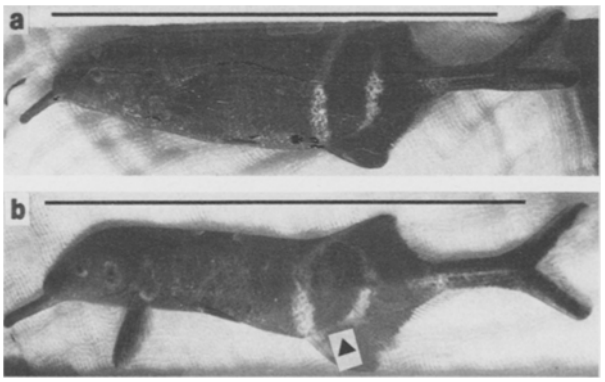


Figure 4. Change in external sexual characteristic in *G. petersii* #5. *a* Day 1 post-implant and *b* day 60 post-implant. Note indentation in base of anal fin by day 60 (arrowhead). Length of black bar: 10 cm. Emaciated appearance of fish is probably due to prolonged hormone treatment [13].

levels [4]. Fish exposed to acute or chronic stress from confinement, capture, and handling exhibited stimulation of the hypothalamo-pituitary-interrenal axis, marked by increased ACTH and cortisol levels, and suppression of the hypothalamo-pituitary-gonadal axis, marked by suppression of plasma androgens [9, 10]. Such environmentally induced hormone responses could explain the previously reported EOD sex difference in *G. petersii*. These differences were: a) accentuated by atypically high aquatic conductivity levels (above 700 µS/cm) [8], b) immediately abolished following physical restraint [7], and c) eliminated within 48 h following sudden, large conductivity changes [8]. Comparable sex-related alterations in EOD as a function of water conductivity were reported in *Pollimyrus isidori* [11]. Testosterone treatment effected other changes in the fish's EOD activity (fig. 3). 1) Frequently, the fish emitted trains of 2–6 multiple discharges that were identical in form and duration and were separated by 6.1-ms intervals (fig. 3a), suggesting autostimulation of the electric organ. Control fish never exhibited such EOD activity. 2) Treated fish exhibited characteristic variations in the ascending portion of the initial positive phase (fig. 3b). 3) On day 60, fish #5 switched between two pulseforms, the elongated EOD (as shown in fig. 1b on day 35) and a discharge of the same duration, best described as a succession of two biphasic pulses with the second lasting twice as long as, and exhibiting about 4 times the peak-to-peak amplitude of the first (fig. 3c). These changes in the generation of EODs could be related to a testosterone-induced increase in electrocyte membrane surface [4].

Testosterone treatment also resulted in external morphological changes. Figure 4 shows the appearance of an indentation in the dorsal margin of the anal fin in fish #5 [12]. The other three 17α-T implanted fish also exhibited this change, whereas none of the control subjects did. In several species of mormyrid fish, such indentation is characteristic of large, mature males. Although the extent of the hormone effects could, in part, be due to pharmacological doses, it is evident that gonadal hormones do influence the electro-communication system in *G. petersii*. Further studies will be required to resolve the issue of a hormone-dependent natural sex difference in the EOD of this species.

1 Acknowledgments. This study was supported by grants from NIMH (NRSA 1F31MH09664) and Sigma Xi to R.E.L., and PSC-CUNY Research Award Program (RF 665405) to P.M. We thank A. H. Bass and T. B. Perera for assistance with surgical techniques and data acquisition. We are particularly grateful to C. F. Harding for assistance with all stages of this project, and J. Atz, V. Blüm and an anonymous reviewer for critical comments on the manuscript.

2 Author for reprint requests.
3 Hopkins, C. D., Science *176* (1972) 1035.
4 Bass, A. H., J. Neurobiol. *17* (1986) 131.
5 Bass, A. H., and Hopkins, C. D., J. comp. Physiol. A *156* (1985) 587.
6 Kramer, B., and Westby, G. W. M., Experientia *41* (1985) 530.
7 Landsman, R. E., Jou, S. H. and Moller, P., in: Reproductive Physiology of Fish, 1987. Eds D. R. Idler, L. W. Crim and J. M. Walsh. Proc. Third Intern. Symp. Reprod. Physiol. Fish, St. John's, Newfoundland 1987.
8 Landsman, R. E., Effects of conductivity change on the EOD of *G. petersii* (1988) in prep.
9 Landsman, R. E., Harding, C. F., Chaudhuri, M., and Perera, T., Overcrowding and handling stress on steroid levels in *Tilapia* (1988) in prep.
10 Sumpter, J. P., Carragher, J., Pottinger, T. G., and Pickering, A. D., in: Reproductive Physiology of Fish, 1987. Eds D. R. Idler, L. W. Crim and J. M. Walsh. Proc. Third Intern. Symp. Reprod. Physiol. Fish. St. John's, Newfoundland 1987.
11 Bratton, B. O., and Kramer, B., (Abstr.) 1st International Congress of Neuroethology Tokyo 1986.
12 Preserved, AMNH 79248.
13 Hildemann, W. H., J. exp. Zool. *126* (1954) 1.

0014-4754/88/100900-04$1.50 + 0.20/0

Clonogenic growth of acute non-lymphocytic leukemia cells in serum-free medium

P. A. Bernabei, V. Santini, O. Dal Pozzo, R. Bezzini, V. Gattei, R. Saccardi, G. Rombolà and P. Rossi Ferrini

U.O. Ematologia, USL 10/D, Università degli Studi di Firenze, Policlinico di Careggi, I-50134 Firenze (Italy)
Received 9 May 1988; accepted 1 July 1988

Summary. We devised a serum-free medium for growth of leukemic colony-forming units (CFU-L), enriched with albumin, transferrin, lipids, insulin, hydrocortisone and oligoelements. Blast cells from 15 patients affected by acute non-lymphocytic leukemia were grown in this medium in the presence of human placental conditioned medium obtained under serum-free conditions (sfHPCM). Their clonogenic growth was comparable with that obtained in a serum-containing system. Furthermore, when serum-free cultures were carried out in absence of sfHPCM, either CFU-L growth was prevented or, if clones were obtained, the cultures showed a marked decrease in clonogenicity, indicating their strict dependence on growth factors.
Key words. Acute non-lymphocytic leukemia; serum-free medium; tumor cell cloning.

The in vitro culture systems employed in the study of hemopoiesis are generally supplemented with animal sera. Since serum may contain growth factors and substances capable of modulating their activity as well as molecules with inhibiting activity, the use of serum-free culture systems is required. Such methods, which were originally described for the study

RPMI 1640 powder	5 mg/ml	(Flow, UK)
TC 199 powder	5.5 mg/ml	(Gibco, Europe)
Bovine serum albumin*	10 mg/ml	(Boehringer Mannheim, WG)
Human transferrin**	80 µg/ml	(Gibco, NY)
Insulin	3 µg/ml	(Gibco, NY)
Glutamine	300 µg/ml	(Merck, WG)
Soy-bean lipids	160 µg/ml	(Boehringer Mannheim, WG)
Cholesterol	96 µg/ml	(Sigma, WG)
Sodium selenite	17.3 µg/ml	(Gibco, NY)
Hydrocortisone	1×10^{-6} M	(Gibco, Europe)
α-thioglycerol	7.5×10^{-5} M	(Sigma, WG)
Trace elements mixture (Se, Si, Mn, Mo, V, Ni, Sn)	10 µl/ml	(Gibco, Europe)
$NaHCO_3$	1.17 mg/ml	

* Purified according to Iscove et al.[2]; ** Fully iron-saturated.

of murine hemopoiesis[1-4], are currently also being used for normal human bone marrow cultures[5-7].

Tumoral cell lines have been grown in serum-free media in both liquid and semisolid cultures[8-10], whereas neoplastic cells from patients, in primary culture, grow with difficulty in these conditions. On the other hand, it is well known that to obtain the successful clonogenic growth of fresh leukemic cells, particular caution is needed, even in the presence of serum[11,12]. In this report we present a serum-free system for clonogenic culture of blast cells from patients affected by acute non-lymphocytic leukemia (ANLL), which is fully comparable with standard serum-containing methods.

Materials and methods. ANLL blast cells were obtained, after informed consent, from bone marrow (10 samples) or peripheral blood (5 samples) of 15 adult patients. Mononucleated cells obtained by 1.077 g/ml Fycoll-Hypaque density gradient centrifugation, were washed twice in phosphate buffered saline and counted. Triplicate samples of 1.10^5 cells were plated in petri dishes, 35 mm diameter, in a final volume of 1 ml per dish, in semisolid media containing agar 0.3% and gentamicin 50 µg/ml, under each of the 3 following conditions:

1) Serum-containing medium, consisting of Iscove's modified Dulbecco medium (IMDM), fetal calf serum (FCS) 10% (v/v) and 5% (v/v) human placental conditioned medium produced under serum-free conditions (sfHPCM) (see below) as a source of colony stimulating factor(s) (CSF).

2) Serum-free medium (SFM), whose formulation is presented in the table, supplemented with 5% (v/v) of sfHPCM.

3) SFM, not supplemented with sfHPCM.

Cultures were incubated at 37 °C in a fully humidified atmosphere of 5% CO_2 in air and scored for clone count after 7 days, defining leukemic colony forming units (CFU-L) as aggregates of more than 20 cells. Single CFU-L from all the cultures, in some cases all the CFU-L scored, were picked up, placed on microscope slides, fixed with glutaraldehyde 3% and stained with Harris's hematoxylin or processed for Su dan black. α-naphthyl-acetate esterase and AS-D-naphthol chloroacetate esterase. Morphological observation of CFU-L cells from all kinds of cultures indicated the absence of mature cells such as myelocytes, metamyelocytes, polymorphonucleated and mature monocytes. Cytochemical staining revealed a concordance between the patterns observed in the fresh blasts from patients and in related CFU-L picked up both from IMDM + FCS and SFM cultures.

sfHPCM preparation was performed by a serum-free modification of the method proposed by Nicola and Metcalf[13]. Briefly, pieces of about 1 cm^3 of placental tissue were placed in flasks containing 20 ml of IMDM supplemented with: purified bovine serum albumin 10 mg/ml, fully saturated human transferrin 80 µg/ml, cholesterol 15.6 µg/ml, soy-bean lipids 160 µg/ml, α-thioglycerol 7.5×10^{-5} M and gentamicin 50 µg/ml. After 7 days of incubation conditioned medium was collected, roughly filtered, centrifuged at 12,000 × g for 10 min, 5 times concentrated by ultrafiltration with a membrane-cutoff of 10,000 daltons and filtered again through 0.22-µm millipore membranes. This sfHPCM supported the optimal growth of normal day 7 and day 14 CFU-GM at a concentration of 5% in IMDM + FCS 20%, comparable with the growth supported by 10% giant cell tumor conditioned medium[14,15].

Results and discussion. The choice of a combination of Tc 199 and RPMI 1640 media, instead of IMDM, for CFU-L serum-free cloning, was determined by the lower glucose content of this mixture, which probably provides an optimal metabolic environment for immature hemopoietic cells. Indeed, their growth is reduced by high levels of glucose[16] and their G1-S transition has been shown to be blocked by glycolysis terminal products and oxidable substrates[17]. Albumin was added as a carrier for lipid, hormones and metals, and as a probable detoxifying agent[10]. Soy-bean lipids and free cholesterol were provided in a quantity allowing a clonal growth nearly equalling that achievable in cultures containing 20% FCS[2]. Insulin was also present, with the role of both modulating cell metabolism and stimulating DNA synthesis[18]. Hydrocortisone has usually been considered important for long-term cultures[3], but it has been reported recently that this hormone promotes the formation of normal CFU-GM in serum-containing[19] and in serum-free cultures[20]. The presence of transferrin is accounted for by its relevance in the maturating process of granulocytic precursors in vitro[21]. Receptors for this protein are also expressed in non-erythroid cells passing into the S phase[22], which suggests a strict Fe requirement in growing cell populations. Trace elements are essential for cell growth[23], as is selenium, which is a component of the protective system of glutathione peroxidase[24].

sfHPCM was used as an optimal source of growth factors. Its stimulatory effect on ANLL cell growth in vitro is fully comparable with the association of recombinant GM-CSF and G-CSF[25]. sfHPCM has recently been produced under serum-free conditions, in IMDM without any supplement[5], but it seems sensible to suppose that provision of albumin, lipids and Fe may improve metabolic conditions for cells in culture, even if they are not proliferating, but only producing growth factors.

CFU-L were obtained from 11 of the 15 samples studied. The serum-free system developed always supported CFU-L growth when this growth was successful in the presence of serum. In two cases the number of CFU-L was even higher in SFM than in IMDM + FCS. Although there was a large variation between individual samples in the frequency of CFU-L, the number of CFU-L obtained under sfHPCM stimulation in the serum-free system appeared to be linearly correlated with the number of CFU-L obtained in serum-containing cultures (fig. 1).

We scored as CFU-L aggregates of 20 or more cells, as suggested elsewhere[11,26,27], considering that leukemic cells

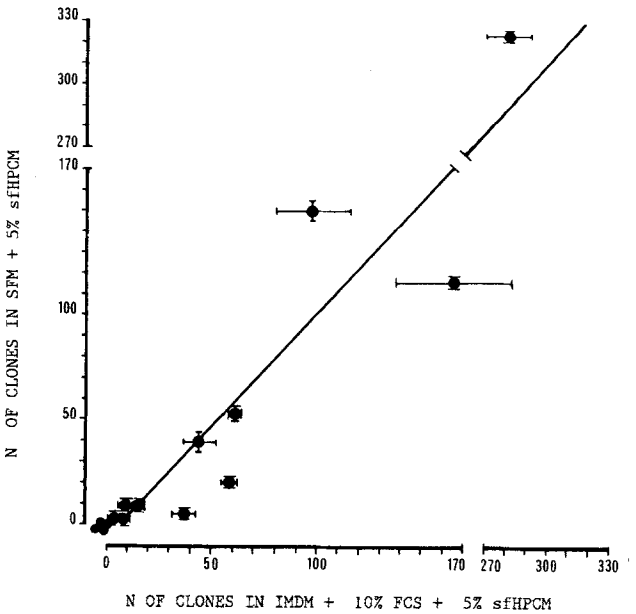


Figure 1. CFU-L growth obtained in IMDM + FCS + sfHPCM vs SFM + sfHPCM for 15 primary ANLL. Results are expressed as the mean number of clones ± SD obtained from triplicate observations. The equation of the correlation line is:
$y = 1.08\,x - 7.3$.
The correlation coefficient r is equal to 0.96 and the y intercept is not significantly different from 0.

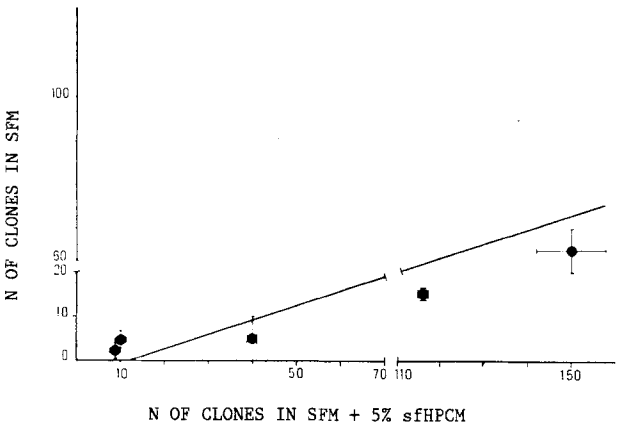


Figure 2. CFU-L growth obtained in SFM + sfHPCM vs SFM alone for 5 primary ANLL. Results are expressed as the mean clones number ± SD obtained from triplicates. The equation of the correlation line is:
$y = 0.335\,x - 4.2$.
The correlation coefficient r is equal to 0.85.

may have a limited growth potential, and that serum-free conditions may delay or affect the production of larger colonies. Successful CFU-L growth was obtained either from bone marrow or peripheral blood cell suspensions, as reported for serum-containing methods. The morphological and cytochemical observation of CFU indicated the leukemic nature of the cells grown in every culture, virtually excluding the presence of normal CFU growth also, in bone marrow samples. Clonal growth from leukemic cells deriving from peripheral blood is unlikely to be contaminated by normal residual CFU-GM.

Cultures carried out in SFM without sfHPCM showed a clonal growth in 5 specimens, in any case poorer than in the presence of sfHPCM. For these cases a linear relationship was observed between the number of CFU-L obtained with or without sfHPCM in serum-free medium (fig. 2), lending further support to the notion that the requirement for growth factors is critical, as has been shown in serum-containing media [28 - 30].

The clonal growth in the absence of colony stimulating activity is consistent with data reporting autonomous CSF secretion in some cases of ANLL [31]. It must be pointed out that adherent cells were present in our conditions of culture, which possibly played a role in the production of factors promoting CFU-L growth. The use of agar may also account for an aspecific stimulatory effect on mononucleated blood cells, which are responsible for production of CSF [32], but no difference between agar and methylcellulose has been noticed in leukemic clonogenic growth in the presence of serum [33].

The absolute failure of CFU-L growth either in the presence or in the absence of serum, observed in four samples, is a challenging question to be answered, and introduces again the wide field of leukemic cells dishomogeneity, which is worthy of further and deeper investigation.

Acknowledgments. This work was supported by a grant of the Italian National Research Council, Special Project 'Oncology', contract number 86.00308.44; M.P.I. 40 %; A.I.L.-Firence; Lega Italiana per la Lotta contro i Tumori, sez. Firenze (Fellowship to V.S., 1987); and A.I.R.C. (Fellowship to V.G.).

1 Guilbert, L. J., and Iscove, N. N., Nature *263* (1976) 595.
2 Iscove, N. N., Guilbert, L. J., and Weyman, C., Exp. Cell Res. *126* (1980) 121.
3 Eliason, J. F., Exp. Hemat. *12* (1984) 559.
4 Eliason, J. F., and Odartchenko, N., Proc. natl Acad. Sci. USA *82* (1985) 775.
5 Drouet, X., Charbord, P., Neel, H., and Baillou, C., Exp. Hemat. *13* (1985) 1133.
6 Lu, L., Welte, K., and Gabrilove, J., Cancer Res. *46* (1986) 4357.
7 Ohara, A., Suda, T., Saito, M., Miura, Y., Okabe, T., and Takaku, F., Exp. Hemat. *15* (1987) 695.
8 Eliason, J. F., Fekete, A., and Odartchenko, N., Rec. Results Cancer Res. *94* (1984) 267.
9 Taketazu, F., Kubota, K., and Kajigaya, S., Cancer Res. *44* (1984) 531.
10 Barnes, D., and Sato, G., Analyt. Biochem. *102* (1980) 255.
11 Dicke, K. A., Spitzer, G., and Ahearn, M. J., Nature *259* (1976) 129.
12 Lowenberg, B., Swart, K., and Hagemeijer, A., Leuk. Res. *143* (1981) 149.
13 Nicola, N. A., Metcalf, D., Johnson, G. R., and Burgess, A. W., Leuk. Res. *2* (1978) 313.
14 Di Persio, J. F., Brennan, J. K., Lichtman, M. A., and Speiser, B. L., Blood *51* (1978) 507.
15 Di Persio, J. F., Brennan, J. K., Lichtman, M. A., Abboud, C. N., and Kirkpatrick, F. H., Blood *56* (1980) 717.
16 Graff, S., Moser, H., Rastner, O., Graff, A. M., and Tannenbaum, M., J. natl Cancer Inst. *34* (1965) 511.
17 Dello Sbarba, P., Cipolleschi, M. G., and Olivotto, M., Exp. Hemat. *15* (1987) 317.
18 Vetter, U., Schlickenrieder, J. H., Zapf, J., Hartmann, W., Heit, W., Hitzler, H., Byrne, P., Gaedicke, G., Heinze, H., and Teller, W. M., Leuk. Res. *10* (1986) 1201.
19 Barr, R. D., Falbo, M., and Koekebakker, M., Leuk. Res. *10* (1986) 397.
20 Wagemaker, G., in: Bone Marrow Transplantation, p. 1. Eds R. Van Bekkum and B. Lowenberg. I. Dekker, New York, Basel 1986.
21 Evans, W. H., Wilson, S. M., and Mage, M. G., Leuk. Res. *10* (1986) 429.
22 Schneider, C., J. Cell Sci. *3* (Suppl) (1985) 139.
23 Ham, R. G., and McKeehan, W. L., Meth. Enzymol. *58* (1978) 44.
24 McKeehan, W. L., Hamilton, W. G., and Ham, R. G., Proc. natl Acad. Sci. USA *73* (1976) 2023.
25 Begley, C. G., Metcalf, D., and Nicola, N. A., Leukemia *1* (1987) 1.
26 McCulloch, E. A., Clinics Hemat. *13* (1984) 503.

27 Kelleher, C., Miyauchi, J., Wong, G., Clarks, S., Minden, M. D., and McCulloch, E. A., Blood *69* (1987) 1498.
28 Hoang, T., Nara, N., Wong, G., Clark, S., Minden, M. D., and McCulloch, E. A., Blood *68* (1986) 313.
29 Vellenga, E., Delwel, H. R., Touw, I. P., and Lowenberg, B., Exp. Hemat. *15* (1987) 652.
30 Vellenga, E., Young, D. C., Wagner, K., Wiper, D., Ostapovicz, D., and Griffin, J., Blood *69* (1987) 1771.
31 Young, D. C., and Griffin, J. D., Blood *68* (1986) 1178.
32 Hoang, T., Iscove, N. N., and Odartchenko, N., Exp. Hemat. *9* (1981) 499.
33 Kubota, K., Preisler, H. D., Sagawa, K., and Minowada, J., Cancer Res. *41* (1981) 3052.

0014-4754/88/100903-04$1.50 + 0.20/0

Autonomous fluorescence of ascidian blood cells with special reference to identification of vanadocytes

H. Michibata[a], J. Hirata[a]*, T. Terada[b] and H. Sakurai[c]

[a]*Biological Institute, Faculty of Science, Toyama University, Gofuku 3190, Toyama 930 (Japan),* [b]*Department of Chemistry, Toyama College of Technology, Hongocho 13, Toyama 930-11 (Japan), and* [c]*Faculty of Pharmaceutical Sciences, University of Tokushima, Sho-machi 1, Tokushima 770 (Japan)*
Received 21 April 1988; accepted 18 July 1988

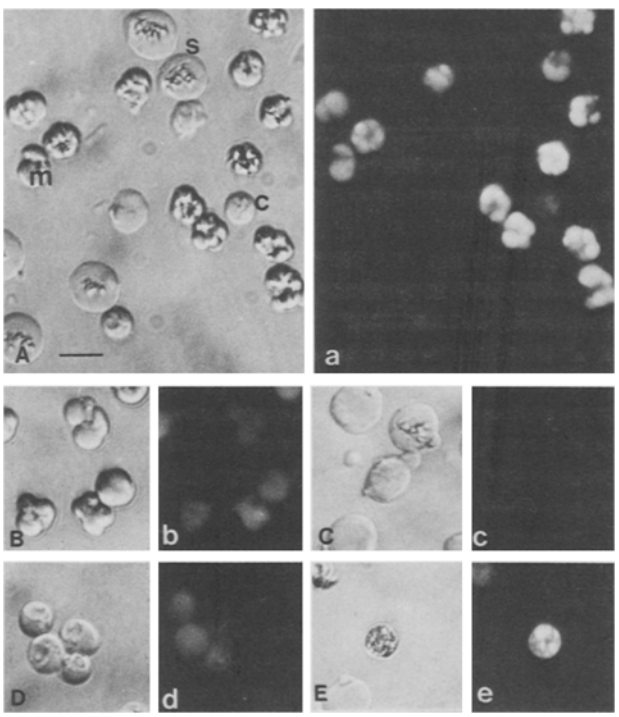
Summary. A tunichrome that has been suggested to be involved in the accumulation of vanadium ions in ascidian blood cells produces an autonomous fluorescence upon excitation with blue-violet light. However, we have found that signet ring cells, which contain large amounts of vanadium, do not fluoresce upon such excitation. The strongest fluorescence due to the tunichrome was observed in morula cells, which do not contain vanadium.
Key words. Ascidian; tunicate; vanadium; vanadocyte; blood cell, fluorescence; vanadium accumulation.

Ascidians belong to the family Ascidiidae and are known to concentrate vanadium ions from seawater to levels as high as one million times those in seawater[1,2]. They have between six and nine different types of blood cells. Among these various cells, the morula cell has been proposed to be the vanadocyte that contains a high level of vanadium ions[3-5]. A tunichrome with a possible involvement in the accumulation of vanadium has been isolated from ascidian blood cells[6]. This substance has been reported to emit a specific autonomous fluorescence after excitation with blue light[7]. The strongest fluorescence in blood cells that could be ascribed to the tunichrome was observed in the morula cells[8]. However, we have recently verified that the morula cell contains no vanadium, whereas the signet ring cell contains a very large amount of vanadium. Therefore, the actual vanadocyte is most likely the signet ring cell[9]. In this study, we examined the fluorescence emitted from the various blood cells in order to ascertain whether the fluorescent tunichrome takes part in the accumulation of vanadium.

Materials and methods. Ascidia ahodori were collected from the seawater tank of the Ushimado Marine Biological Station, Okayama University, Ushimado, Okayama, Japan. They were maintained in a temperature-controlled aquarium in our laboratory. Blood, drawn by making an incision through the lower part of the tunic and puncturing the mantle, was suspended in Ca^{2+}- and Mg^{2+}-free artificial seawater to avoid clotting. Living specimens were observed by multi-microspectrophotometry (MMSP) in an Olympus system which was equipped with a mercury lamp and a fluorescence optics unit.

Results and discussion. The tunichrome, which can be extracted from the ascidian blood cells of *A. nigra* and which has been proposed to be involved in the accumulation of vanadium ions[1,6,8], is known to fluoresce at 532 nm and 581 nm after excitation at 488 nm[7]. Blood cells that fluoresce under these conditions are, therefore, considered to be the sites of the tunichrome, as a result of studies of cellular fluorescence properties[7,8].

In the present experiment, the morula cells, the compartment cells, and the orange cells emitted autonomous fluorescence after excitation with blue-violet light composed of line spectra at 405 nm and 435 nm and wide spectrum at 490 nm (using a BG-12 filter). The fluorescence emitted from the morula cell was cut by O515 barrier filter, indicating that its wavelength was longer than 515 nm. The compartment cell



Blood cells of *Ascidia ahodori* were observed with a light (A) and a fluorescence microscope (a). The morula cell (m), signet ring cell (s) and compartment cell (c) were shown. The detailed observation was made in clusters of each blood celltype. The morula cells fluoresced strongly upon excitation with blue light (B and b) but signet ring cells did not fluoresce (C and c). The fluorescence was also observed in the compartment cells (D and d) and orange cell (E and e). Amoebocyte, granular amoebocyte and lymphocyte did not emit fluorescence (not shown). Photographs marked with (A–E) and (a–e) are those observed with a light and a fluorescence microscope, respectively. Scale bar indicates 10 μm.