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Authors: Kaneko, Aya, Uyama, Taro, Moriyama, Yoshinori, and Michibata, Hitoshi

Source: Zoological Science, 12(6) : 733-739

Published By: Zoological Society of Japan

URL: https://doi.org/10.2108/zsj.12.733

ZOOLOGICAL SCIENCE 12: 733–739 (1995)

Localization, with Monoclonal Antibodies and by Detection of Autonomous Fluorescence, of Blood Cells in the Tissues of the Vanadium-Rich Ascidian, *Ascidia sydneiensis samea*

AYA KANEKO, TARO UYAMA, YOSHINORI MORIYAMA and HITOSHI MICHIBATA*

Mukaishima Marine Biological Laboratory, Faculty of Science and Laboratory of Marine Molecular Biology, Graduate School of Science, Hiroshima University, Mukaishima-cho, Hiroshima 722, Japan

ABSTRACT—Ascidians have several types of blood cells which have been reported to be involved in various functions. However, it is still unclear what type of blood cell participates in each of various functions and in which tissue each type of blood cell localize in ascidians. As a necessary step to make clear the important and intractable problems, the localization of four different types of blood cells was examined using two kinds of cell marker, monoclonal antibodies and autonomous fluorescence. Consequently, it was revealed that signet ring cells were the only blood cells that were localized in the connective tissues around the alimentary canal and the amoebocytes were mainly distributed in the transverse vessels of the branchial basket. The morula cells and compartment cells were distributed under the epidermis of the mantle around the visceral region.

INTRODUCTION

Ascidians have several types of blood cells which have been reported to be involved in various functions, such as nutrition [1], allogeneic reaction [2, 3], formations of the tunic [4–6] and of gonad [7], and the accumulation of heavy metals [8–10]. However, it is still unclear what type of blood cells participates in each of various functions except for a few cases and in which tissue each type of blood cell localize in ascidians.

The establishment of reliable cell markers for the recognition of different types of blood cells is, therefore, a necessary step to working out important and intractable problems such as the functional characterization of each cell type, cell lineage relations amongst the different types of blood cells, and the determination of haematopoietic sites, because it is difficult to interpret any possible interrelationships or developmental series based only on the light microscopical examination of the blood cells.

Hereupon, ascidians, in particular those that belong to the suborder Phlebobranchia, are known to accumulate high levels of vanadium ions from seawater and reduce them to vanadium ions in the +3 oxidation state in their blood cells [11]. There appear to be from nine to eleven different types of blood cells that fall into six categories on the basis of their morphology: haemoblasts, lymphocytes, leucocytes, vacuolated cells, pigment cells and nephrocytes [12]. Vacuolated cells, accounted for about 80% of the total population of blood cells in *A. sydneiensis samea*, were further classified into at least 4 different types, signet ring cell, morula cell, compartment cell, and small compartment cell. Among which signet ring cells were identified as the vanadocytes that contained a high level of vanadium [13, 14]. For that reason, we have paid special attention to vacuolated cells [15, 16]. Thus, a monoclonal antibody, S4D5, which recognized the vanadocytes specifically was produced [17]. However, the problem about the cell lineage and localization of the vanadocytes in ascidians remains open.

The present experiments, employing immunocytochemical techniques and based on autonomous fluorescence emitted from each type of blood cell, were planned to examine the problem described above as the first step. It was consequently revealed that four different types of blood cells localized in different sites.

Accepted August 19, 1995
Received June 23, 1995
* All correspondence should be addressed.

MATERIALS AND METHODS

Materials

Ascidians, *Ascidia sydneiensis samea*, were collected at the Otsuchi Marine Research Center, Ocean Research Institute, the University of Tokyo, at Iwate, Japan. The ascidians were maintained in an aquarium in our laboratory at 18°C.

Monoclonal Antibodies

In the present experiments, two kinds of monoclonal antibody, namely, S4D5 that was specific to signet ring cells, the so-called vanadocytes [14], and C2A4 that was specific to amoebocytes, were used to examine the localization of blood cells in the adult tissues.

C2A4 was prepared for the present set of experiments as follows.

As described previously [17], blood cells were separated from serum by centrifugation at 300×g for 10 min and then suspended in 1 ml of ice-cold PBS (phosphate buffered saline), which consisted of 136.9 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, and 1.8 mM KH_2PO_4, pH 7.2. The suspended cells were sonicated with an ultrasonicator (Sonics & Materials Inc., USA) at 40 kHz and 8 W for 10 sec and an aliquot of 0.5 ml of the sonicated suspension, which corresponded to 10^7 cells and contained about 20 mg of protein, was injected intraperitoneally into female BALB/c mice. Injections were repeated 4 times at intervals of 2 weeks to boost the titer of antibodies. The mice were sacrificed three days after the last injection. Blood and spleen were collected immediately. The blood was used for preparation of polyclonal antiserum and spleen cells were fused with myeloma cells by the protocol of Galfre *et al.* [18]. The fused cells (hybridomas) that produced antibodies were picked up one by one and cloned. The antibodies produced by the cloned hybridomas were assayed to obtain a monoclonal antibody that specifically recognized the vacuolar amoebocyte, which cell type was easily characterized by a number of large vacuoles, occasional lipid droplets, and a prominent broad ruffled membrane [19]. Thus, a monoclonal antibody, C2A4, was prepared.

Immunohistochemical Localization of Vanadocytes and Amoebocytes

Adult ascidians, 2 to 3 cm in length, under anesthesia with *L*-menthol, were fixed in methanol for 15 min at −20°C and then in ethanol for 15 minutes at −20°C. Fixed samples were embedded in polyester wax (BDH Chemicals Ltd., England). These samples were serially sectioned at 4 μm from the anterior to the posterior end in transverse sections and the sectioned samples were then mounted on micro-coverslips. After removal of the polyester wax with absolute ethanol, the micro-coverslips were washed three times with PBS and were then immersed in 100 μl of S4D5 or C2A4 hybridoma culture medium for 1 hr at room temperature. As controls, some coverslips were immersed in the antiserum and in non-immune serum.

Each coverslip was washed with PBS for 30 min at room temperature, and was then incubated for 30 min with 6 μl of fluorescein isothiocyanate-conjugated antibodies prepared in sheep against mouse IgG (Organon Teknica Co., Philadelphia, USA), which had been diluted 100-fold in PBS. Then, the coverslips were washed with PBS for 30 minutes at room temperature and mounted in 80% glycerol. Samples were examined under a Nomarski differential-interference microscope equipped with an epifluorescence optic unit (Olympus Co., Ltd., Tokyo, Japan).

Immunoblot Analysis of Antigens

Blood cells were suspended in 62.5 mM Tris-HCl buffer solution that contained 5% 2-mercaptoethanol, 10% glycerol, 0.5 μM PMSF (phenylmethylsulfonyl fluoride) and 2.3% SDS (sodium dodecyl sulfate) and were sonicated with an ultrasonicator at the same conditions as described above. The resultant suspension was subjected to electrophoresis in a 12.5% polyacrylamide gel that contained 2% SDS. The gel was blotted electrophoretically onto a nitrocellulose membrane in the usual way. After the membrane was incubated with 0.01 unit/ml endoglycosidase F (Sigma Chemical Co., USA) for 1 hr at 37°C in an acetic acid buffer solution containing 130 mM CH_3COOH and 185 mM CH_3COONa at pH 5.0 in order to digest polysaccharides contained, it was exposed to the supernatant of culture medium of C2A4 hybridomas. As controls, a few strips of paper were exposed to immune and non-immune mouse serum. Each strip of nitrocellulose membrane was washed three times in PBS and incubated with biotinylated anti-mouse IgG (Amersham Inc. plc., England) and streptavidin-horseradish peroxidase complex (Amersham Int. plc., England). The membrane washed with PBS was exposed to 3, 3'-diaminobenzidine tetrahydrochloride according to the standard procedure [20].

Detection of Blood Cells on the Basis of Their Autonomous Fluorescence

Since several types of blood cells are known to emit autonomous fluorescence [15, 21, 22], the localization of compartment cells and morula cells, two types of vacuolated blood cells, was examined on the basis of their autonomous fluorescence after excitation with ultraviolet light composed of bright-line spectra at 334 and 365 nm. Fixed and sectioned samples, as described above, were mounted on micro-coverslips and were examined after removal of the polyester wax.

RESULTS

Monoclonal Antibody C2A4 Specific to Amoebocytes

A hybridoma cell line designated C2A4 was newly prepared to secrete a monoclonal antibody that specifically recognized vacuolar amoebocytes among several types of blood cells (Fig. 1). Almost all of the vacuolar amoebocytes were exclusively recognized by the antibody. The antigenicity was localized in the cytoplasm but not in the pseudopodia of the vacuolar amoebocytes.

In order to identify the antigenic peptide that was recognized by the C2A4 antibody, proteins extracted from the blood cells were subjected to immunoblot analysis as reported previously [17], after treatment with endoglycosidase F. The monoclonal antibody reacted with a single band of protein that corresponded to a molecular mass of about 200 kDa after electrophoresis on a non-reducing SDS-polyacrylamide gel (Fig. 1). No band was detectable without prior treatment with the glycosidase. The monoclonal antibody specific to the signet ring cells, S4D5, has been reported to recognize a protein with a molecular mass of about 45 kDa [17].

Localization of Blood Cells, as Revealed by Monoclonal Antibodies

To examine the localization of blood cells, the ascidians were serially sectioned from the anterior to the posterior end in transverse sections and the sectioned samples were then reacted with two kind of monoclonal antibodies, S4D5 and C2A4. The results revealed by the monoclonal antibodies are schematically represented in Figure 2, including those obtained by detection of autonomous fluorescence descried on the next chapter.

Immunoreactivity against the monoclonal antibody specific to signet ring cells (S4D5) was observed in the connective tissues around the alimentary canal (ac), where blood cells were densely distributed, as shown in Figure 3. Signet ring cells, 10–12 μm in diameter, are characterized by a single, fluid-filled vacuole which fills up the entire cell and displaces both the nucleus and the cytoplasm to the periphery [16].

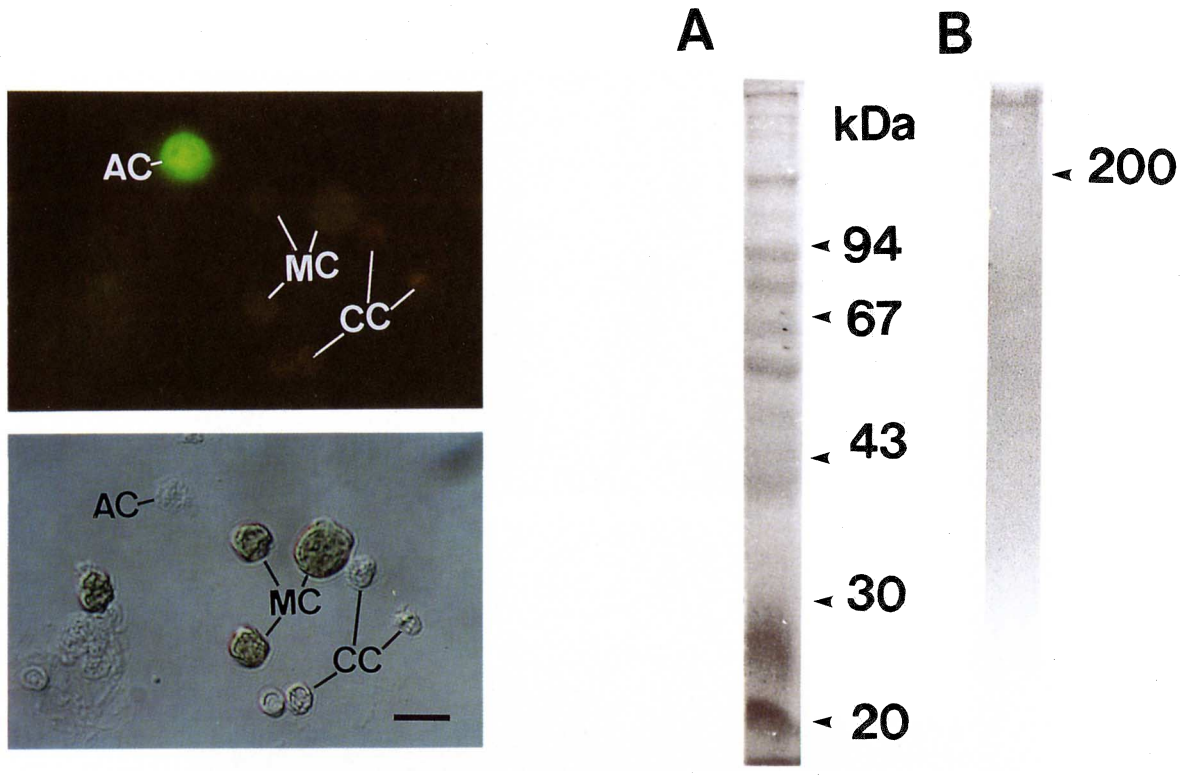
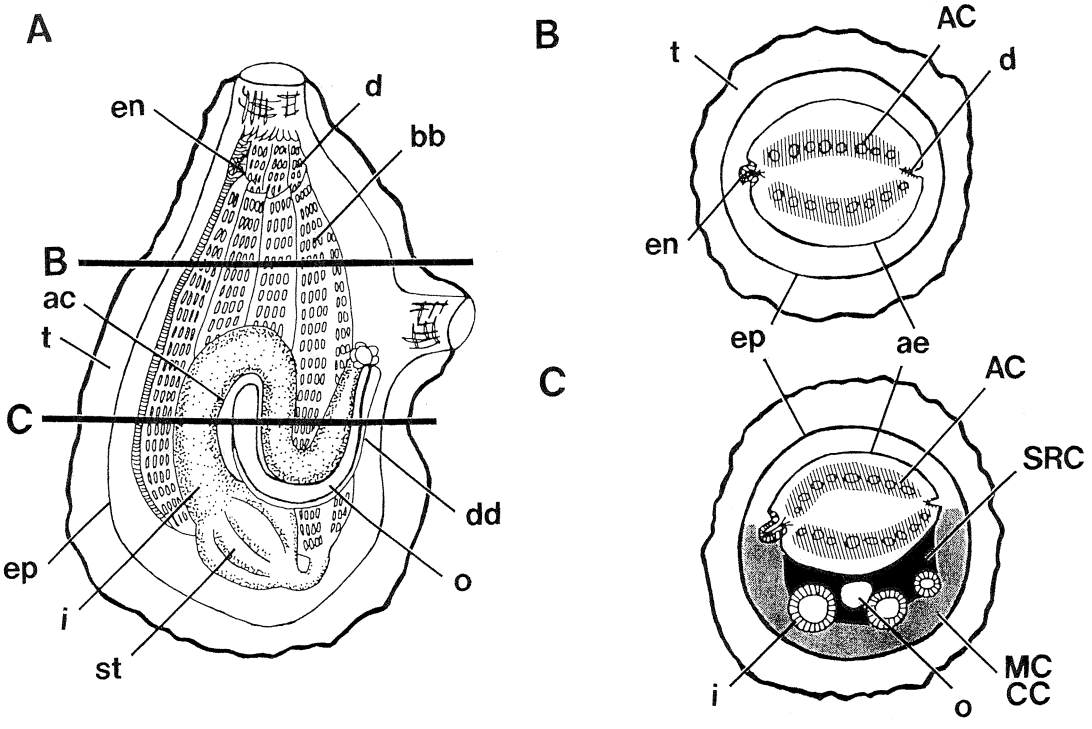


FIG. 1. A monoclonal antibody, C2A4 prepared newly, reacted specifically with the vacuolar amoebocytes among several types of blood cell and the antigen recognized by the monoclonal antibody was subjected to immunoblot analysis. The upper photograph on the left hand shows a fluorescence micrograph and the lower one on the left hand shows a Nomarski differential-interference micrograph. AC, vacuolar amoebocyte; MC, morula cells; CC, compartment cells. Scale bar=10 μm. Two lanes on the right hand indicate SDS-PAGE (A) and immunoblot analysis (B), respectively. A homogenate of blood cells was subjected to SDS-PAGE. The separated proteins were blotted onto a nitrocellulose membrane and were decorated with the C2A4 antibody. The antibody recognized a single band that corresponded to a protein with a molecular mass of about 200 kDa, after treatment with endoglycosidase F.



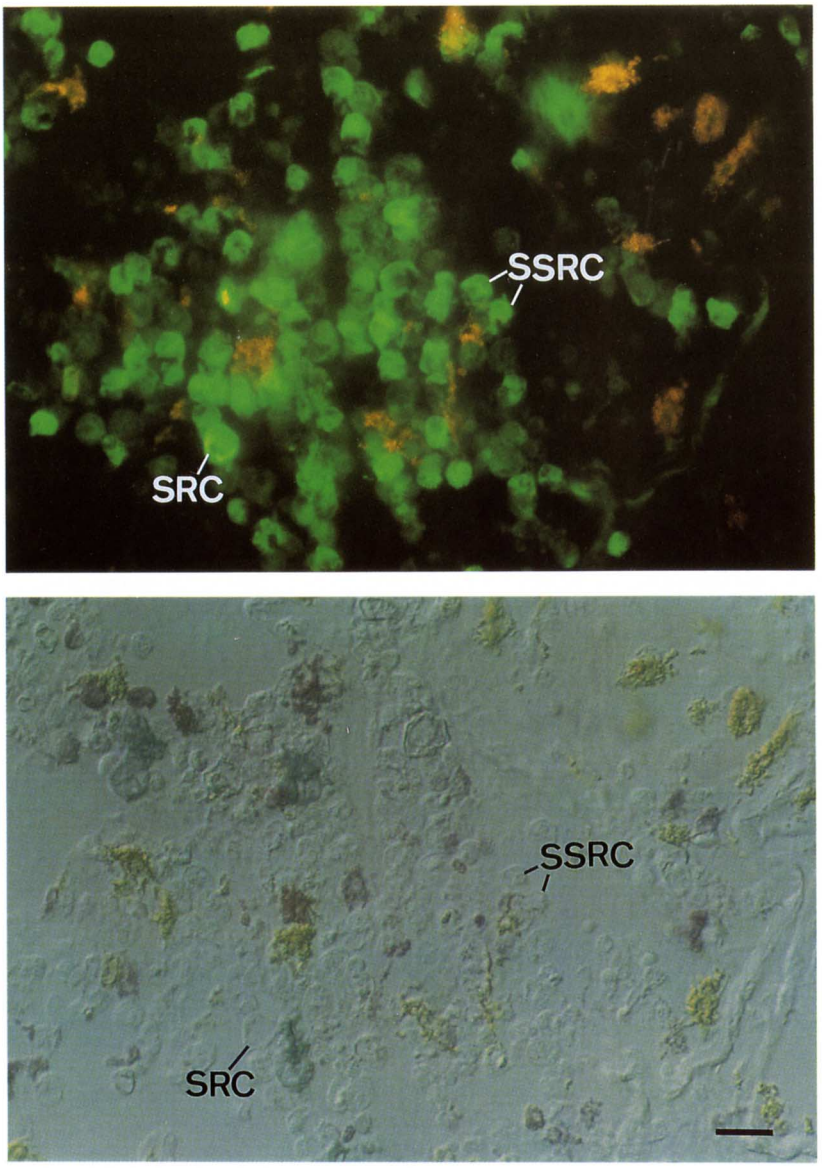


FIG. 3. The localization of signet ring cells as revealed by indirect immunofluorescence microscopy. Clusters of dozens of signet ring cells that were reactive with a monoclonal antibody (S4D5) were observed in the connective tissues around the alimentary canal. Smaller blood cells, resembling signet ring cells, were found in this area but were less reactive with the S4D5 monoclonal antibody. Autonomous fluorescence of orange-yellow was emitted from a kind of pigment cells. The upper photograph shows a fluorescence micrograph and the lower one shows a Nomarski differential-interference micrograph. SRC, signet ring cells; SSRC, smaller signet ring cells. Scale bar=10 μm.

Such cells formed clusters of dozens of cells. These cells were identified clearly by indirect immunofluorescence staining and by the binding of the S4D5 monoclonal antibody manifested over a ring of cytoplasm. Furthermore, smaller blood cells, 8–10 μm in diameter, resembling signet ring cells, were also found in this area. However, these cells did not react strongly with the S4D5 monoclonal antibody.

On the other hand, amoebocytes have been classified into three different types, namely, vacuolar, granular and refractive amoebocytes [16]. The C2A4 monoclonal antibody recognized the vacuolar amoebocytes, which were characterized by a large number of vacuoles in the cytoplasm and by a ruffled membrane at the leading edge. A large number of vacuolar amoebocytes were localized in the transverse vessels of the branchial basket and a small number was observed both in the coelom and the connective tissues

FIG. 2. The localization of four types of ascidian blood cells, revealed by the monoclonal antibodies and by detection of autonomous fluorescence, is shown schematically. Signet ring cells (SRC) are localized in the connective tissues around the alimentary canal (Fig. 1C) and the vacuolar amoebocytes (AC) are distributed in the branchial basket (Figs. 1B and 1C). The morula cells (MC) and compartment cells are distributed in the epidermis of the mantle around the visceral region (Fig. 1C). d, dorsal lamina; bb, branchial basket; o, oviduct; st, stomach; ep, epidermis; i, intestine; ac, alimentary canal; en, endostyle; ae, atrial epithelium; t, tunic; dd, ductus deferens.

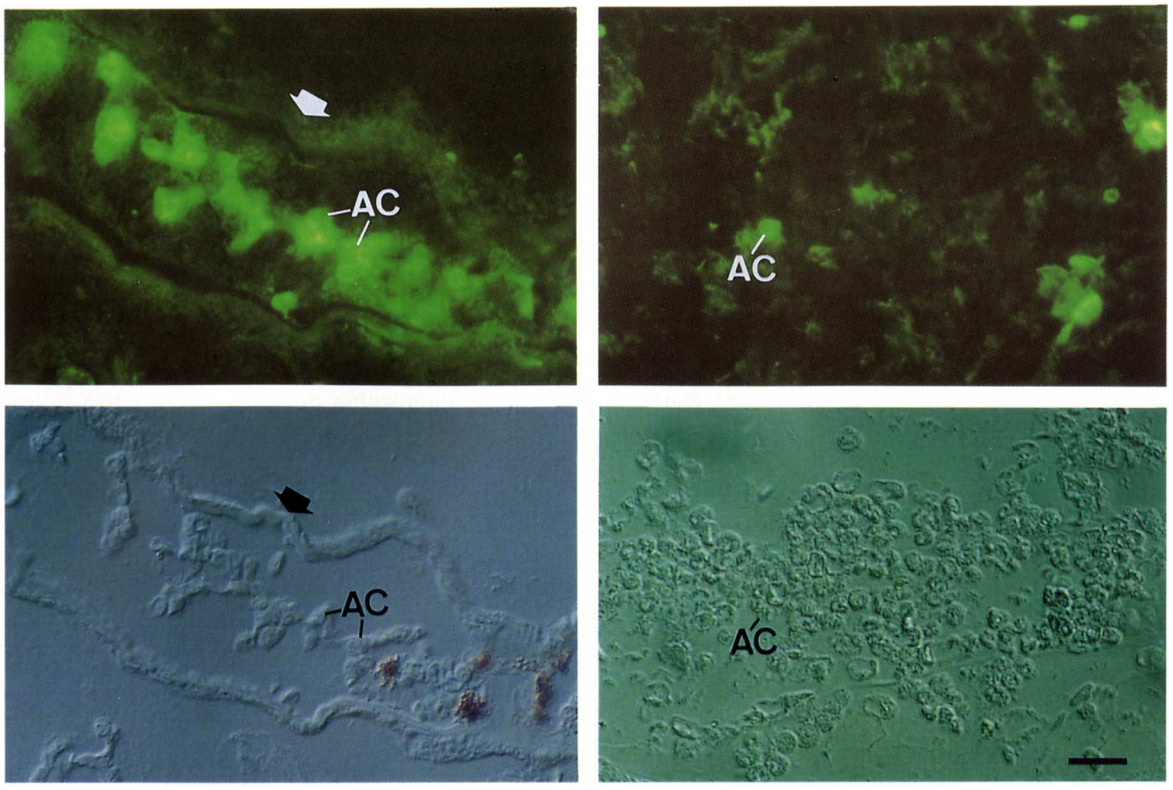
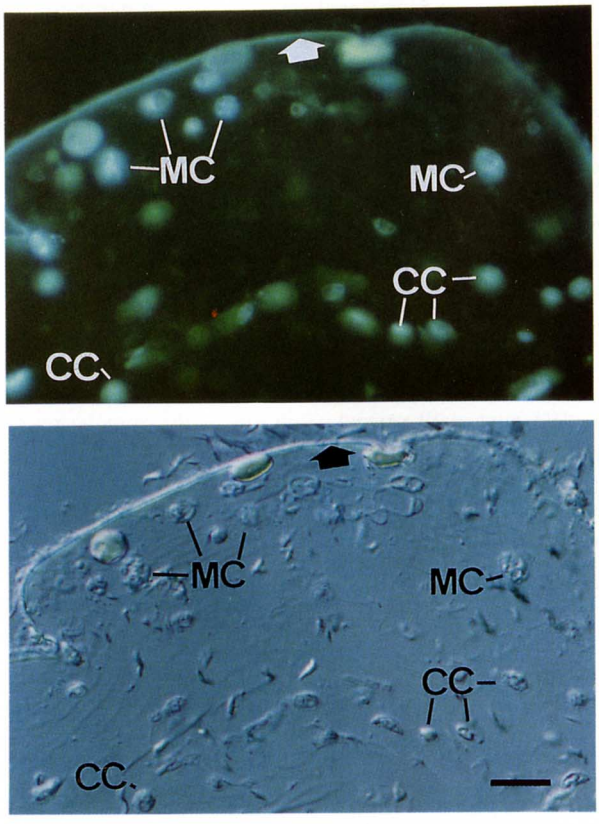


FIG. 4. Blood cells, flowing in the transverse vessels of the branchial basket (the left photograph), were stained for indirect fluorescence with a monoclonal antibody specific to amoebocytes (C2A4). A small number of vacuolar amoebocytes was also observed in the connective tissue around the alimentary canal (the right photograph). The upper photographs show fluorescence micrographs and the lower ones show Nomarski differential-interference micrographs. AC, vacuolar amoebocytes. Arrow indicates the transverse vessels. Scale bar = 10 μm.



around the alimentary canal (Fig. 4). The cells recognized by the monoclonal antibody located in the transverse vessels of the branchial basket (bb in Figs. 2 and 4) but no antigenicity was observed in the cells located in the coelom and connective tissues around the alimentary canal.

Localization of Blood Cells as Revealed by Autonomous Fluorescence

The color of the autonomous fluorescence has been reported to be specific to each type of living blood cells in the ascidian [16]. The morula cells and compartment cells emit blue-green fluorescence and blue fluorescence, respectively, from their vacuoles, and they are, therefore, easily distinguishable. However, when these blood cells are fixed with alcohol, the fluorescence derived from morula cells and compartment cells changes to blue and bright blue, respectively. Nonetheless, these blood cells remain distinguish-

FIG. 5. The autonomous fluorescence emitted from morula cells and compartment cells revealed their localization. Morula cells, emitting blue-green fluorescence, were distributed under the epidermis of the mantle around the visceral region. The compartment cells, emitting blue fluorescence, were observed both under the epidermis (arrow) and in the coelom (not shown). The upper photograph shows a fluorescence micrograph and the lower one shows a Nomarski differential-interference micrograph. MC, morula cells; CC, compartment cells. Scale bar = 10 μm.

able from the other types of blood cells.

On the basis of such fluorescence, the morula cells were shown to localize mainly just beneath the epidermis (ep) of the mantle around the visceral region, emitting bright fluorescence (Fig. 5), but none were observed around the alimentary canal. The compartment cells were distributed not only under the epidermis but also in the coelom.

DISCUSSION

The present experiments clearly revealed the localization of four different types of blood cells in ascidian tissues, on the basis of results obtained with two kinds of cell markers, monoclonal antibodies and autonomous fluorescence. Signet ring cells were the only blood cells that were localized in the connective tissues around the alimentary canal and the amoebocytes were mainly distributed in the transverse vessels of the branchial basket. The morula cells and compartment cells were distributed under the epidermis of the mantle around the visceral region.

There are several reports on the localization of blood cells in ascidians [23–26]. According to these earlier reports, haematogenic activity is observed in three main areas of ascidians: ① in the connective tissues around the alimentary canal; ② in the pharyngeal wall and transverse vessels of the branchial basket; and ③ in discrete nodules located in the body wall. Actually, Ermak [25, 26] demonstrated that haematogenic tissues were abundant in the pharyngeal wall and around the gut-loop (alimentary canal) in *Ciona intestinalis*, belonging to the same Phlebobranchia as *A. sydneiensis samea* used in the present experiment, using autoradiography labeled with tritiated thymidine. In *Chelyosoma productum* and *A. ceratodes*, most haematogenic tissues were around the gut and organized into small nodules containing several cells which incorporated tritiated thymidine. Moreover, in *Styela clava* belonging to Stolidobranchia most lymph nodules, which consisted of one or two groups of labeled cells with tritiated thymidine, occurred in the pharyngeal and body walls.

As judged from the pattern of distribution of each type of blood cells in the present experiments, the localization site of signet ring cells corresponds to site ① described above. There is a strong likelihood that the signet ring cells are renewed in the connective tissues around the alimentary canal for two reasons: the dense localization of signet ring cells and the existence in this area of similar but smaller cells, which could be precursor cells and some of which reacted with the monoclonal antibody specific to the signet ring cells. Observation by Ermak [25], that lymph nodules contained haemoblasts were surrounded by maturing blood cells in various stages of differentiation, also supports our finding. That is, it seems that the precursor cells of signet ring cells are in the connective tissues.

The site of localization of the amoebocytes coincided with site ② described above. Most of amoebocytes were densely localized in the transverse vessels of the branchial basket as shown in Figures 2 and 4, where the cells recognized by the monoclonal antibody of C2A4, though no antigenicity was observed in the cells located in the coelom and connective tissues around the alimentary canal. However, further evidence is needed to determine the renewal site of the amoebocytes.

On the basis of autonomous fluorescence, it was revealed that localization site of the morula cells was beneath the epidermis of the mantle around the visceral region and that of the compartment cells was in the epidermis and coelom. Concerning the localization site of morula cells, Endean [27] reported that morula cells can be observed in various stage of disintegration within the tunic and in association with tunic microfibrils. More direct evidence suggesting morula cell participation in tunic formation was offered from studies on tunic regeneration during wound healing [6, 24]. Recently, Hirose *et al.* [28] found that many morula-like tunic cells aggregated in the tunic around the epidermis and suggested the possibility that the morula-like tunic cells originated from morula cells of haemocytes which passed through the epidermis. On the other hand, since not only the site of localization but also the function of compartment cells have scarcely been reported, this report might be the first step to clarify those problems.

Present knowledge of the cell lineages of ascidian blood cells is inadequate [12]. On the basis of cytological observations, various relationships between the different types of blood cells have been proposed. Endean [27] proposed that the lymphocytes might be the stem cells that differentiate to signet ring cells via vacuolar amoebocytes and that morula cells differentiate from signet ring cells via compartment cells. Thereafter, Ermak [25] suggested, on the basis of tracer experiments with tritiated thymidine, that the lymphocytes might be a primitive type of blood cell and the vacuolated blood cells did not divide. Scippa *et al.* [29, 30] proposed that, since signet ring cells, vacuolar and granular amoebocytes and compartment cells all differentiated at early stages of development while morula cells appeared at later stages, the morula cells did not differentiate from the signet ring cells. However, more direct evidence is needed to clarify the cell type that corresponds to the stem cells and the way how the stem cells differentiate to the peripheral types of blood cells.

We have paid special attention to the signet ring cells since they have been shown to be involved in the remarkable accumulation of high levels of vanadium from seawater [13–15, 31] and, moreover, the signet ring cells comprise from 32% to 44% of the total population of blood cells [22]. We showed recently that an antigen that was recognized by a monoclonal antibody specific to signet ring cells first appeared in juveniles just after metamorphosis, at the same time as significant accumulation of vanadium occurred during embryogenesis [32]. However, the site at which signet ring cell are renewed in adult ascidians remained to be determined. The present study seized an opportunity to open the key of this problem.

ACKNOWLEDGMENTS

The authors express their hearty thanks to the staff of the Otsuchi Marine Research Center, Ocean Research Institute, the University of Tokyo at Otsuchi, Iwate, Japan, for cooperation in collecting the biological materials. Their thanks are also due to Mr. Noriyuki Abo for his efforts to keep the ascidians. This work was supported in grants-in-aid from the Ministry of Education, Science and Culture, Japan and in part by grants from the Salt Science Foundation, the Asahi Glass Foundation and the Electric Technology Foundation of Chugoku to H. M. and T.U.

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