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Application of Backscattered Electron Imaging to Enzyme Cytochemistry of Phytohemagglutinin-Stimulated Blastoid Cells

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Abstract Backscattered electron imaging (BEI) mode of scanning electron microscope (SEM) was applied to study cytochemical reactions of acid phosphatase, peroxidase, nonspecific esterase, and a silver-staining method for nuclei of human blastoid cells in phytohemagglutinin (PHA)-stimulated culture. Acid phosphatase activity was detected within the lysosomes and sometimes in the endoplasmic reticulum of PHA blastoid cells and monocytes. Peroxidase-positive granules after staining with osmium were seen in blastoid cells with good contrast and resolution in the BEI mode of SEM. Nonspecific esterase activity was also demonstrated in the BEI mode after incubation of the cells in Hanker's medium and staining with osmium. These enzyme activities were generally visible at the identical sites of blastoid cell surface to those secondary electron image modes. The surface structure of these cells was always well-preserved in spite of the short fixation and the cytochemical procedure, and comparable to conventional SEM controls. Silver staining permitted recognition of the nuclear shapes and the fine chromatin patterns within the cultured cells.

Key Words: Backscattered electron imaging, Scanning electron microscopy Lymphocyte blastogenesis, Enzyme cytochemistry, Phytohemagglutinin

Introduction

The secondary electron imaging (SEI) mode of scanning electron microscope (SEM) offers useful information about the surface topography, while interior morphological details of the specimens are hardly recognizable. Backscattered electrons (i.e., electrons from the primary electron beam) emerge in part from the surface, but mostly from within the specimens. The intensity of backscattered electron imaging (BEI) mode increases with the atomic number (Z) of the target of the incident beam[1]. Such BEIs in SEM of biological specimen reflect the topographical distribution and the concentration of high atomic number substances within the cells[2,3]. Therefore, comparison of the SEI and the BEI of identical sites makes possible the direct correlations between surface morphology and the localization of enzyme activity within blood cells stained with heavy metal[4-8].

On the other hand, since the first report on

This paper is dedicated to Emeritus Professor Kazuhiko Awaya, the former president of Yamaguchi University, on the occasion of his retirement from Yamaguchi University.

the development of phytohemagglutinin (PHA)-simulated blastoid cells by Nowell[9], the appearance of blastoid cells from small lymphocytes has become widely established[10,11] and their surface morphological characteristics are well documented by SEM observations[12,13]. However, very little information is available about the direct correlations of the surface morphology and the localization of enzyme activity in different blastoid cell types accompanying lymphocyte activation and proliferation. In this context, the purpose of this study is to show the applicability of the BEI mode in SEM to enzyme cytochemistry in PHA blastoid cells and further elucidate the correlation of the BEI-SEM images with light and transmission electron microscopic (TEM) images.

Materials and Methods

Lymphocyte culture

Human peripheral blood lymphocytes from normal healthy volunteers were separated on Ficoll-Isopaque (Lymphoprep, Nyegaard & Co. A/S, Oslo) density gradient and cultured as reported previously[14,15]. Briefly, lymphocytes (1.5×10^6) were suspended in 1 ml TC-199 medium (Gibco) containing 20% fetal calf serum (Gibco) and incubated with phytohemagglutinin (PHA-P, Difco, 10μg/ml) for 3 days at 37℃. After washing three times in Hanks' balanced salt solution, the cultured cells were allowed to attach to glass cover slides or carbon planchets (Oken, Tokyo) pretreated with 1% aquous poly-L-lysine for light and scanning electron microscopy respectively. The cells, attached to a glass slide, were stained with Giemsa stain. Aliquots of samples were prepared for transmission electron microscopy.

Enzyme cytochemistry for light and scanning electron microscopy

Acid phosphatase (ACPase): The attached cells were fixed with 1.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.2) for 30 min. The samples for light microscopy were incubated in a medium[16] containing naphthol AS-TR phosphate as substrate and hexazotized pararosaniline as coupler in Michaelis acetate buffer (pH 5.0) for 1 hr at 37℃. Other samples for electron microscopy were immersed in Barka and Anderson medium[17] for 30 min at 37℃.

Peroxidase: The cells were fixed with 1.0% glutaraldehyde in 0.1M cacodylate buffer (pH 7.2) for 30 min and incubated in Graham and Karnovsky medium[18] for 1 hr at 37℃. After incubation, the samples for the BEI-SEM were immersed in 0.5% osmium tetroxide for 3 min.

Esterase: The cells were fixed with 1% glutaraldehyde in 0.1M cacodylate buffer (pH 7.4) for 10 min. The samples for light microscopy were reacted for nonspecific esterase using α-naphthyl acetate (Sigma) as substrate and hexazotized pararosaniline as coupler according to the method of Knowles et al.[19] Other samples for electron microscopy were incubated for 3 hours at room temperature in Hanker's medium[20] containing naphthyl-thiol-acetate (Polyscience, Warrington, PA) as substrate and Fast blue BB (Sigma) as coupler. After incubation, they were also treated with 0.5% osmium tetroxide for 3 min.

Control experiments for the enzymes: The reaction medium was altered respectively as followed: 1) Substrate (β-glycero-phosphate, 3,3'-diaminobenzidine-4HCl (DAB) and naphthyl-thiol-acetate) was omitted. 2) Sample was treated with NaF (10mM), KCN (100mM) and E-600 (10mM) in the final concentration before being incubated in the reaction medium. 3) Sample was incubated for 60 min at 60℃ before incubation.

Silver reduction staining

The cells were fixed with 2.5% glutaraldehyde in 0.1M cacodylate buffer (pH 7.2) for 2 hrs at 4℃ and incubated in a Gomori methenamine silver solution for nuclear staining as recommended by Becker and Sogard[5]. Incubations were repeated four times (45 min each) at 56℃, followed each time by a rinse in a large volume of distilled water. Finally, the samples were placed for 20 min in a 5% solution of sodium thiosulfate and rinsed in distilled water.

Backscattered electron imaging (BEI) of SEM.

All samples treated as described above for SEM cytochemistry were rinsed in distilled water, dehydrated in ascending concentration of ethanol, transferred to t-butyl alcohol for three changes and then dried in a freeze dryer (ID-2, RMC-Eiko Co. Tokyo, Japan). For control observation, some samples were processed for SEI mode only with or without cytochemical staining. Dried samples were mounted on aluminum stabs with a carbon paste. They were uniformly coated with carbon by evaporation to

a thickness of about 30 nm to prevent artifacts due to charging and examined in a JEOL JSM -820 or Hitachi S-800 SEM equipped with a backscattered electron detector. The BEI micrographs were taken under accelerating voltage of 20-25 kV and those of SEI at 10 kV as described previously[21-24].

Transmission electron microscopy

The cells were fixed in suspension and then treated with cytochemical techniques similar to those described above for SEM. After incubation, cells were rinsed in buffer, spun to obtain a thin pellet and postfixed for 1 hr in 2% osmium tetroxide. After dehydration, the cell pellets were routinely embedded in Epon.[25] Ultrathin sections were obtained with a diamond knife, stained with uranyl acetate or without further staining and examined using a JEOL JEM 100 CX TEM.

Results

Blastoid cell morphology

The smear obtained from PHA-stimulated culture at 3 days showed clumps of cells containing large mononuclear forms, mainly some types of blastoid cells interspersed with small lymphocytes (Fig. 1). The cytoplasm of PHA blastoid cells stained with Giemsa solution was intensely basophilic and contained some azurophilic granules and clear vacuoles.

Acid phosphatase (ACPase)

ACPase activity in PHA blastoid cells, partly in small lymphocytes was seen as granular reaction products (Fig. 2). Such ACPase reaction products were clearly recognized as highlights in round or irregular shapes in the BEI mode of SEM (Fig. 3). In some cells processed for ACPase reaction, the cell outline and sometimes faint images of surface projections were also visible, but these were usually distinguishable from the highlights of intracytoplasmic reaction products (Fig. 5). The surface structure of the cells observed in the SEI mode of SEM was always well-preserved in spite of the applied ACPase reaction (Fig. 4) and cytochemically comparable to conventional SEM controls without the cytochemical procedure (Fig. 6). In TEM observation the reaction products for ACPase activity could be seen within the lysosomes, phagosomes and sometimes in the endoplasmic reticulum and Golgi lamella (Figs. 7 and 8). Their cytoplasm often contained globular inclusions, possibly lipid bodies in addition to ACPase-positive lysosomes (Fig. 8).

Peroxidase

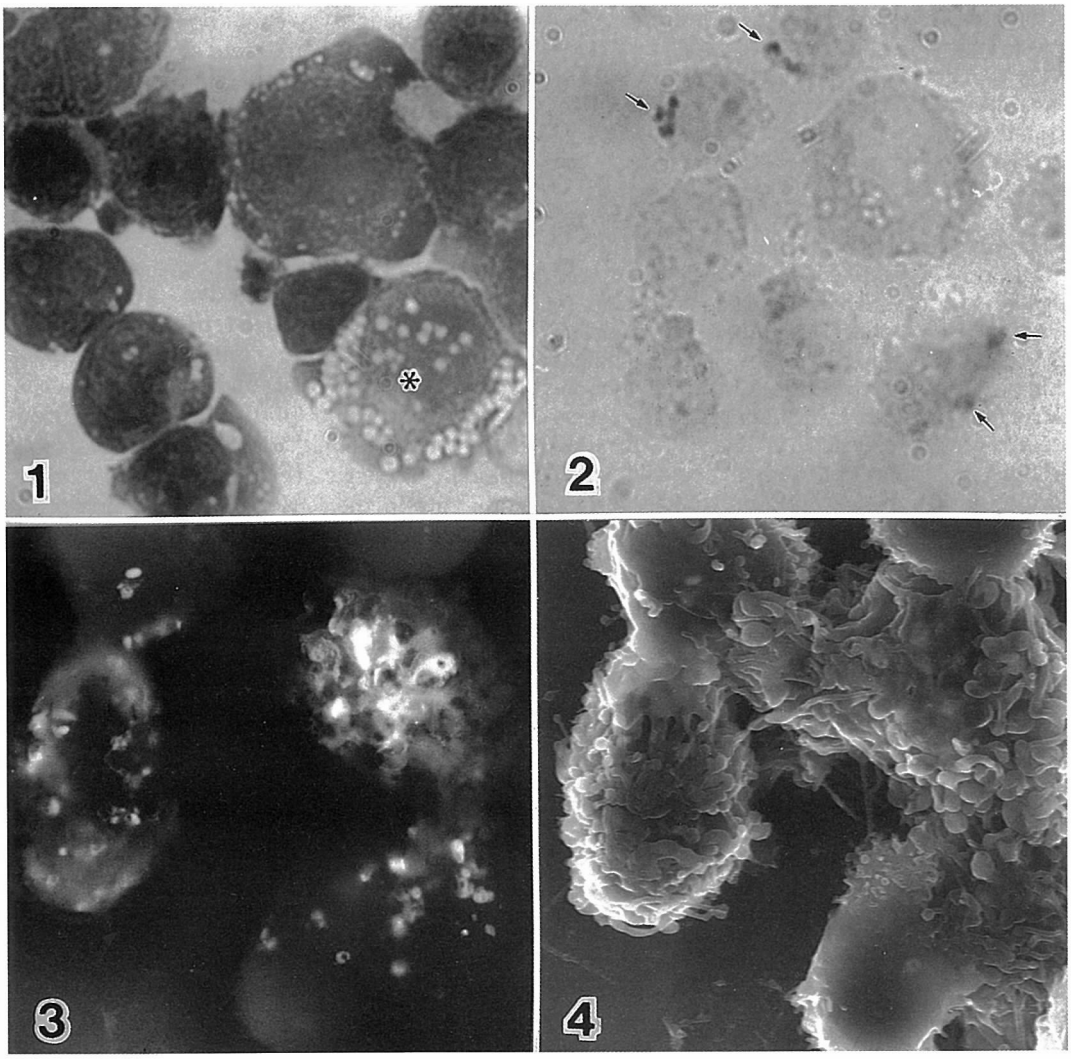
Large peroxidase-positive granules as DAB deposits were seen in PHA blastoid cells in striking contrast to small lymphocytes by light and electron microscopy (Figs. 9 and 10). The reaction products for peroxidase activity took a variable number of dense, round or oval forms as highlights in the BEI (Fig. 11). Such granules were generally visible at the identical sites of the cell surface to those in the SEI mode (Fig. 12). In control samples, where DAB or H_2O_2 were omitted or KCN added in the incubation medium, no such granules were seen.

Esterase

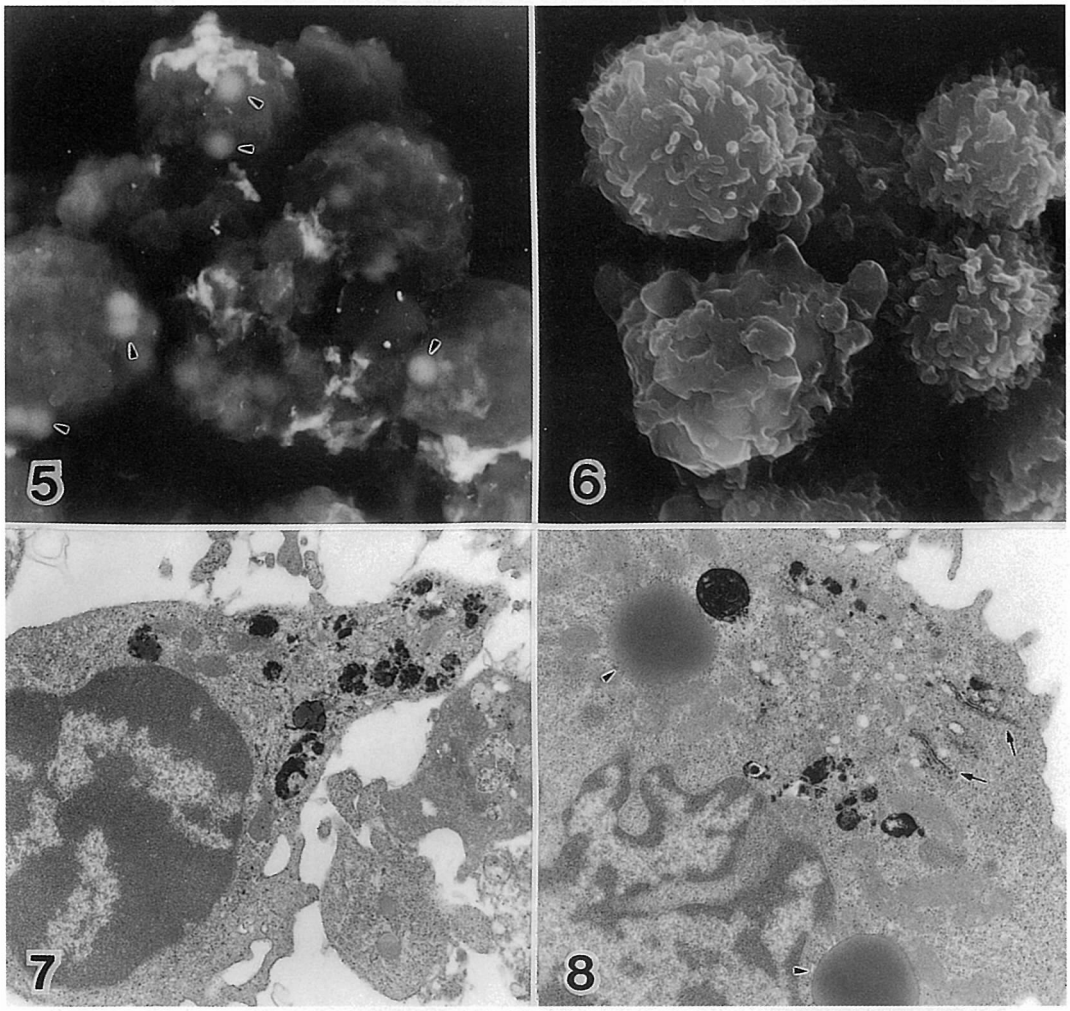
Nonspecific acid α-naphthyl acetate esterase activity was detected in small lymphocytes, PHA blastoid cells and monocytes, but not in polymorphonuclear leukocytes. The esterase-positive PHA blastoid cells and monocytes contained granular or dot-like and diffuse reaction products respectively, whereas the majority of esterase-positive small lymphocytes displayed a dot-like staining pattern adjacent to the cell membrane (Fig. 13). The reaction products for esterase activity as the azo-dye-aryl complexes formed granular shapes as highlights in the BEI mode (Fig. 14). Two contiguous granules easily gave a fused BEI image if one tried to increase the contrast over certain limits. In control samples, where the substrate was omitted or E-600 added in the medium, no such reaction products were seen.

Silver reduction staining

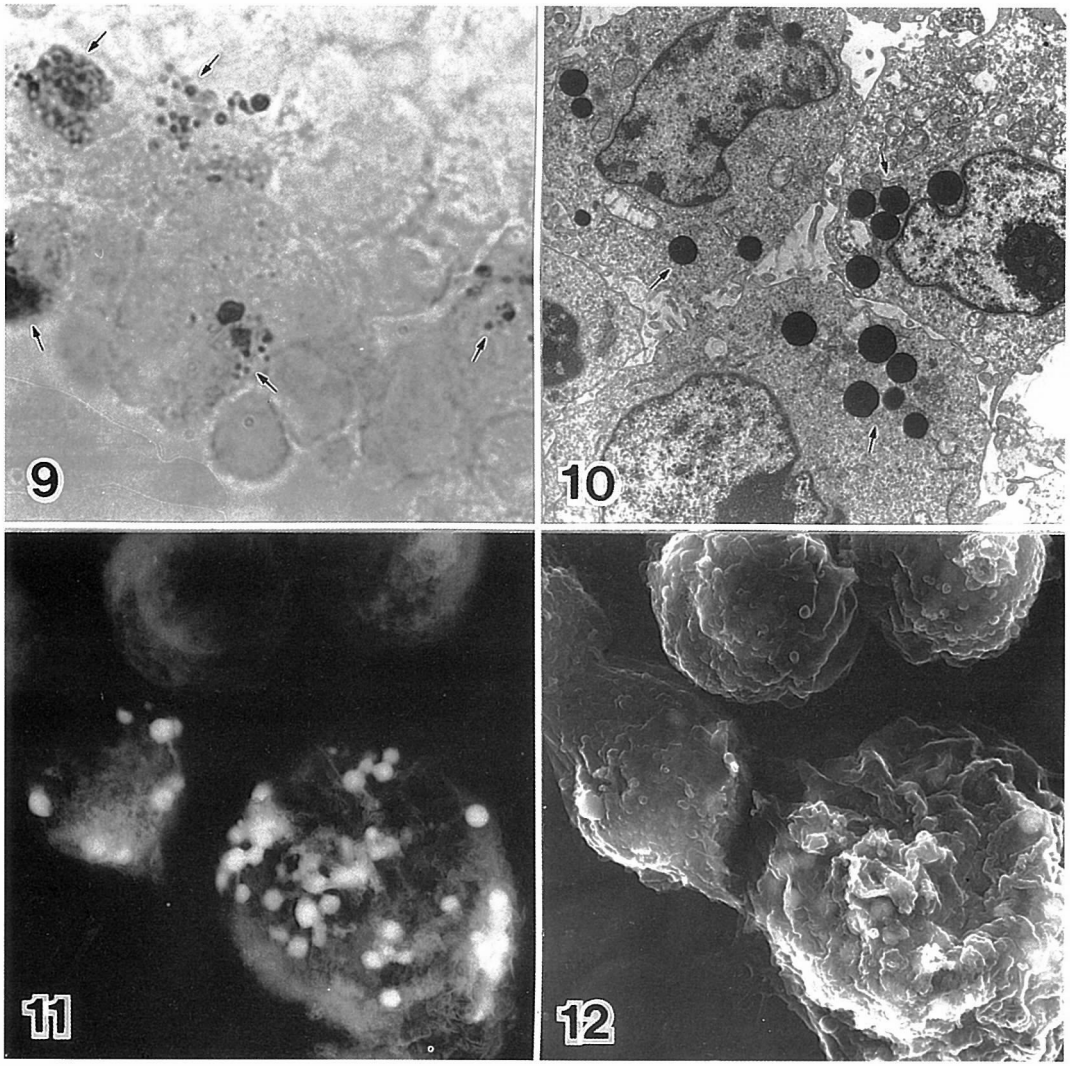
The silver staining for nuclear chromatin revealed nuclear contours of cultured cells (Fig. 15). The nuclear sizes and the fine chromatin patterns were also seen in the BEI. The concentration of the silver depositions



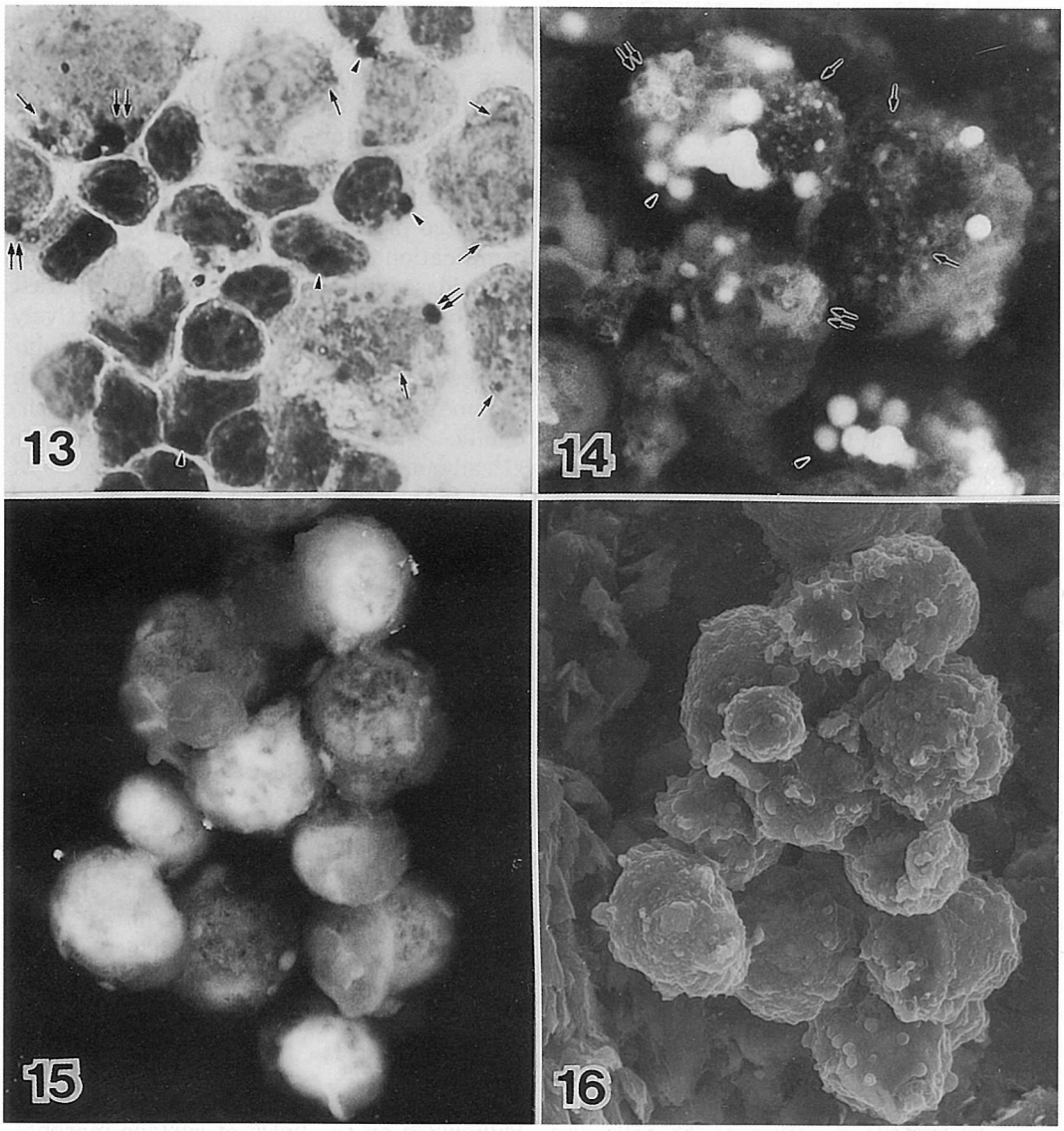
Figs. 1-4 Light and SEM micrographs of PHA-stimulated cells cultured for 3 days. Fig.1 Giemsa-stained smear. Note different types of PHA blastoid cells. The blastoid cell (asterisk) at the lower-right side of the micrograph contains many clear vacuoles. × 2400. Fig.2 ACPase preparation using azo-dye method. ACPase-positive granules (arrows) are visible. × 2400. Fig.3 BEI-SEM of ACPase preparation using lead method. Lead depositions are seen inside the cells as highlights. Fig.4 SEI-SEM of the same cells as in Figure 3. The cell surface is well-preserved showing microvilli and short ridges in spite of the applied cytochemical procedure. × 8500.



Figs. 5-8 SEM and TEM micrographs of PHA-stimulated cells cultured for 3 days. Fig.5 BEI-SEM of the cells after ACPase reaction. The faint, granular images may be lipid bodies (arrowheads). × 8300. Fig.6 SEI -SEM of the cells without cytochemical procedure. The cell surfaces are comparable to those seen in Figure 4. × 8300. Figs. 7 and 8 TEM of PHA blastoid cells showing ACPase-positive reactions within lysosomes and sometimes in the Golgi lamella (arrows). × 12,000.



Figs. 9-12 Light-, TEM and SEM micrographs of PHA-stimulated cells after peroxidase reaction. Figs. 9 and 10 Peroxidase-positive cytoplasmic granules (arrows) are visible. × 2200, × 4200. Fig.11 BEI mode in SEM. Such peroxidase-positive granules are clearly seen as highlights. The surface ruffles are faintly visible. Fig.12 SEI mode of the same cells as in Figure 11. × 6000.



Figs. 13 and 14 Light-and SEM micrographs of PHA-stimulated cells after esterase reaction. Fig.13 Nonspecific acid α-naphthyl acetate esterase. The blastoid cells contain granular (arrows) or dot-like (double arrows) reaction products, while the majority of esterase-positive lymphocytes displays dot-like staining pattern (arrowheads). Counterstained with methyl green. × 2200. Fig.14 BEI-SEM of the cells after incubation in Hanker's medium for esterase activity. Numerous granular (arrows) or dot-like (double arrows) reaction products are visible as highlights, while strong, globular highlights (arrowheads) could be interpreted as a deposition in lipid bodies at the cell surface after osmium treatment. × 5500.

Figs. 15 and 16 BEI- and SEI-SEM micrographs of PHA-stimulated cells with silver staining. Fig.15 BEI mode in SEM. The nuclear shapes and the fine chromatin patterns are demostrated as highlights. Fig.16 SEI mode of the same cells as in Figure 15. The cells surface preservation is satisfactory even after silver staining. × 4500.

was considerably higher in the nuclei, correlating perfectly with what was observed in the SEI mode although a certain degree of cytoplasmic staining was always observed by TEM. The preservation of the cell surface was also adequate even after silver staining (Fig. 16).

Disscussion

The identification of blastoid cells in mitogen-stimulated lymphocyte sulture by conventional SEM in the SEI mode alone is frequently difficult and requires establishing a direct control at the level of each individual cells by light and electron microscopic cytochemistry. The use of the BEI mode in SEM is more useful for observation of intracytoplasmic organelles in whole cells teated for enzyme cytochemistry. The present study demonstrates direct correlatlion of surface morphology and interior enzyme activity of PHA blastoid cells when observed in the SEI and BEI modes.

Large cells in PHA-stimulated culture at 3 days, which are blastoid cells and monocytes from the initial cell inoculum, have intense ACPase activity. Such cells are characterized by the presence of lead precipitates as irregular shaped particles which probably correspond to lysosomes, phagosomes or endoplasmic reticulums. The insoluble precipitates of the lead phosphate produced by the enzyme reaction generate an intense backscattered electron signal (Lead, $Z=82$) and lead ions do not bind any appreciable amount to nonspecific cellular components. Moreover, the surface structure of the cells was always well-preserved in spite of the short fixation and cytochemical reaction and comparable to conventional SEM controls. ACPase activity in human leukemic cells has also been demonstrated in the BEI of SEM[6-8]. Such lead-based reaction for ACPase activity, therefore, may be the best available cytochemical method for the BEI study.

Graham and Karnovsky method[18] is originally recommended for TEM to demonstrate proxidase activity. This method is based on the deposition of DAB-osmium complexes at the sites of proxidase activity. Since osmium inevitably binds to cell membrane structures and also fatty substance during long osmification, the samples should have very brief osmium treatment. In the present material, therefore, the BEI contrast is due primarily to sample composition. The effective contrasting element, osmium ($Z=76$), is deposited preferentially in spite of the short osmification at the sites of DAB reaction for peroxidase activity. Topographic contrast may occasionally be evident in selectively stained structures although some globular bodies are slightly highlighted. Such enzyme activity, which is present in the azurophilic granules of myeloid cells[26], was already demonstrated in the BEI mode of SEM in rat bone marrow[4,5] and human blood leukocytes[6-8].

The esterase method, which utilizes the osmiophilic properties of the azo-dye-aryl complexes[20], has been developed for the TEM. This method was applied to the ultrastructural study of white blood cells by Payne et al.[27] In the recent BEI-SEM study using this method, Soligo et al.[7] have demonstrated that numerous granules stained with Hanker's medium for nonspecific esterase activity are visible in human leukemic monoblast. In the present BEI-SEM study, many granular or dot-like osmium deposites are obtained in PHA blastoid cells, occasionally in small lymphocytes. This method allows a precise localization of the sites of the esterase activity but has the same disadvantages already discussed for peroxidase BEI cytochemistry.

The identification of polymorphonuclear leukocytes on the basis of surface morphology only by conventional SEM in the SEI mode is little difficult. The most widely used staining technique for the nucleus is the Gomori silver methenamine method, first demonstrated in the BEI mode by Becker and Sogard[5]. The detail of the nuclear contour within cultured cells can be observed in the BEI mode although the intensity of the BEI signal varies with the degree of chromatin condensation. Especially, the nuclei of blastoid cells with dispersed chromatin appear less contrasted but the BEIs make it possible to recognize the shapes of their nuclei. Sometimes an unpredicable cytoplasmic

staining masks the details of nuclear contours. It should be emphasized that the best way for the nuclear morphology in the BEI mode must be the use of thinly spread cells rather than spherical blood cells.

The same cultured cells during the period of proliferation can display various surface morphologies under the influence of metabolic cell cycle. The existance of such morphological and cytochemical heterogeneity among activated cells has been noted[10.11.28]. The intensity of the nonspecific esterase activity, for example, in mitogen-stimulated lymphocytes varies considerably according to blastoid transformation and sometimes the enzyme is widely distributed in their cytoplasm[29]. However, it is fairly difficult to explain to what extent the morphological and cytochemical heterogeneity reflects varying degrees of activation of the cells.

The deeply localized ones inside the cells can not be seen, especially in the cells displaying a complex surface structure. The intensity of the BEI signal is higher when it originates from a structure located close to the cell surface. Highly contrasted granules within the cells stained for peroxidase activity, for example, are frequently seen mixed with less contrasted ones. The latter are probably located deeper inside the cells. Therefore, less contrasted in the BEI mode should not necessarily be interpreted as indicating a weaker enzyme activity. The intensity of enzyme activity, in a strict sense, should be determined cytochemically by combined BEI-SEM and TEM observation.

As illustrated in this paper for enzyme activity and nuclear staining, the direct correlation between the presence of intracytoplasmic organelles having a given enzyme activity and the surface morphology can be established at the level of cultured cells in the BEI and SEI mode of SEM. Cytochemistry in the BEI mode of SEM may provide us with useful method for identification of lymphoid and myeloid subpopulations concerning cytoplasmic and nuclear maturation of the individual cells. This SEM cytochemistry seems to offer interesting possibilities, *that is*, the three dimensional viewing of BEI-SEM micrographs can provide unique morphological information from combined cytochemical and immunological analysis.

References

1) Wells, O.C.: Backscattered electron image (BEI) in the scanning electron microscope (SEM). *Scanning Electron Microsc.*, I: 747-771, 1977.

2) Watanabe, S. and Ohishi, T.: Labeling methods of lymph node cells for a scanning electron microscopy. *In* T. Takeuchi, K. Ogawa, S. Fujita (eds.), *Histochemistry and cytochemistry.* Tokyo: Japan Society of Histochemistry and Cytochemistry, 1972, p. 167-168.

3) Abraham, J.L. and DeNee, P.B.: Scanning -electron-microscope histochemistry using backscattered electrons and metal stains. *Lancet,* **1**: 1125, 1973.

4) Becker, R.P. and DeBruyn P.P.H.: Backscattered electron imaging of endogenous and exogenous peroxidase activity in rat bone marrow. *Scanning Electron Microsc.,* II: 171-178, 1976.

5) Becker, R.P. and Sogard, M.: Visualization of subsurface structures in cells and tissues by backscattered electron imaging. *Scanning Electron Microsc.,* II: 835-870, 1979.

6) Soligo, D. and DeHarven, E.: Ultrastructural cytochemical localization by backscattered electron imaging of white blood cells. *J. Histochem. Cytochem.,* **29**: 1071-1079, 1981.

7) Soligo, D., Pozzoli, E., Nava, M.T. and Polli, N., Lambertenghi-Deliliers, G. and DeHarven, E.: Cytochemical methods for the backscattered electron imaging mode of scanning electron microscopy: Further applications to the study of human leukemic cells.*Scanning Electron Microsc.,* Ⅳ: 1795-1802, 1983.

8) Soligo, D., DeHarven, E., Pozzoli, E., Nava, M.T., Polli, N. and Lambertenghi-Deliliers, G.: Scanning electron microscope cytochemistry of blood cells. *Scanning Electron Microsc.,* II: 817-825, 1985.

9) Nowell, P.C.: Phytohemagglutinin: An initiator of mitosis in cultures of normal human leukocytes.*Cancer Res.,* **20**: 462-466, 1960.

10) Elves, N.W.: In vitro lymphocyte transformation and antibody formation. *In The lymphocytes.* Lloyd-Luke Ltd, London, 1966, p.196-225.

11) Ling, N.R. and Kay, J.C.: *Lymphocyte stimulation,* North-Holland Publishing Co./American Elsevier Publishing Co. Oxford and New York, 1975, p.97-102.

12) Newell, D.G. and Roath, S.: The surface morphology of mitogen-stimulated human peripheral blood lymphocytes. *Br. J. Haematol.*, **39**: 615-622, 1978.
13) Cuschieri, A. and Mughal, S.: Surface morphology of mitogen-activated human lymphocytes and their derivatives in vitro. *J. Anat.*, **140**: 93-104, 1985.
14) Kato, S.: Studies on inhibition of human lymphocyte blastogenesis by hydrocortisone in vitro. I. Effect of hydrocortisone on lymphocyte response to phytohemagglutinin. *Bull. Yamaguchi Med. Sch.*, **24**: 1-13. 1977.
15) Kato, S., Awaya, K., Nakatsukasa, Y. and Okuda, Y.: Blastogenic property of lectin from semen jequirity (*Abrus precatorius L.*). *Bull. Yamaguchi Med. Sch.*, **28**: 27-34, 1981.
16) Goldburg, A.F. and Barka, T.: Acid phosphatase activity in human blood cells. *Nature*, **196**: 267, 1962.
17) Barka, T. and Anderson, P.J.: *Histochemistry, Theory and Practice and Bibliography*, Hoeber Harper & Row, New York, 1965, p. 238-244.
18) Graham, R.C. and Karnovsky, M.J.: The early stages of absorption of injected horseradish peroxidase in the proximal tubules of mouse kidney: ultrastructural cytochemistry by a new technique. *J. Histochem. Cytochem.*, **14**: 291-302, 1966.
19) Knowles, D.M., Hoffman, T., Ferrarini, M. and Kunkel. H.G.: The demonstration of acid α-naphthyl-acetate esterase activity in human lymphocytes: usefulness as a T-cell marker. *Cell Immunol.*, **35**: 112-123, 1978.
20) Hanker, J.S., Katzoff, L., Rosen, H.R., Seligman, M.L. Uelo, H. and Seligman, A.M.: Design and synthesis of thiolesters for the histochemical demonstration of esterase and lipase via formation of osmiophilic diazo thioethers. *J. Med. Chem.*, **9**: 288-291, 1966.
21) Kato, S. and Miyauchi, R.: Enzyme-histochemical visualization of lymphatic capillaries in the mouse tongue: Light and electron microscopic study. *Okajimas Folia Anat. Jpn.*, **65**: 391-404, 1989.
22) Kato, S.: Enzyme-histochemical identification of lymphatic vessels by light and backscattered image scanning electron microscopy. *Stain Technol.*, **65**: 131-137, 1990.
23) Kato, S.: Enzyme-histochemical demonstration of intralobular lymphatic vessels in the mouse thymus. *Arch. Histol. Cytol.*, **53** (Supplement): 87-94, 1990.
24) Kato, S. and Gotoh, M.: Application of backscattered electron imaging to enzyme histochemistry of lymphatic capillaries. *J. Electron Microsc.*, **39**: 186-190, 1990.
25) Kato, S. and Schoefl, G.I.: Microvasculature of normal and involuted mouse thymus. Light- and electron-microscopic study. *Acta Anat.*, **135**: 1-11, 1989.
26) Bainton, D.F. and Farguhar, M.G.: Differences in enzyme content of azurophil and specific granules of polymorphocuclear leukocytes. II Cytochemistry and electron microscopy of bone marrow cells. *J. Cell Biol.*, **39**: 299-317, 1968.
27) Payne, B.C., Kim, H., Pangalis, G.A., Rothman, A. and Rappaport, H.: A method for the ultrastructural demonstration of non-specific esterase in human blood and lymphoid tissue. *Histochem. J.*, **12**: 71-86, 1980.
28) Cuschieri, A., Mughal, S. and Kharbat, B.A.: The fate of phytohemagglutinin-activated human lymphocytes following their peak proliferative activity. *J. Anat.*, **140**: 79-92, 1985.
29) Kato, S. and Kurihara, K.: Nonspecific esterase activity and E-rosette formation of activated T lymphocyte (in Japanese). *Igakunoayumi*, **117**: 1076-1079, 1981.