Changes in Glycogen Content of Neutrophils in Eel, *Anguilla japonica* by Bacterial Infection

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Glycogen content of neutrophils in the eel which were injected with some extraneous materials such as formalin-killed cells, 2% solution of casein and physiological saline were determined and their locomotion and phagocytosis were also examined. The eel injected with bacteria had the highest content of neutrophil glycogen, ranged from 74.6 μg to 105.9 μg per 10^7 neutrophils. The values in those injected with 2% solution of casein and physiological saline were 60.1-66.9 μg and about 45 μg , respectively. In the locomotion of the neutrophils, the neutrophils from the eel injected with 2% solution of casein migrated best, and those from the eel injected with bacteria did in the next place. The bacterial phagocytosis of the neutrophils was examined *in vitro* in the whole blood. Glycogen-rich neutrophils showed the most remarkable phagocytosis, though the locomotion was not promoted.

Introduction

Neutrophils are believed to play an important role in defence against bacterial infections. In mammals neutrophils are a major phagocytic cells and appear rapidly at an inflammatory site. In addition it is known that neutrophils in mammal include a large amount of glycogen in their cytoplasm and their glycogen are the energy source for motility (KAKINUMA, 1976; WAGNER, 1947).

We described that the neutrophils in the peripheral blood of the eel injected with bacteria showed changes in PAS reaction and these PAS positive substances in the neutrophils were identified as glycogen by salivary digestion test (NAGAMURA and WAKABAYASHI, 1983).

In the present study, we investigated the changes in glycogen content in the neutrophils of the eel injected with various extraneous materials like bacteria, and examined how the glycogen is used as a source of energy by examining the locomotion and phagocytosis of the neutrophils.

Materials and Methods

Experimental Fish

Eel (*Anguilla japonica*, 150-250 g of body weight) cultured in the Fisheries Laboratory of Tokyo University was used.

Extraneous Materials

The extraneous materials used were formalin-killed cells of *Vibrio anguillarum* and of *Edwardsiella tarda*, and 2% solution of casein as well as physiological saline as a control.

Blood Samples

Blood samples were taken from the eel which were injected with formalin-killed cells of bacteria, 2% solution of casein and physiological saline before 12 hrs or 24 hrs. Most of the whole blood cells were used for neutrophil isolation and the rest were prepared for the blood smears, which were stained with May-Grunwald Giemsa (MGG) and Periodic-acid Schiff reaction (PAS).

Isolation of the Neutrophils

Neutrophils were separated from the peripheral blood by using Ficoll-Metrizoate solution (Fig. 1). One ml of undiluted blood were carefully loaded onto Ficoll-Metrizoate solution as the separation medium and the tube was centrifuged for 30 min at

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Fig. 1. Schematic representation of the discontinuous density Ficoll-metrizoate used for blood cell separation.

Fig. 2. Neutrophils separated by using a Ficoll-metrizoate solution.

Fig. 3. Membrane filter apparatus.

1500 rpm.

The whole blood cells were divided into three layers by centrifugation (Fig. 2). Fraction 1 contained thrombocytes and lymphocytes, Fraction 2

mainly neutrophils and Fraction 3 erythrocytes. The neutrophils in Fraction 2 were collected with pasteur pipette and their cells were washed three times with KRP.

Glycogen Content of Neutrophils

Glycogen content of neutrophils were determined by Montgomery's method.

Locomotion of the Neutrophils

Locomotion of the neutrophils were determined in vitro by a modification Boyden chamber method. Figure 3 showed the scheme of millipore filter apparatus to examine the locomotion of neutrophils. The upper chamber was filled with neutrophil suspension and the lower chamber was filled with the activated serum as a chemotactic substance. After preparation, this apparatus was incubated at 26°C for 1 hr.

After incubation, the millipore filter was removed from the apparatus and then stained with hematoxylin. The migration distance of 100 neutrophils was measured under a microscopy.

Phagocytosis of the Neutrophils

The bacterial phagocytosis of neutrophils were examined in vitro using the whole blood cells. The whole blood cells were incubated with the bacterial suspension of *E. tarda* and shaken at 26°C gently. After incubation, the blood smears were prepared at regular intervals and then stained with MGG and PAS.

The bacteria phagocytized by neutrophils were carefully observed microscopically.

Results

Glycogen Content of Neutrophils

The result of the determination of glycogen content of neutrophils are shown in Table 1. As shown in this table, the glycogen content of neutrophils was highest in the eel injected with bacteria, and it ranged from 74.6 μg to 105.9 μg per 10^7 neutrophils. In the eel injected with 2% solution of casein, it ranged from 60.1 μg to 66.9 μg . Neutrophils of the control fish injected with physiological saline had the lowest glycogen content of about 45 μg .

Locomotion of the Neutrophils

The migration distance of neutrophils in the eel

Table 1. Glycogen content of neutrophils of eel injected with various irritants

Irritant	Mean percentage positivity					Glycogen content ($\mu\text{g}/10^7$ cells) Phenol- H_2SO_4
	I	II	III	IV	V	
Physiological saline	0	87.5	10.0	2.5	0	44.6
	0.5	66.5	30.5	1.5	1.0	47.4
2% Casein* ¹	2.0	61.5	35.5	1.0	0	60.1
	0	60.0	39.0	1.0	0	66.8
	0.5	24.5	69.5	5.5	0	66.9
<i>V. anguillarum</i> * ²	0	25.0	58.5	16.0	0.5	74.6
	1.5	18.5	37.5	37.5	5.0	98.0
	0	9.0	35.5	52.0	3.5	88.3
	0	16.5	22.5	60.5	1.5	78.4
<i>E. tarda</i> * ²	0	26.0	47.5	26.5	0	77.5
	1.0	19.5	33.0	46.5	0	84.6
	0.5	6.0	22.5	67.5	3.5	103.3
	0	2.5	26.0	66.0	5.5	105.9

*¹ These fish were injected intraperitoneally with 0.1 ml of 2% solution of casein 12 hrs before bleeding.

*² These fish were injected intraperitoneally with 1 mg of formalin-killed cells of *V. anguillarum* and of *E. tarda* respectively 12 hrs before bleeding.

Fig. 4. Migration of neutrophils toward zymosan-activated serum. The neutrophils were obtained from eel which was injected with 2% solution of casein.

injected with formalin-killed bacteria, 2% solution of casein and physiological saline showed Fig. 4, Fig. 5 and Fig. 6, respectively. The neutrophils in the eel injected with 2% solution of casein showed a marked migration. In the fish injected with formalin-killed bacteria, the neutrophils migrated considerably. In the control fish injected with phys-

iological saline, in comparison with neutrophils in the eel injected with 2% solution of casein or formalin-killed bacteria, the migration distance of the neutrophils varied widely.

Phagocytosis of the Neutrophils

The bacterial phagocytosis were examined in

Fig. 5. Migration of neutrophils toward zymosan-activated serum. The neutrophils were obtained from eel which was injected with formalin-killed cells of *E. tarda*.

Fig. 6. Migration of neutrophils toward zymosan-activated serum. The neutrophils were obtained from eel which was injected with physiological saline.

vitro using the whole blood cells in the eel injected with formalin-killed cells of *E. coli*, with 2% solution of casein and with physiological saline. As shown in Table 2, after incubation for 30 min, the neutrophils in the eel injected with the formalin-killed cells of *E. coli* showed a marked phagocytosis and their phagocytic rate were 40% and 56%.

Whereas, the phagocytic rate of the neutrophils in the eel injected with physiological saline were 16% and 17%. In the case of 2% solution of casein, it was the lowest.

The phagocytosis of the neutrophils against bacteria are shown in Fig. 7. The neutrophil phagocytized bacteria actively 30 min after incubation

Fig. 7. Phagocytosis of the neutrophil. 1: After incubation for 30 min, MGG stain. 2: After incubation for 30 min, PAS reaction. 3: After incubation for 12 hr, MGG stain. 4: After incubation for 12 hr, PAS reaction.

Table 2. Phagocytic activity of leukocytes from eel against *E. tarda* at 26°C

Irritant	30 min		4 hrs	
	Phagocytic rate (%)	Phagocytic index	Phagocytic rate (%)	Phagocytic index
Physiological saline	17	0.43	97	9.47
	16	1.09	96	5.37
2% Casein* ¹	8	0.09	100	9.46
	1	0.01	99	9.60
<i>E. coli</i> * ²	40	1.32	89	4.95
	56	2.40	94	6.70

*¹ These fish were injected intraperitoneally with 0.1 ml of 2% solution of casein 12 hrs before bleeding.

*² These fish were injected intraperitoneally with 1 mg of formalin-killed cells of *E. coli* 12 hrs before bleeding.

(Fig. 7-1) and the cytoplasm of their neutrophils were stained well with PAS (Fig. 7-2). After 12 hr incubation, their neutrophils had vacuoles, where the phagocytized bacteria were observed and they became swollen, the PAS reaction of neutrophils phagocytizing bacteria became less prominent gradually (Fig. 7-3, 4).

Discussion

It is known that neutrophils in mammals contain a large amount of glycogen in their cytoplasm (KAKINUMA, 1976; WAGNER, 1947).

WAGNER (1947) reported that glycogen content of the white blood cells was 4.23 μg per 10^6 cells. In the control fish of eel, Glycogen content of neutrophils was about 45 μg per 10^7 cells, its value did not show clear difference in comparison with those of mammals. However, it is an interesting fact that the neutrophils in the eel injected with the bacterial cells have considerably larger amount of glycogen in cytoplasm than those in the eel injected with 2% solution of casein or physiological saline.

In locomotion of the neutrophils, the neutrophils in the eel injected with 2% solution of casein tend to migrate best and in the next place those of the eel injected with bacteria, followed by the control. In spite of the neutrophils in the eel injected with bacteria had a large amount of glycogen, they migrated less actively than those from the eel in-

jected with 2% solution of casein. Therefore, it is speculated that the locomotion of neutrophils does not necessarily depend on a large amount of glycogen in their cells. Whereas, in phagocytosis of the neutrophils, the neutrophils in the eel injected with the formalin-killed cells of *E. coli* showed a marked phagocytosis after incubation for 30 min. From those fact, it is probable that the glycogen is used as an energy source for phagocytosis rather than for their locomotion. And it is to be noted that the phagocytosis is likely to be taken place non-specifically, from the fact that the neutrophils in the eel injected with *E. coli* responded quickly for the administration of *E. tarda*.

Thus, the neutrophils play a major role in primary response against bacterial infections.

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