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Comparative Studies on the Internal Defense System of Schistosome-Resistant and -Susceptible Amphibious Snail *Oncomelania nosophora*
1. Comparative Morphological and Functional Studies on Hemocytes from Both Snails

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ABSTRACT—Two morphologically distinct blood cell types (hemocytes), Type I and Type II were found coexisting in hemolymph from two kinds of snails, *Oncomelania nosophora* strain, viz. from the Nirasaki strain (schistosome-resistant snail) and the Kisarazu strain (schistosome-susceptible snail). Ten min after inoculation of SRBC, the majority of Type I cells from Nirasaki strain flattened and spread over the surface of the glass plate by extending pseudopodia. In the Kisarazu strain, Type I cells adhered to the surface of substrate with spike-like filopodia, but did not form spreading lamellipodia. Type I cell from the Nirasaki strain phagocytosed SRBC but that from the Kisarazu strain did not. The starting time of recognition of foreign materials was slightly different in the Type I hemocytes from the two strains. Type II cells from both strains were round and lymphocyte-like. Ten or sixty min after incubation, Type II cells from neither strain adhered to the surface of substrate or SRBC, and did not phagocytose SRBC. Type II cells from the Nirasaki strain were quite similar to those from the Kisarazu strain. We concluded that Type I cells from the schistosome-resistant snail, Nirasaki strain, possessed higher phagocytic activity than those from the susceptible snail, Kisarazu strain, despite the morphological similarities of the hemocytes from both strains.

Key words: *Oncomelania nosophora*, hemocyte, ultrastructure, adherence, phagocytosis

INTRODUCTION

The immune system of invertebrates lacks inducible immunoglobulin. Instead, lectins are believed to play the role of recognition molecules. Like in vertebrates, in most invertebrates, invasion of their bodies by foreign materials elicits a cellular response. In particular, molluscs possess a very effective immune system which can dispose of a variety of foreign particles and organisms. In the terrestrial slug, macrophages (hemocytes) can recognize and phagocytose biotic and abiotic non-self materials (Furuta *et al*., 1987; Yamaguchi *et al*., 1988; Furuta *et al*., 1990), which are agglutinated and opsonized by body surface mucus (Furuta *et al*; 1995; Yuasa *et al*., 1998). Moreover, studies on freshwater snails and terrestrial slugs revealed that they could recognize and reject allografts by encapsulation (Jourdane and Cheng, 1987; Yamaguchi *et al*., 1999). In view of these facts, the mobilization of defense mechanisms of these molluscs against incompatible trematodes parasites may be considered to be a normal reaction, since comparatively, these parasites are more non-self than allografts. However, the parasites usually are not destroyed by the internal defense system of certain molluscs and can use their host as a place for nutrition, growth and proliferation.

The subtleties of the intermediate host-parasite relationship, although recognized, are not fully understood, but evidently should be viewed in terms of compatibility or incompatibility (Arfaa *et al*., 1989; Joubert *et al*., 1991; Anger and Granath, 1995).

The amphibious snail, *Oncomelania nosophora,* is an obligate intermediate host for the human blood fluke, *Schis-*

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tosoma japonicum. Recently, we found that in a host-parasite relationship there were strains of the snail, *O. nosophora* that were schistosome-resistant and -susceptible snails to *S. japonicum* originating from Mindoro Island, Philippines (Kirinoki *et al.*, 2000). Resistant snails were derived from marshes near Nirasaki city and susceptible ones from marshes near Kisarazu city. In resistant snails, the parasite is killed by macrophage-like hemocytes after encapsulation by the hemocytes. The strain of *O. nosophora* susceptible to *S. japonicum* is capable of encapsulating schistosomes, but within one or two days the capsule disappears and normal development of the parasite follows (Kirinoki *et al.*, 2000).

The objective of this study is to identify whether strain specific cellular factors in hemolymph of both strains of *O. nosophora* differ in recognition, adherence and phagocytosis of non-self materials by their hemocytes.

MATERIALS AND METHODS

Animals

Amphibious snails, *Oncomelania nosophora* which were collected in Nirasaki (Yamanashi Prefecture) and Kisarazu (Chiba Prefecture) were used in the experiments. Nirasaki is about 200 km distant from Kisarazu. The water system of habitats in Nirasaki is not connected with that in Kisarazu. Therefore both snails from Nirasaki and Kisarazu are considered geographic strains. Snails from Nirasaki or Kisarazu are hereafter abbreviated Nirasaki strain or Kisarazu strain. We used 150 snails from each strain for the experiments. The snails were maintained on a wet filter paper placed in Petri dishes. The dishes were covered with a stainless steel mesh and placed in a cabinet supplying fresh humidified air at 25–27°C. The snails were kept for one month or more to adapt to this condition. At the time of the experiments, adult snails of both strains were 6 to 7.5 mm in shell length and 2 to 3 mm in shell diameter.

Collection of hemolymph

The snails were maintained on a wet filter paper containing antibiotics (200U/ml penicillin and 200 μg /ml streptomycin; Bio Whittaker, Inc. Walkersville, USA) at 25°C for a few days. Hemolymph collected by gently crushing a shell was dropped on a square glass plate (10×10mm Matsunami Tokyo, Japan) and then an equal volume of saline-antibiotic (SA) solution was added to each glass. SA solution consisted of 1.16M NaCl, 0.15M Na_2HPO_4, 0.15M KH_2PO_4, 100U/ml penicillin and 100 μg/ml streptomycin, and was adjusted to pH 7.2.

Cell adherence experiments

Fresh hemolymph was dropped on the square glass plate and then mixed with equal volumes of SA solution. These preparations were left horizontally in a moist atmosphere at 25°C for 10 or 60 min. After incubation, the preparation was roughly rinsed with SA solution and then hemolymph cells (hemocytes) adhering to the plate were fixed and stained with May-Grunwald-Giemsa. The cells were observed by light microscopy.

Non-self recognition by hemocytes

Sheep red blood cells (SRBC) fixed 10% formalin (Nippon Bio-Test Lab. Inc. Tokyo, Japan) were used as foreign materials. SRBC were washed five times with PBS and resuspended at a concentration of 1% with PBS. 1% SRBC and SA solution were dropped on the glass plate (10×10mm) which was placed in a plastic dish. Fresh hemolymph equal in volume to the SA solution was added to the plate and left horizontally in a moist atmosphere at 25°C for 60 min. The ratio of SRBC to hemocytes was approximately 10:1. After incubation, they were stained with Giemsa. The adherence rate (%) was calculated by the ratio of SRBC-adhering hemocyte numbers to the total hemocyte number. The adherence rate was analyzed using Student's *t* test. A value of *p* less than 0.05 was considered to indicate a statistically significant difference.

Phagocytosis of SRBC by hemocytes

A hole was bored in the shell of the snail with a drill (0.8 mm in diameter). About 5 μl suspensions of 1% SRBC were injected into the hemocoel of the snail through the hole using a micropipet. Each inoculated snail was placed in a Petri dish and maintained on a wet filter paper at 25°C. Twenty hours after inoculation of SRBC, the hemolymph was collected by gently crushing a shell, and then the hemolymph was dropped on the plastic dishes and SA solution equal in volume to the hemolymph was added. The dishes were kept horizontally at 25°C for 60 min.

Transmission electron microscopy

The plastic dishes were rinsed carefully with PBS and the hemocytes on the dishes were fixed gently with 0.1% glutaraldehyde in SA solution for 20 hr at 25°C. They were post-fixed with 1%

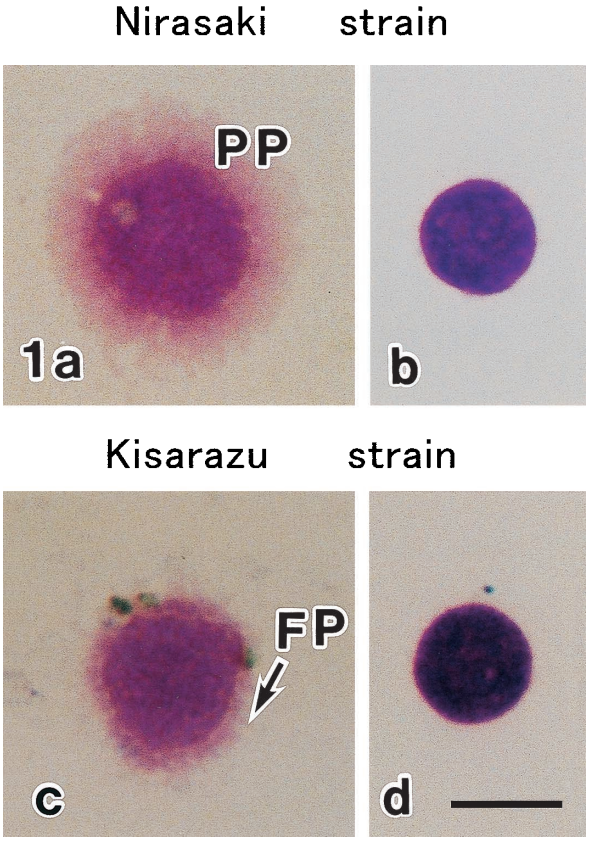

Fig. 1. a, b, c, d. Light micrograph of Type I cell (a, c) and Type II cell (b, d) stained with May-Grunwald-Giemsa, 10 min after incubation. (a) Type I cell (macrophage-like cell) from Nirasaki strain flattens and spreads over the surface of the substrate by extending pseudopodia (PP). (b) Type II cell (lymphocyte-like cell) with deeply-stained nucleus from the Nirasaki strain. (c) Type I cell (macrophage-like cell) from the Kisarazu strain adhere to the substrate with spike-like filopodia (FP) but do not form spreading lamellipodia. (d) Type II cell (lymphocyte-like cell) from the Kisarazu strain is quite similar to that of the Nirasaki strain. Bar=5 μm

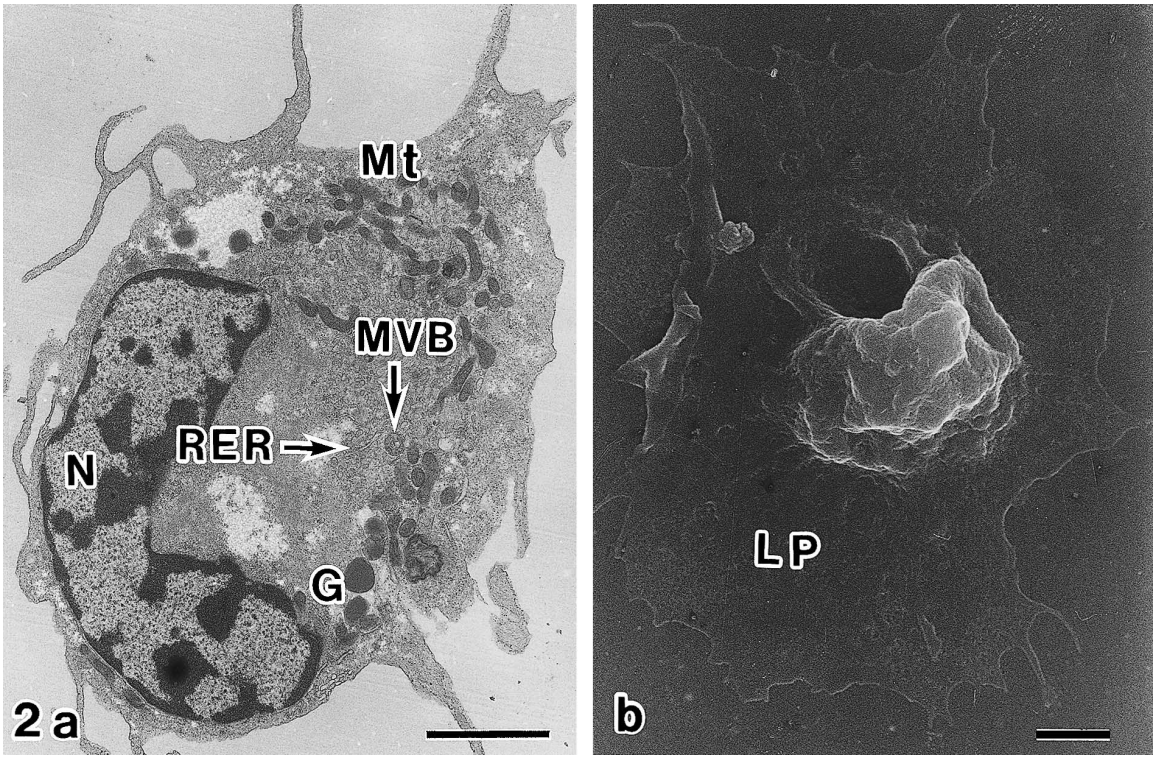

Fig. 2a, b. Type I cell (macrophage-like cell) from the Nirasaki strain, 60 min after incubation. (a) The spreading cell contains a kidney-shaped nucleus (N), mitochondria (Mt), rough endoplasmic reticulum (RER), multivesicular body (MVB) and fine granules (G) which appeared to be lysosomes. (b) SEM micrograph of Type I cell becoming flatter and broader by extension of lamellipodia (LP). Bar=2 μm

OsO_4 in PBS for 60 min. They were dehydrated, and then embedded in Epon 812. The blocks were stripped from the plastic dishes by heating with a gas burner. The specimens, the cells attached to the dish, were horizontally sectioned on a Super NOVA ultramicrotome. Ultrathin sections were stained with uranyl acetate and lead citrate, and examined with a JEM-1210 (JEOL, Tokyo) transmission electron microscope at 80 kV.

Scanning electron microscopy

As mentioned above 1% SRBC, SA solution and the hemolymph were dropped on several round glass plates (13mm in diameter, Oken, Tokyo, Japan), and left horizontally in a moist atmosphere at 25°C for 60 min to allow the cells to adhere to the glass plate. After incubation, the glass plates were processed through a series of scanning electron microscopy procedures, including fixation (0.1% glutaraldehyde in SA solution at 25°C for 20 hr), dehydration, critical point drying and coating with Au. They were examined with a Hitachi S-4700 scanning electron microscope at 15 kV.

RESULTS

Morphology of hemocytes

Hemocytes of Nirasaki strain

Two morphologically distinct blood cell types (hemocytes), Type I and II were found in hemolymph from the Nirasaki strain. Type I cells which spread over the substrate by pseudopodia were macrophage-like and Type II cells which possessed a large round nucleus and slightly cytoplasm were lymphocyte-like. Type II cells were less than Type I cells, the proportion of Type II cells to Type I cells

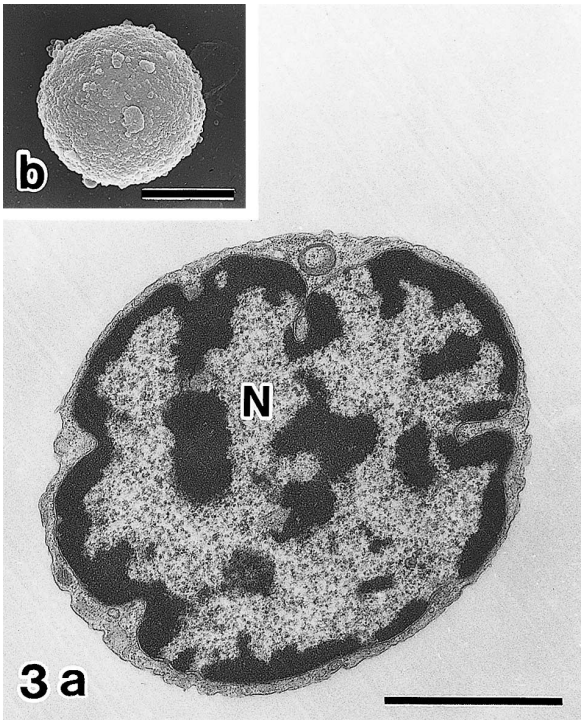

Fig. 3a, b. Type II cell (lymphocyte-like cell) from Nirasaki strain, 60 min after incubation. (a) TEM micrograph of Type II cell showing large round nucleus (N), free ribosomes and scanty cytoplasm. (b) SEM micrograph of a round Type II cell. Type II cell from Kisarazu strain was quite similar to that of the Nirasaki strain. Bar=2 μm

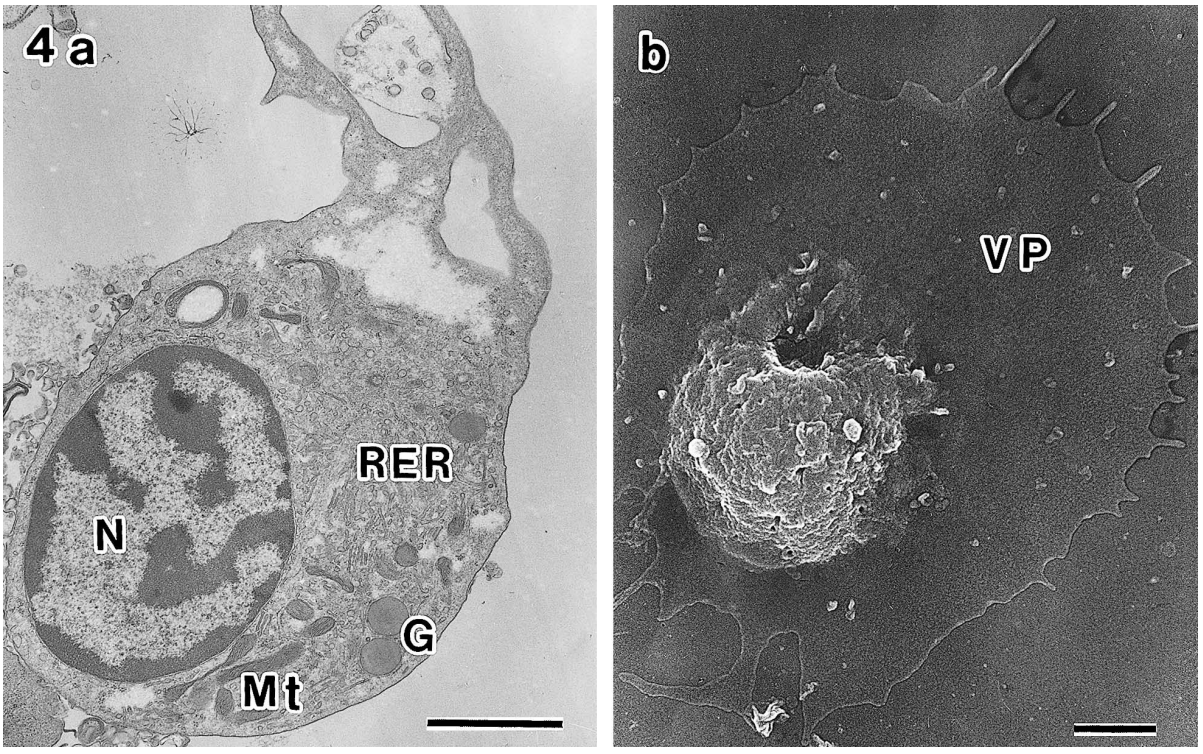

Fig. 4a, b. Type I cell from Kisarazu strain, 60 min after incubation. (a) The cell contains a round nucleus (N), mitochondria (Mt), rough endoplasmic reticulum (RER) and granules (G). (b) SEM micrograph of Type I cell. The cell possesses veil-like pseudopodia (VP). Bar=2 μm.

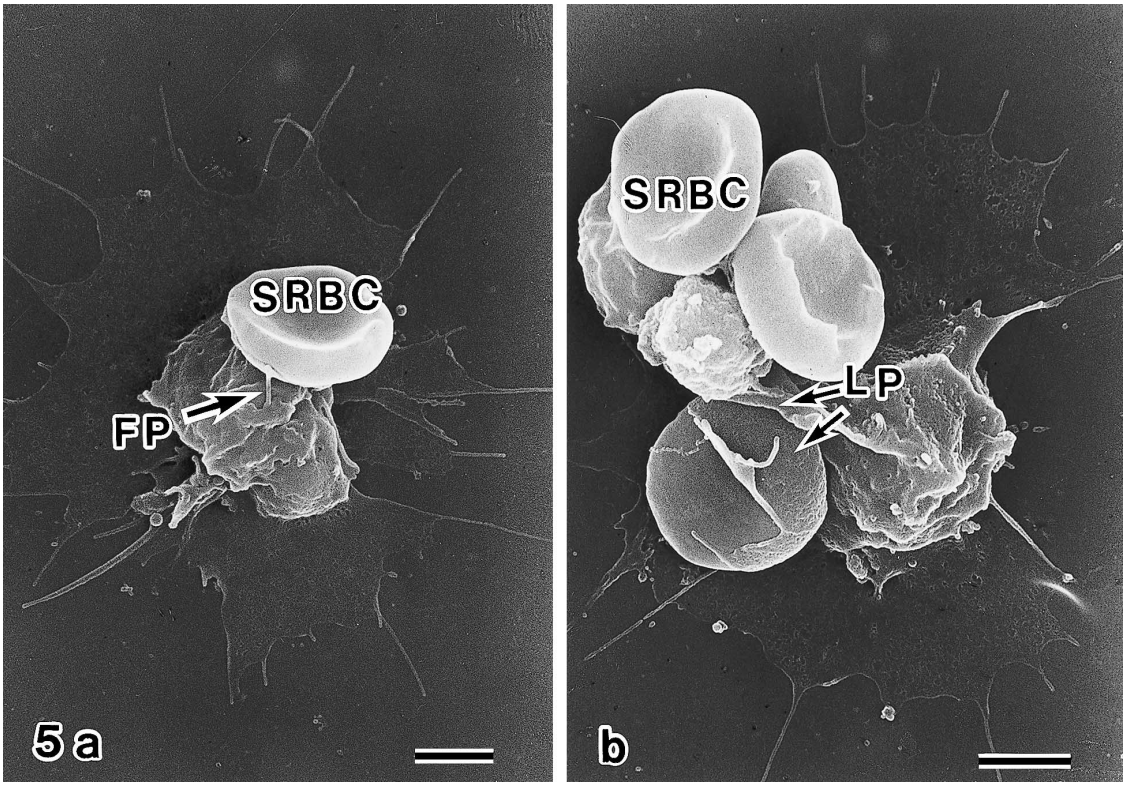

Fig. 5a, b. SEM micrograph of adherence to SRBC of Type I cell from the Nirasaki strain, 60 min after incubation. (a) The filopodia (FP) elongate and adhere to SRBC. (b) The lamellipodia (LP) elongate and adhere to SRBC. Bar=2 μm

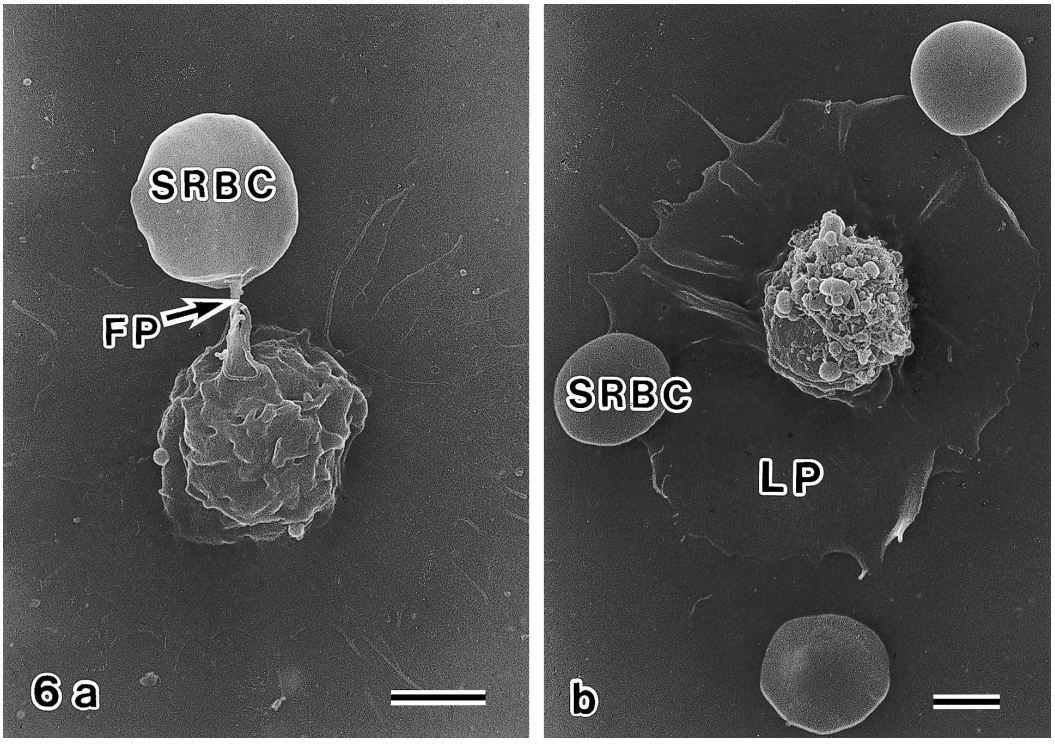

Fig. 6a, b. SEM micrograph of Type I cell from the Kisarazu strain, 60 min after incubation. (a) The cell slightly adheres to SRBC by filopodia (FP). (b) Type I cell cannot adhere to SRBC. LP; lamellipodia, Bar=2 μm

being about 1:7. Ten min after incubation, the majority of Type I cells flattened and spread over the surface of the glass plate by extending pseudopodia (Fig. 1a). Sixty min after incubation, the cells became flatter and broader by lamellipodia with supporting ribs and measured 14±3 μm in diameter. The shape of the nuclei of spreading Type I cells varied considerably, from round or oval to kidney-shaped and lobulated. The cytoplasm contained mitochondria, rough endoplasmic reticulum, multivesicular body and fine granules which appeared to be lysosomes (Fig. 2 a, b). Type II cells (lymphocyte-like) were usually spherical or ovoid (5 μm in diameter) and the scanty cytoplasm was devoid of elaborate organelles, but contained small amounts of mitochondria and free ribosomes surrounding a large round nucleus (Fig. 3 a, b). The cells adhered slightly to the substrate surface and formed only slight amounts of pseudopodia, if at all. In blood smears, the nucleus was large round and was deeply stained with routine method (Fig.1b). Moreover, the nucleus to cytoplasm ratio was high.

Hemocytes of Kisarazu strain

Two categories of hemocytes were also recognized in the Kisarazu strain: (1) Type I; macrophage-like cell, (2) Type II; lymphocyte-like cell (Fig. 1 c, d). The proportion of Type II cells to Type I cells was about 1:7. Ten min after incubation, Type I cells adhered to the surface of substrate with spike-like filopodia, but did not form spreading lamellipodia (Fig. 1 c). Sixty min after incubation, the cell possessed a veil-like pseudopodia (Fig. 4 a, b), measuring 12±3 μm in diameter. It contained a nucleus, mitochondria, rough endoplasmic reticulum and granules (Fig. 4 a). Type II cells were about 5 μm in diameter with a high nucleus to cytoplasm ratio. Sixty min after incubation, Type II cells from the Kisarazu strain were quite similar to those of the Nirasaki strain in morphology and size.

Function of hemocytes

In gastropod molluscs, at least one kind of hemocyte is highly phagocytic and phagocytosis is a major line of

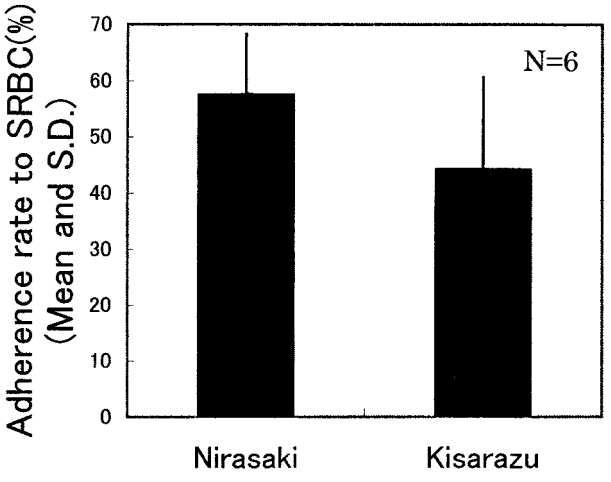

Fig. 7. Adherence to SRBC by Type I cell of the Nirasaki strain and Kisarazu strain. Significantly more SRBC were adhered to by hemocytes of the Nirasaki strain (*t*- test. $p < 0.05$). N refers to the number of replicates.

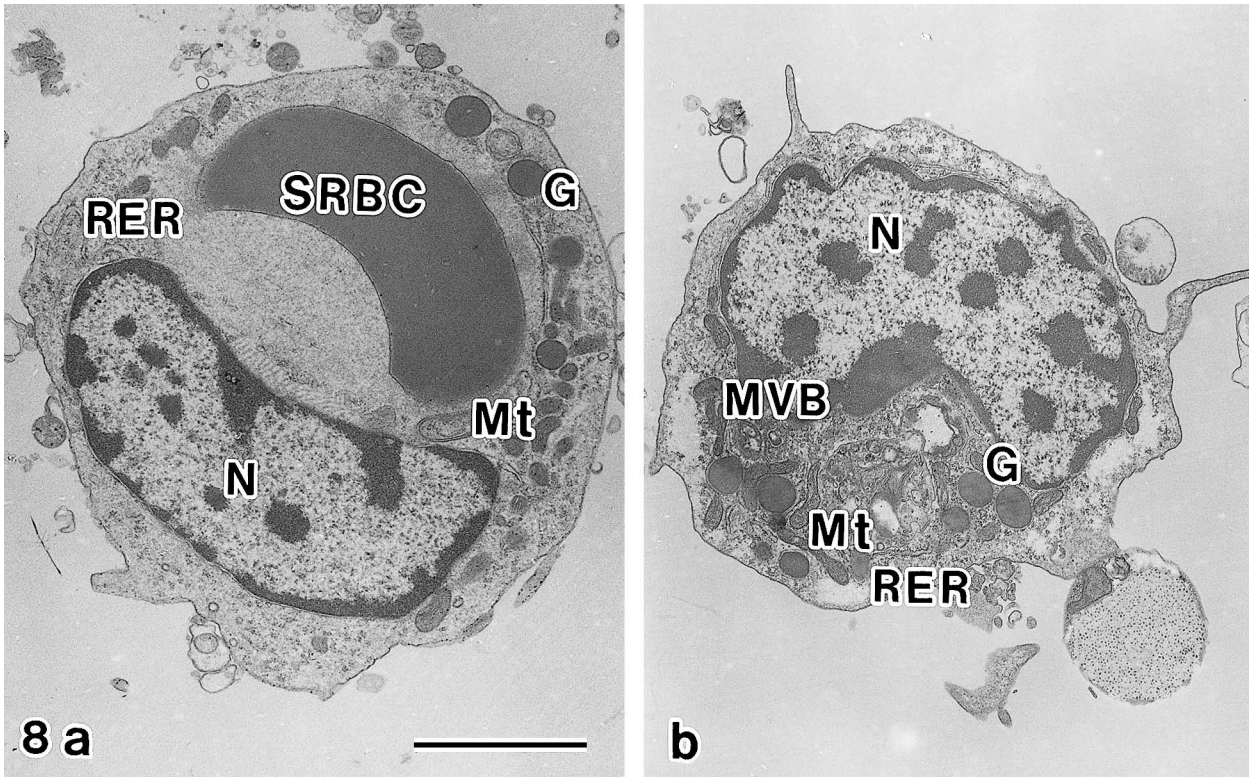

Fig. 8a, b. (a) Type I cell from Nirasaki strain phagocytoses SRBC, 24 hr after inoculation of SRBC. (b) Type I cell from Kisarazu strain does not phagocytose them, 24 hr after inoculation. N; nucleus, Mt; mitochondria, RER; rough endoplasmic reticulum, G; granule, MVB; multivesicular body, Bar=2 μm

defense against invading microorganisms. The initial steps in phagocytosis are first the recognition of foreign particles and then subsequent adhesion.

Rate of adherence to SRBC

Sixty min after inoculation of SRBC, many SRBC surrounded the majority of Type I cells from the both strains, but did not surrounded Type II cells. However, this phenomenon did not appear to be in a state of rosette formation because SRBC were freely distributed with no definite pattern. Nirasaki strain Type I cells could adhere to SRBC, but not Type II cells. The lamellipodia or philopodia of Type I cells from Nirasaki strain elongated to and adhered to SRBC (Fig. 5a, b). However, philopodia of both Type I and Type II cells from Kisarazu strain could not, or only slightly, elongate to SRBC even at 60 min after inoculation (Fig. 6a, b). The rate of adherence to SRBC of the Nirasaki strain was significantly higher than that of Kisarazu strain ($p<0.05$) (Fig. 7).

Phagocytosis

Type I Nirasaki strain cells phagocytosed SRBC, 24 hr after inoculation of SRBC and SRBC were enclosed in phagosomes. But at 24 hr after inoculation of SRBC, Type I cells from Kisarazu strain did not phagocytose them (Fig. 8 a, b). Type II cells from neither strain could phagocytose SRBC.

DISCUSSION

The present study on the ultrastructure and function of hemocytes from two kinds of *Oncomelania nosophora* strains, viz. from the Kisarazu strain (schistosome-susceptible snail) and from the Nirasaki strain (schistosome-resistant snail) is the first report on this subject. In previous experiments, we found that *Oncomelania nosophora* which is an obligate intermediate host for the human blood fluke, *Schistosoma japonicum* was divided into two strains; a resistant strain and a susceptible strain for schistosome (Kirinoki *et al*., 2000). The resistant snails originated from marshes near Nirasaki and the susceptible ones were from marshes near Kisarazu. To clarify the differences between these two snail strains, we first demonstrated the classification and function of blood cells (hemocytes) in the two kinds of the snail. Our observations revealed that the amphibious snail, *Oncomelania nosophora* possessed two types of hemocytes, i.e. Type I cell (macrophage-like) and Type II cell (lymphocyte-like) in the circulating hemolymph. Type I cells were approximately 13 μm in diameter, when allowed to spread on a glass substrate. Type II cells were round and measured approximately 5 μm in diameter. The cells did not adhere to the surface of the substrate. Type I cells of Nirasaki strain were capable of recognizing and ingesting foreign materials. On comparing observations (LM, TEM and SEM) of Type I cells of both strains, morphological dif-

ferences between them were not significant. However, there were some differences in the adherence rate to SRBC.

In gastropods, numerous studies have been conducted on hemocytes (Cheng et al. 1969; Sminia 1972, 1980; Yoshino 1976; Van der Knaap 1981; Furuta et al., 1986, 1987, 1990). The number of cell types in the circulating hemolymph varies among different groups of molluscs. According to Jeong and Heyneman (1976), in the freshwater snail, *Biomphalaria glabrata,* the most common cell is a granulocyte which possesses a strong phagocytic response toward a variety of particles, in the virtual absence of snail hemolymph. In addition to these reports, Sminia and Barendsen (1980) concluded that only one type of hemocyte, the amoebocyte, existed in the freshwater snails, *Lymnaea stagnalis*, *Biomphalaria glabrata* and *Bulinus truncatus* and the amoebocytes constituted a morphologically and functionally heterogeneous population of cells, ranging from round cells with the morphological characteristics of young cells to highly phagocytic spreading cells with a prominent lysosomal system. Our observation about hemocyte-type and behavior agree to the report of Sminia and Barendsen (1980). Type II cell morphologically is an immature cell-type, thus we also consider that the cell is a young type of phagocytic spreading cell type, because we have observed in preliminary experiments when the snails are treated with large amount of SRBC, Type II cells gradually decrease in number in hemolymph (data not shown). This phenomenon indicates that Type II cells (round cells) are young cells which still have the capacity to develop and change into Type I cells which are active in phagocytosis. *Biomphalaria glabrata* can be classified into two strains; a resistant strain and a susceptible strain for *Schistosoma mansoni* infection, and hemocytes of schistosome-resistant and susceptible *Biomphalaria glabrata* can recognize different antigens on the surface of the parasites (Anger and Granath, 1995).

In our observation, Type I cells from Nirasaki strain possessed high phagocytic activity. Though the cells from Kisarazu strain could recognize foreign materials, they did not phagocytose them. This fact may show the first step of the difference between schistosome-resistant and schistosome-susceptible snails. When a schistosome penetrates a potential intermediate host-snail, the initial hemocyte response probably involves a directed movement towards the parasite, although there is no definitive evidence for this. Once hemocytes are recruited into the vicinity of the parasite, the next critical step is to establish contact with the parasites tegument. In host-parasite combinations, in the case of susceptible snail, although hemocytes are found adjacent to the parasite, they fail to make such contact. If a parasite is encapsulated, within several days the capsule disperses and normal development of the parasite follows. In the case of resistant snail, killing of the parasite is the result of an encapsulation response by macrophage-like hemocytes.

Thus, besides the cellular factor, probably unique recognition factors are present in resistant hemolymph. We are therefore now investigating humoral factor(s).

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