Chemotactic and Leukocytosis-inducing Activities of Eel Complement

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(Received November 20, 1987)

Biological activities of eel complement activated through the alternative pathway, namely, chemotactic activity and leukocytosis-inducing activity, were investigated.

The normal eel sera were mixed with zymosan or formalin-killed cells of selected bacteria (*Escherichia coli*, *Edwardsiella tarda* or *Vibrio anguillarum*) and incubated at 25°C for 60 min. The sera were separated from zymosan or bacterial cells by centrifugation. And then, this treated sera were heated at 50°C for 30 min.

Chemotactic activity of the treated sera were determined by the modified Boyden chamber method. The distance of eel neutrophil migration toward the treated sera were significantly different from that toward the inactivated sera.

The peripheral neutrophils increased in number rapidly 6-9 hr after the intraperitoneal injection with the treated sera. There appeared to be the leukocytosis-inducing activity in the treated sera.

These biological activities of eel complement indicated in this study may play an important role in the non-specific defence mechanism of eel.

For the non-specific defence mechanism of mammals in the early stage of infection, it is thought that the serum components, for example, C-reactive protein, transferrin, interferon, lysozyme and complement, are very important. Complement is especially critical, because it possesses various biological activities by the activation through the alternative pathway (JOHNSTON, 1977). These activities include opsonization, bactericidal activity, chemotactic activity and leukocytosis-inducing activity. The non-specific defence mechanism of fish is thought to be similar to that of mammals (INGRAM, 1980).

In earlier papers, we reported that eel complement exhibited the bactericidal activity and opsonization through the alternative pathway (IIDA and WAKABAYASHI, 1983; MORITOMO *et al.*, 1988). The purpose of this study was to demonstrate the biological activities of eel complement, namely, chemotactic and leukocytosis-inducing activities, through the alternative pathway.

Materials and Methods

Sera and neutrophils

Eels, *Anguilla japonica*, weighing approximately 200 g were maintained in 100 l aquarium at 25°C. After acclimation for 5-7 days, the fish were bled from bulbus arteriosus. Sera were separated by centrifugation and pooled from three fish. A half of the sera was stored at -80°C until used, while the rest was inactivated by heating at 50°C for 30 min.

In order to isolate neutrophils for chemotactic activity test, blood was taken from bulbus arteriosus by means of heparinized syringe. The fish had been intraperitoneally injected with 2% solution of casein 12 hr before being bled, because NAGAMURA and WAKABAYASHI (1985b) reported that neutrophils of the fish intraperitoneally injected with the casein solution were activated in migration. The procedure for isolation of neutrophils from peripheral blood was followed that used by NAGAMURA and WAKABAYASHI (1985a). The isolated neutrophils were washed three times with Hanks' balanced salt solution and resuspend-

ed at a density of 10^5 – 10^6 cells/ml in Engle's MEM with the inactivated eel sera (final concentration 1%). The rate of neutrophils in the suspended cells was more than 60%.

Preparation of treated sera

A mixture of 1 ml of the normal sera and 25 mg of zymosan, which is known as an activator for the alternative complement pathway, or 10 mg of formalin-killed bacteria (*Escherichia coli* IAM 1239, *Edwardsiella tarda* FPC 381 or *Vibrio anguillarum* SG 7701) were incubated at 25°C for 60 min with occasionally shaking. The sera was separated from zymosan or bacterial cells by centrifugation. This treated sera was heated at 50°C for 30 min and stored in refrigerator.

In vitro chemotactic activity

In vitro migration of neutrophils toward the treated sera was measured by using a modified Boyden chamber as described by NAGAMURA and WAKABAYASHI (1985b). This consisted of two unattached chamber separated by a filter (3 μ m pore size, Millipore Filter Corporation, USA), with neutrophil suspension in the upper chamber and with the treated sera (final concentration 1% in Eagle's MEM) in the lower chamber. The migration distances of 10 cells of the farthest neutrophils in the filter were measured under a microscope.

The *in vitro* chemotactic activity test was done at 2 different bacterial densities. In the first experiment, the bacterial density in the normal sera was 10 mg/ml, while in the second experiment: 100 mg/ml.

Leukocytosis-inducing activity

A small amount of blood was sampled from caudal vessel every 3 hr for 24 hr after the intraperitoneal injection with the treated sera (0.2 ml/fish). The blood smears were air-dried and stained with May-Grünwald Giemsa and Periodic acid Schiff reagent. The number of neutrophils relative to 5000 erythrocytes was counted under a microscope.

Results

In vitro chemotactic activity

In the first experiment, the migration distance

Fig. 1. Migration distance of isolated eel neutrophils toward the following sera: A, incubated with zymosan; B, incubated with *Escherichia coli*; C, incubated with *Edwardsiella tarda*; D, incubated with *Vibrio anguillarum*; E, inactivated. Neutrophils used at each experiment were isolated from one fish.

of neutrophils toward the sera incubated with zymosan was 128 μ m, and those toward the sera incubated with *E. coli*, *E. tarda* or *V. anguillarum* were 127, 123 and 127 μ m, respectively. On the other hand, the migration distance of neutrophils toward the inactivated sera was 113 μ m. In the second experiment, the migration distance of neutrophils toward the sera incubated with zymosan, *E. coli*, *E. tarda* or *V. anguillarum* were 116, 125, 114 and 124 μ m, respectively, and that toward the inactivated sera was 105 μ m (Fig. 1). In the first and second experiments, the distance of neutrophil migration toward the sera incubated with zymosan or the bacterial cells were significantly different from that toward the inactivated sera by Mann-Whitney U test ($P < 0.01$). The treated sera had the chemotactic activity.

Leukocytosis-inducing activity

Peripheral neutrophils increased in number

generated by activating fish complement has been demonstrated (GRIFFIN, 1984). SUZUKI (1986) indicated that chemotactic response of eel neutrophils was induced by the reaction of the blood plasma with zymosan, Sephadex and inulin, which are activators for the alternative complement pathway. In the present study, we also showed that eel neutrophils migrated directionally toward the sera incubated with zymosan or bacterial cells. This result suggested that eel complement has C5a-like substance.

In mammals, C3e derived from C3c fragment of C3 has the ability to evoke leukocytosis, and C3e differs from C3a and C5a in several respects (GHEBREHIWET and MÜLLER-EBERHARD, 1979). Moreover, FEARON and WONG (1983) indicated that the leukocyte which had a receptor against C3e was neutrophil. Most of Japanese eel granulocytes seemed to be neutrophil (HOSHINA, 1962), and the tendency of change with time in the number of peripheral neutrophils in the present study was similar to that in mammals reported by GHEBREHIWET and MÜLLER-EBERHARD (1979). This facts implied that there was C3e-like activity

NAKANO *et al.* (1981) purified C5 fragment from fish complement. Their report supports that fish complement might possess chemotactic ability similar to mammalian C5a. In fact, chemotaxis

in the activated eel sera. After bacteria infect eel organ, peripheral neutrophils increases in number (NAGAMURA and WAKABAYASHI, 1983). The C3e-like substance is thought to be partly related to this phenomenon.

As mentioned above, when eel complement is activated through the alternative pathway, the biological activities, that is, chemotactic activity and leukocytosis-inducing activity, are generated. These activities may play an important role in the non-specific defence mechanism of eel against bacterial infections.

MORITOMO *et al.* (1988) reported that opsonization of *E. tarda* with normal eel sera was not observed. But in this study, the sera incubated with *E. tarda* cells possessed the biological activities. From these results, it is suggested that although eel complement is activated by *E. tarda* cells, opsonin of eel complement can not adhere to *E. tarda* cell surface.

Acknowledgements

We express our sincere thanks to Dr Douglas P. ANDERSON, United States Fish and Wildlife Service, for reviewing the manuscript. This work was partly supported by a grant from the Ministry of Education, Science and Culture, grant No. 61480067.

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