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Interaction between Human Eosinophils and Schistosomula of *Schistosoma japonicum in vitro*: IgG Dependent Cell-Adherence and Killing of the Parasites

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Introduction

The adherence of eosinophils to schistosomula *in vitro* has been studied most intensively with *Schistosoma mansoni*. The schistosomula larvae of the parasite are damaged by eosinophils after cell-fusion (Caulfield *et al.*, 1980). This evidence has been demonstrated in several animal species including human (Vadas *et al.*, 1980), baboon (Butterworth *et al.*, 1976), mouse (Kassis *et al.*, 1979), and rat (Capron *et al.*, 1978). The cytotoxicity of the cells was dependent on IgG (Butterworth *et al.*, 1977), IgE (Capron *et al.*, 1981), or complement (McLaren *et al.*, 1979).

Although much information is known about the functions of eosinophils on schistosomula of *S. mansoni*, little information is available concerning *S. japonicum*. It is interesting to learn the defence mechanisms of the host agaist *S. japonicum* because shcistomiasis japonica is thought to be the most severe type of infection, both clinically and pathologically, among shistosome infections in man. In the present study, we demonstrated that human eosinophils adhered to schistosomula of *S. japonicum in vitro* in the presence of patients' serum and that the cells killed some of the parasites.

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Materials and Methods

Eosinophils and neutophils. Heparinized venous blood was collected from patients with eosinophilia (of unknown etiology 2 patients, filariasis 1 patient, paragonimiasis 1 patient, and Kimura's disease 1 patient), before treatment. Eosinophils and neutrophils were purified by the method of Gärtner (1980), in brief, five parts of blood was mixed with one part of 6 % w/v dextran (Dextran 250, Pharmacia, Uppsala, Sweden) in 0.15 M NaCl and left at 37°C for 40 min to allow sedimentation of the erythrocytes. The leucocyte-rich supernatant was collected and washed twice with Hanks' balanced salt solution (HBSS), then resuspended in Percoll (Pharmacia) solution, of which specific gravity (sp. gr.) was adjusted to 1.070 with HBSS containing 10 % fetal calf serum (FCS, Gibco, Grand Island, NY, USA). The suspension was layered on the top of gradients made by Percoll and HBSS. Each sp. gr. was 1.100, 1.090, 1.085, and 1.080. The gradients were centrifuged at 1,600 g for 20 min at room temperature. Eosinophils were rich in the lowest layer and neutrophils in the second lowest one. These layers were collected with Pasteur pipettes then washed 3 times with HBSS and resuspended in a culture medium (Eagle's MEM, Handaibiken, Osaka, Japan, containing 10 % heat-inactivated FCS, 100U/ml penicillin, 100 μg/ml streptomycin). When the percentage of eosinophils was lower than 80 %, the cell fraction was re-applied to the gradients, so that the purities of eosioophils

and neutrophils used were 80–96 % and more than 96 %, respectively.

Schistosomula. Cercariae of *S. japonicum* of Yamanashi-strain (Yamanashi, Japan), maintained in this laboratory by passage through outbred mice and the *Oncomelania nosophora* snail, were applied to an apparatus for skin-schistosomula as described by Clegg and Smithers (1972). Schistosomula were collected after 3 h incubation, then resuspended in a culture medium after being washed twice.

Serum. Serum from patients with schistosomiasis japonica was obtained from Leyte Island, Philippines (kindly supplied by Dr. Kawanaka, NIH of Japan).

A COP-positive pooled serum was used for the experiments. A control serum was collected from healthy Japanese adults (both sexes). All serum samples were inactivated at 56°C for 40 min. Absorption of serum with anti-immunoglobulin serum was accomplished as follows: based on preliminary studies, no precipitating band was obseved in immunoelectrophoresis when one part of patient's serum was absorbed with 14 parts of anti-human-γ-chain goat serum (MBL, Nagoya, Japan) or with one part of anti-μ-chain goat serum (MBL). The patient's serum was incubated with either each anti-serum in their respective ratios or an equivalent amount of normal goat serum (MBL) at 37°C for 30 min, then dialyzed overngiht against 1,000 fold MEM (with 3 changes) at 4°C to remove NaN_2 (anti-microbial regent in anti-serum). The final dilution of each absorbed serum to the original serum in culture was 1 : 100.

Culture and microscopical assay. Aliquots of 160 μl, containing 100 schistosomula, were dispensed into 13 X 100 mm round-bottomed polystyrene tubes (Falcon, Oxnard, CA, USA). Serum was added, according to the design of each experiment, in volumes of 20 μl (30 μl for absorbed serum), then incubated at 37°C for 30 min. After pre-incubation, effector cells were added in 20 μl of culture medium and further incubated in 5 % CO_2 humidified atomosphere at 37°C. In some experiments, fresh serum (autologous to leucocyte donor) was added to a final concentration of 20 % as complement source. Each treatment was performed in duplicate or triplicate. After various incubation periods, about one half the volume of supernatant in the culture tube was discarded by suction and the residual contents were put on glass slide (without coverglass) after gentle agitation. A total of 25 worms per slide (except for marginal ones) was examined microscopically in a warm (28–30°C) room. Cell attachment to the parasite was quantified by counting the number of organisms with 10 or more cells and 5 or more (including 10 or more) cells. Mortality of the worms was evaluated by examination of mobility over a 1 min period. Some preparations were fixed with methanol and stained with Giemsa solution (Merk, Darmstadt, Germany).

Infectivity of cultured schistosomula. To examine the infectivity of schistosomula cocultivated with antibody and eosinophils, all contents of each tube was injected into the peritoneal cavity of ddY strain male mice after 48 h of incubation. The mice were killed 8 weeks after onset of infection or immediately after death (death occured later than 6 weeks) to collect the worms by perfusion from the portal and mesenteric veins of the mice.

Statistics. Each value represents mean ± S. E. of three to five independent experiments and the significance of difference between groups was assessed by the Student's t-test.

Results

Time course of adherence of eosinophils and neutrophils to schistosomula. Ninety seven percent of schistosomula were covered with 5 or more cells when incubated with patient's serum and eosinophils for 3 h, whereas 21.3 % of the organisms were attached by 5 or more cells when incubated with control (healthy) serum and eosinophils. Gradual decreases in the percentages of cell attached organisms were observed (Fig. 1A). Schistosomula were covered with cells surrounding the body except near the oral sucker, where only a few cells, if any, were attached

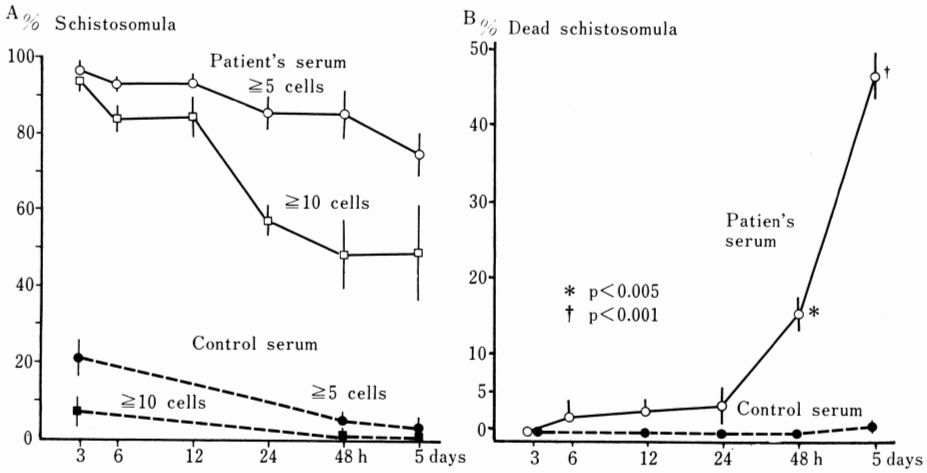

Fig. 1 Time course of eosinophil adherence and cytotoxicity
Serum dilution=1 : 10 and target-to-cell ratio=1 : 1,000

(Fig. 2-a). Stained preparations showed that the adhering cells were eosinophils (Fig. 2-d). These cells had intact shapes at an early stage of incubation, then degranulation, swelling and denucleation were observed within 48 h (Fig. 2-e). Therefore it was difficult to count the exact number of adhered cells. The decrease in the number of cell-covered organisms did not merely mean the detachment of the cells. Neutrophils adhered to schistosomula in the presence of patient's serum in an early stage of incubation. Although schistosomula covered with 5 or more cells was 91.0 % at 3 h, it decreased to 4.2 % by 48 h (Table 1). In the case of neutrophils, cells seemd to be detached from organisms because cell components were not seen around the schistosomula at 48 h.

Time course of killing of schistosomula. The percentages of mortality of schistosomula after various incubation periods are shown in Fig. 1 B. The killing of the organisms was not significant until 24 h. A significant number of death of worms was observed at 48 h ($p<0.005$) when incubated with patient's serum and eosinophils, whereas no organism was killed up to 48 h when cultured with control serum and eosinophils. After 5 days of incubation, the killing rate of schistosomula rose to 48 % and 2 %, respectively. After 48 h of incubation, all dead schistosomula were covered with 5 or more cells (Fig. 2-b), while alive schistosomula were divided into four types ; 1) bearing no or only a few cells, 2) bearing 5 or more cells and severely damaged, 3) bearing many cells and moving vigorously, and 4) bearing a capsule-like substance that was attached with several cells (Fig. 2-c).

Effect of serum concentration and various the target-to-cell ratios on adherence and cytotoxicity of eosinophils. Based on the above data, cell-adherence and cytotoxicity were examined at the same time after 48 h of incubation in further experiments. A 1 : 100 dilution of patient's serum (target-to-cell ratio was 1 : 1,000) resulted in a cell adherence and schistosomula killing pattern that was similar to the 1 : 10 dilution. At a 1 : 1,000 dilution, only 24 % of the organisms had 5 or more cells attached to their surface, further, the number of organism covered with 10 or more cells and the killing rate of schistosomula were only at the control (control serum and eosinophils) level. Cell adherence patterns did not differ among three cultures with target-to-cell ratios of 1 : 5,000, 1 : 1,000, and 1 : 100 (serum dilution was 1 :

Table 1 Cell-adherence to and killing of schistosomula of *Schistosoma japonicum* in various cultures

Dilution of serum	Target-to-cell ratio	% Schistosomula*		%Dead schistosomula*
		≧5 cells	≧10 cells	
Patient's serum				
1 : 10	Eos† 1 : 1,000	86.3± 5.9	48.8± 9.3	16.1±2.3‖
1 : 100	Eos 1 : 1,000	61.3± 7.0	34.9± 2.9	11.0±1.8‖
1 : 1,000	Eos 1 : 1,000	24.0± 8.3	2.0± 1.2	0±0
1 : 10	Eos 1 : 5,000	77.0±17.0	66.0±22.0	25.0±3.0‖
1 : 10	Eos 1 : 100	73.9± 4.6	46.4± 3.9	4.2±0.2‖
1 : 10	Eos 1 : 10	19.0±12.5	1.9± 1.2	1.3±1.3
1 : 10	Neu‡1 : 1,000	4.2± 2.0	2.0± 2.0	2.7±1.8
1 : 10	Neu 1 : 5,000	4.0± 1.0	0±0	2.0±2.0
1 : 10	Nil			0±0
1 : 10	Nil +C′§			0±0
1 : 10	Eos 1 : 1,000 +C′§	78.3± 7.3	49.8±11.2	10.3±1.2‖
Control serum				
1 : 10	Eos 1 : 1,000	5.3± 0.7	1.4± 0.7	0±0
Nil	Eos 1 : 1,000	1.7± 1.7	0± 0	0±0

* Examined after 48 h incubation. † Eosinophils. ‡ Neutrophils.
§ Fresh autologous serum (20%). ‖ $p<0.005$

Table 2 Adult worm recovery and mortality of mice inoculated with cultured schistosomula

Cultured with	Exp. 1 (4 animals)			Exp. 2 (4 animals)		
	% Dead schistosomula *in vitro*	% Worm recovery	% Mortality of mice	% Dead schistosomula *in vitro*	% Worm recovery	% Mortality of mice
Patient's serum +eosinophils	8.1±3.1	26.3± 3.2*	0	13.3± 4.3	8.0±3.0*	0
Control serum + eosinophils	0 ± 0	53.0± 5.0	50	0 ± 0	32.7±4.2	50
Patient's serum	1.0±1.0	49.5± 2.7	75	not done		

* $p<0.005$

10). A target-to-cell ratio of 1 : 5,000 produced 25% mortality, whereas only 4.2% of schisto somula were killed at the ratio of 1 : 100, but this data was still statistically significant ($p<0.005$). The presence of complement did not alter the cell adherence pattern nor the observed death rate of schistosomula. Neutrophils detached from worms by 48 h, so that neutrophil-mediated killing of schistosomula was not observed (Table 1).

Infectivity of cultured schistosomula. Adult worm recovery after infection of cultured schistosomula to normal mice showed the same results as those obtained in an *in vitro* assay of killing (Table 2). A significant loss of recovery ($p<0.005$) and the mortality of mice were observed in the group co-cultured with patient's serum and eosinophils compared with that cultured with control serum and eosinophils or with patient's serum only.

Effect of absorption of patient's serum with either anti-μ or anti-γ serum. The immune adherence and cytotoxicity to schistosomula mediated by eosinophils fell to the control level with IgG-depleted patient's serum, while the capacity for killing was not

Table 3 Adherence and cytotoxicity of eosinophils together with absorbed patient's serum

Absorbed with	% Schistosomula with≧5 cells	% Dead schistosomula
Normal goat serum	44.0±9.0	9.0±1.5
Anti-γ-chain	2.3±1.5	0±0*
Anti-μ-chain	55.3±7.6	7.8±1.0

* $p<0.005$

depressed by the absorption of patient's serum with anti-μ serum (Table 3).

Discussion

This study was designed to investigate immune adherence of human eosinophils to schistosomula of *S. japonicum in vitro* and killing of the organisms. Li Hsü *et al.* (1977) reported that immune serum, obtained from rhesus monkey immunized with X-irradiated cercariae of *S. japonicum* then challenged with intact cercariae,mediated cytotoxic effects of not only eosinophils but also neutrophils and lymphocytes. The serum had a high activity to kill about 50 % of schistosomula *in vitro* even after heat-inactivation. With this immune serum, eosinophils killed more than 90 % of schistosomula at day 1. In our system, on the contrary, the serum was obtained from patients who developed a natural infection of *S. japonicum*, therefore the killing activity of the serum was much lower than in the immunized monkey system so that the serum, even with complement, killed no schistosomula after 48 h of incubation (Table 1). At a 1 : 100 dilution or higher concentrations of patient's serum, a significant eosinophil-adherence was observed. Also, at higher target-to-cell ratios of more than 1 : 100, a significnat number of organisms were covered with eosinophils (Table 1). Although the killing of schistosomula was statistically significant under the above conditions, the killing rates were only 4.2 to 25 % which were lower levels compared with the immunized monkey or *S. mansoni* system as reported previously (Vadas *et al.*, 1979).

With regard to low levels of cytotoxicity in our system, two points should be noted. First, the serum from patients might not be so highly immunized. Because of efforts to control *S. japonicum* infections, the incidence and severity of patients with schistosomiasis japonica in Leyte Island have decreased, especially in recent years (Tanaka *et al.*, 1982). Therefore it was difficult to obtain hyperimmune serum from patients with a natural course of infection. Furthermore, in our previous study in mice, it was found that serum collected from primary infected mice after 19 weeks of infection exhibited only a 11-12 % killing rate of schistosomula plus eosinophil-adherence. However, serum collected after 12 weeks of infection exhibited no killing but eosinophil-adherence only. (Kasuya and Ohtomo, 1982). It might be reasonable to assume that such a high level of killing antibody was not induced under conditions such as primary infections or natural infections. Secondly, some of the antigens of the Japanese strain and Philippine strain of *S. japonicum* might be different. Mitsuyama *et al.*(1983) established a hybridoma that secreted IgM antibody binding to schistosomula of the Japanese strain of *S. japonicum* which did not react with the Philippine strain. The cross-reactive cell-adherence and killing were less effective (Kassis *et al.*, 1979).

The decrease of infectivity of cultured schistosomula in mice clearly confirmed the *in vitro* assay of the cytotoxicity of eosinophils (Table 2). Moreover, the adherence and cytotoxicity of eosinophils were dependent on IgG class of antibody (Table 3). These data suggested that the IgG-dependent eosinophil-mediated killing of schistosomula played one of the important roles in defence mechanisms against *S. japonicum* as already reported in

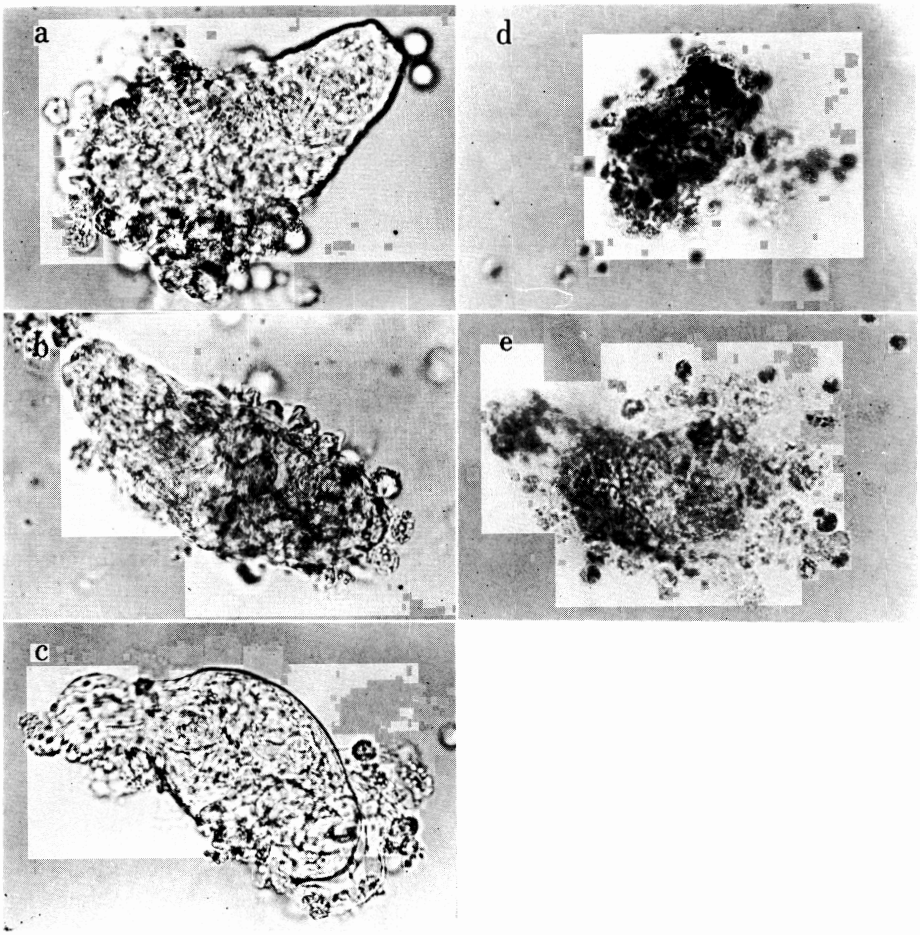

Fig. 2 Microscopical examination of schistosomula cultured with patient's serum and human eosinophils. a, at 3 h; b, at 48 h (dead); c, at 48 h (alive); d, at 6 h (Giemsa stained); e, at 48 h (Giemsa stained).

S. mansoni system. On the contrary, complement-dependent augumentation of cytotoxicity of eosinophils reported in the *S. mansoni* system (McLaren and Ramalho-Pinto, 1979) was not demonstrated in our experiments (Table 1). Neutrophil-mediated killing of schistosomula (Anwar *et al.*, 1979) was not also observed (Table 1). These results differed somewhat from those reported in *S. mansoni* system.

The escape mechanisms of the parasites from immune attacks of the hosts is another important problem. Schistosomula of *S. mansoni*, preincubated in medium with serum (Novato-Silva *et al.*, 1980) or without macromolecule (Dessein *et al.*, 1981) were not damaged by the ADCC mechanism *in vitro*. The parasite acquired host antigens on the surface *in vivo* (Goldring *et al.*, 1977). It seems likely that similar mechanisms exist in *S. japonicum* infections. In the present *in vitro* system, many schistosomula escaped from the ADCC mechanism. Certainly, one reason may be due to the activity of the antibody mentioned above, but schistosomula of *S. japonicum* may possess escape mechanism from ADCC. The capsule-like substance as shown in Fig. 2-c, whose origin is unknown now, was attached with many cells, and the schistosomula, that were not directly adhered with cells, moved vigorously. Maybe, type 1 alive schistosom-

ula described in results (bearing no or only a few cells) were produced in the next step. It is reasonable to hypothesize that schistosomula lost their membrane, against which the ADCC was directed, then acquired a new membrane which was less reactive to the antibodies.

Summary

The effects of serum from patients with schistosomiasis japonica together with human eosinophils and neutrophils on this schistosomula were examined *in vitro*. Adherence of eosinophils on schistosomula and a partial but statistically significant ($p<0.005$) killing rate (4.2-25.0 %) of the organisms were observed. Complement did not augument the adherence and cytotoxicity. Neutrophils adhered to schistosomula only in the early stages of incubation (3 h), then became detached in later stages, so that no neutrophil-mediated killing was observed. The decreased infectivity of co-cultured schistosomula to normal mice confirmed the *in vitro* examination of these killings. Adherence to and killing of the parasites were completely lost when the patient's serum was depleted with IgG.

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ヒト好酸球と日本住血吸虫 Schistosomula との相互作用： IgG 依存の細胞付着および殺虫効果

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日本住血吸虫患者血清の存在下で好酸球もしくは好中球の日本住血吸虫 schistosomula に対する影響を *in vitro* で検討した.

好酸球の schistosomula への付着および，部分的ながら有意 ($p<0.005$) の殺虫率 (4.2～25.0%) を認めた. この反応は補体によつて増強されなかつた. 好中球は培養初期（3時間）には schistosomula に付着したが，その後離脱し，好中球による殺虫効果は認められなかつた.

好酸球と培養した schistosomula をマウスに感染したところ，回収虫体の著しい減少を認めた。この結果は *in vitro* の殺虫効果の判定と一致した.

この細胞付着および殺虫効果は，患者血清より IgG を除去することにより完全に消失した.