. Immunopathol., 12: 223-234, 1986.

Rhee, J. K.: Experimental studies on the second intermediate hosts of *Clonorchis sinensis* III. Observation on the relationship between clavate cells of epidermis and infectivity of metacercariae of *Clonorchis sinensis* in fresh-water fish. Korean J. Parasit., 33: 101-110, 1974 (in Korean).

Rhee, J. K., Baek, B. K., Ahn, B. Z. and Park. Y. J.: The wormicidal substances of fresh water fishes on *Clonorchis sinensis* I. Preliminary research on the wormicidal substance from mucous substance of *Carassius carassius*. Korean J. Parasit., 17: 121-126, 1979 (in Korean).

Rhee, J. K., Baek, B. K., Ahn, B. Z. and Park. Y. J.: The wormicidal substances of fresh water fishes on *Clonorchis sinensis* II. Preliminary research on the wormicidal substance from mucous substances of various fresh water fishes. Kor. J. Parasitol., 18: 98-104, 1980a (in Korean).

Rhee, J. K., Baek, B. K., Ahn, B. Z. and Park. Y. J.: The wormicidal substances of fresh water fishes on *Clonorchis sinensis* III. Seasonal variation on the wormicidal substance of mucous substances of *Carassius carassius*. Korean J. Parasit., 18: 179-184, 1980b (in Korean).

Rhee, J. K., Kim, P. G., Baek, B. K., Lee, S. B. and Ahn, B. Z.: Clavate cells of epidermis in *Cyprinus carpio nudus* with reference to its defence activity to *Clonorchis sinensis*. Korean J. Parasit., 20: 201-203, 1982.

Rhee, J. K., Lim, P. G., Baek, B. K., Lee, S. B. and Ahn, B. Z.: The wormicidal substances of fresh water fishes on *Clonorchis sinensis* IV. Preliminary research on the wormicidal substance from mucus of *Cyprinus carpio nudus*. Kor. J. Parasitol. 21: 21-26, 1983 (in Korean).

Rhee, J. K., Lee, S. B. and Ahn, B. Z.: Rhee, J. K., Baek, B. K., Ahn, B. Z. and Park. Y. J.: The wormicidal substances of fresh water fishes on *Clonorchis sinensis* V. Purification and chemical characterization of clonorchicidal substance from epidermal mucus of *Cyprinus carpio*. Korean J. Parasit., 22: 127-134, 1984a (in Korean).

Rhee, J. K., Lee, S. B. and Baek, B. K.: Seasonal effects on clonorchicidal substances from epidermal mucus of *Cyprinus carpio*, *Ophicephalus argus* and *Parasilurus asotus*. Korean J. Parasit., 22: 135-137, 1984b.

Rim, H. J.: Clonorchiasis in Korea. Kor. J. Parasitol., 28 (Suppl.): 63-78, 1990.

Robinson, R. D. and Arme, C.: *Echinococcus granulosus*: effect of protoscoleces on murine macrophage activation. Helminthologia 23: 201-209, 1986.

Scott, A. L. and Klesius, P. H.: Chemiluminescence: A novel analysis of phagocytosis in fish. Develop. Biol. Standard., 49: 243-254, 1981.

Wehnert, S. D. and Woo, P. T. K.: *In vivo* and *in vitro* studies on the host specificity of *Trypanoplasma salmositica*. J. Wildlife Dis., 16: 183-187, 1980.

Wood, B. P. and Matthews, R. A.: The immune response of the thick-lipped grey mullet, *Chelon labrosus* (Risso, 1826), to metacercarial infections of *Cryptocotyle lingua* (Creplin, 1825). J. Fish Biol., 31 (Suppl. A): 175-183, 1987.

간흡충 탈낭유충에 대한 잉어, 붕어, 참붕어의 보체 살충능 및 식세포 Chemiluminescent 반응 차이

김기홍 · 황윤정 · 권세련 · 조재범

부경대학교 수산생명의학과

간흡충 탈낭유충에 대한 잉어, 붕어, 참붕어의 보체 살충능 및 식세포 chemiluminescent 반응 차이를 조사하였다. 낙동강에서 채집한 참붕어는 모두 간흡충 피낭유충에 중감염되어 있었으나, 진영의 저수지에서 채집한 참붕어 및 낙동강에서 채집한 붕어와 양식 잉어에서는 간흡충 피낭유충이 전혀 검출되지 않았다. 각 실험어의 신선혈청을 이용하여 간흡충 탈낭유충에 대한 살충능을 조사한 결과 간흡충 피낭유충에 중감염된 참붕어 혈청과 붕어의 혈청은 잉어의 혈청에 비해 높은 살충능을 나타낸 반면, 간흡충 피낭유충에 감염되지 않은 참붕어의 혈청은 가장 낮은 살충능을 나타냈다. 각 어종의 혈청을 47°C에서 30분간 처리하여 보체를 불활화시킨 결과 모든 실험어종에서 혈청의 살충능이 완전히 사라졌다. 간흡충 탈낭유충 균질액의 상층액을 각 어종의 두신 식세포에 첨가하여 chemiluminescent(CL) 반응을 측정한 결과 참붕어와 잉어에서 CL반응이 zymosan만을 첨가했을때에 비해 유의적($P<0.05$)으로 감소하였으며, 각 어종별 간흡충 탈낭유충 균질액의 상층액 첨가에 의한 CL반응 감소율은 참붕어의 경우 22.9%, 붕어 9.6%, 잉어 12.4%로 나타났다.

Key words: *Clonorchis sinensis*, Excysted metacercariae, Freshwater fish, Complement, Chemiluminescent response