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SCANNING ELECTRON MICROSCOPE CYTOCHEMISTRY OF BLOOD CELLS

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Abstract

The backscattered electron imaging (BEI) mode of scanning electron microscopy (SEM) has been applied to study various histo-cytochemical reactions in biological specimens since the early seventies. Due to numerous, recent technical improvements the BEI mode of SEM now belongs to the routine of many SEM laboratories.

For cytochemistry, BEI has been mainly used to: visualize intracellular structures and organelles; recognize the different cell types in heterogeneous populations or tissues; study the correlations between enzymatic activities and cell surface features.

We have evaluated the most relevant results obtained in the study of blood cells and the possible future applications of these techniques.

KEY WORDS: Scanning Electron Microscopy, Backscattered Electron Imaging, Cytochemistry, Bloods Cells, Leukocytes.

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Introduction

The backscattered electron imaging (BEI) mode of SEM permits the visualization of intracellular structures stained with heavy metals in intact cells (7), and offers a rapid method for their study, simpler and less time consuming than serial sectioning reconstruction or high voltage electron microscopy. Silver stains have been utilized to recognize nuclear shape and to study chromatin distribution (7,19) as well as nucleoli (22). Mitochondria have been studied in sperm after diamionobenzidine/osmium reactions (7) and in heart cells after lead/copper staining (18). Moreover, the possibility of staining the endoplasmic reticulum by means of enzymatic reactions has been indicated in a previous report (28). Backscattered electron imaging is expected to be applied increasingly in the near future to the study of intracellular structures, because of the availability of improved microscopes, more sensitive detectors and more selective stains. Up to now, however, BEI has been utilized in hematology mainly to identify cytochemically different cell types under the SEM. (4,26,27,28). Our aim is to analyze the usefulness of this technique in the classification of different blood cells and to study the surface features of accurately identified cells. Different BEI methods applicable to the study of blood cells will be illustrated and the significance of the results briefly discussed.

Cytochemical stain

While paraffin sections stained with classical heavy metal light microscopy methods can provide excellent backscattered electron (BE) images (16), many cytochemical procedures for transmission electron microscopy (TEM) can be used for the BEI study of critical point

dried cells or tissues (7,28), sometimes with only slight modifications. Methods based upon the precipitation of silver (Z=47) salts permit to visualize reticular fibers, basement membranes (1,2,15) and nuclei (1,2,7,25,26,27). For the latter, the most widely used is the Gomori silver stain, as modified by Becker and Sogard (7). As stressed by these authors, the critical prerequisites for good silver staining are 1) incubation in a continuously stirred medium to avoid the deposition of reduced silver particles on the sample and, more importantly, 2) the pH of the incubation medium which should be rigorously kept around 8.8/8.9.

Osmium (Z=76) tetroxide complexed with diaminobenzidine (DAB) has been used to visualize granules containing myeloperoxidase (6,7,26,27,28) or esterase (28) in white blood cells, mitochondria containing cytochrome oxidase (7), phagosomes containing horseradish peroxidase (7) and, more recently, to localize tissue antigens with peroxidase labeled antibodies (17).

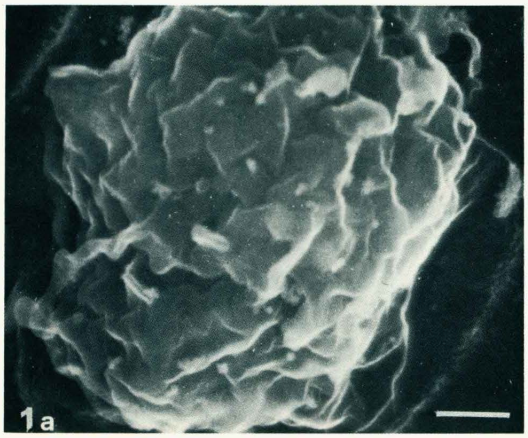
Although osmium has numerous advantages over other heavy metals, its non-specific binding to all lipid containing structures causes problems already discussed in a previous report (27).

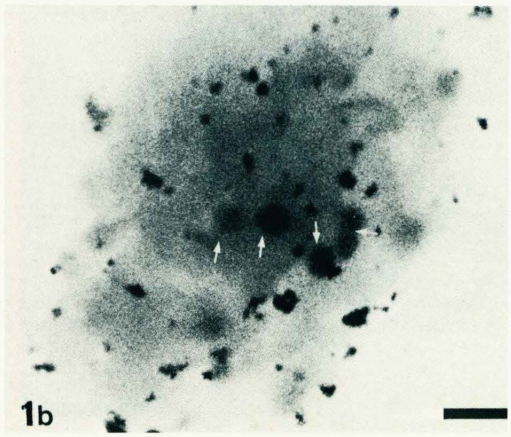
Lead (Z=82) salts have been used to demonstrate acid and alkaline phosphatase activity in different cells and tissues (7,10,24,26,27,28). The intense BE signal produced by lead containing structures and the specific deposition of lead in certain cytoplasmic organelles make this cytochemical method ideal for double stains as first demonstrated in macrophages incubated for acid phosphatase and stained with silver methenamine (7). An acid phosphatase lead method has been recently combined with the immunogold labeling of cell surface antigens; lead and gold markers can be viewed simultaneously, as shown in fig. 1a,b, in the BEI mode.

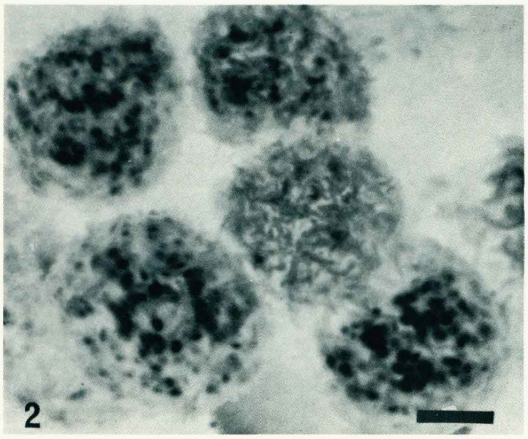
Lead-copper citrate and osmium have been recently used by Le Furgey (18) to visualize myocardial cells mitochondria in the BEI mode of the SEM. Different methods of counterstain or of intensification of the cytochemical reaction products have been recommended for TEM and are generally based on the capacity of DAB to bind different metal ions (3,20). Encouraging results, illustrated in a previous report (28), were obtained in the BE imaging of myeloperoxidase positive granules, post-incubating with cobalt-nickel, gold chloride (28) and more recently with sodium gold chloride (20). Eosinophil peroxidase shown with a DAB medium was intensified by post-treatment with copper nitrate (8).

While the early application of BEI to cytochemistry (6,7) required microscopes equipped with field emission or lanthanum hexaboride cathodes, mainly because of the low sensitivity of the available detectors, the majority of cytochemical reaction products can now be visualized with various commercially available backscattered electron detectors and conventional tungsten cathodes. However, the two more important limiting factors affecting BE imaging remain electron sources and backscattered electron detectors. Field emission microscopes are not available in most biological laboratories and, in our experience, significant difference for backscattered electron imaging between tungsten and lanthanum hexaboride filaments was found only when working at low accelerating voltage. Yet, no significant difference exists among the various types of commercially available detectors, their sensitivity being generally sufficient to visualize cytochemical reaction products. Advantageous, however, are BEI detectors with high speed for easier routine observations in the TV scan mode, or of a particular geometry which facilitates signal mixing (29).

In BEI imaging of biological samples, special attention has to be given to specimen coating. Carbon coating of cell monolayers is compatible with the collection of an adequate secondary signal without noticeable charging artefacts, and of an intense BE signal. Greater problems are encountered, however, when working with tissues; in fact, even with a very heavy carbon coating, it is sometimes impossible to avoid electrostatic charging. Some authors have suggested that a thin layer of gold does not preclude BEI observation, while improving the secondary image (7,25). However, film thickness should be carefully monitored, since the BE signal emitted from intracellular structures can, in our experience, be dramatically obliterated even by a small excess of gold coating. Theoretically at least, other metals recently recommended for high resolution SEM (21) seem more promising. In particular, vanadium (Z=23) and chromium (Z=24) should offer high conductivity and good secondary electron emission, while allowing for the collection of backscattered electron signals originating from intracellular sites.







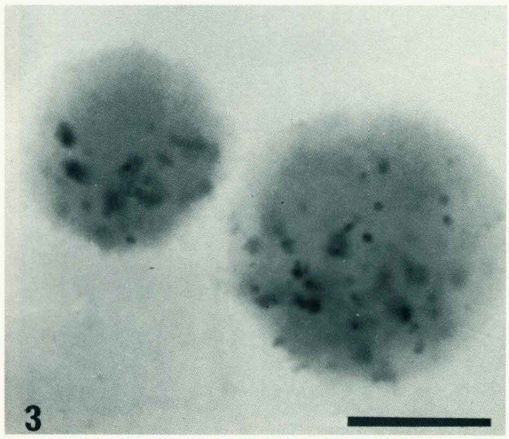


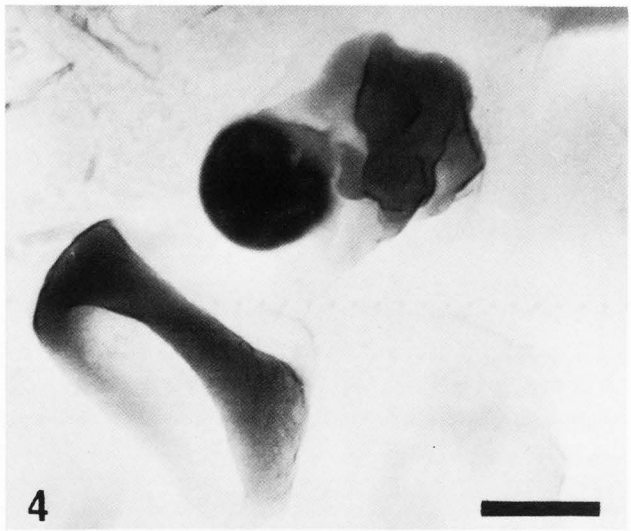
Fig. 1. Acute monoblastic leukemic cell incubated with the Leu M1 monoclonal antibody (Becton-Dickinson) and then reacted with goat anti-mouse immunoglobulins coupled with 40 nm gold particles (GAM G40 Janssen Pharmaceutica, Beerse, Belgium) fixed for 30 min in glutaraldehyde 2% and subsequently incubated in a lead medium for the demonstration of acid phosphatase activity.

a) SEI mode of SEM. Gold particles, generally clumped in small aggregates, are very difficult to recognize on the surface of the cell.

b) BEI mode of SEM. Numerous highly electron scattering gold particles are now visible all over the cell. In addition large and less dense black dots (arrow), representing lead stained lysosomes, show up in the center of the cell apparently underneath the gold labeled surface. Bar = 10 µm

Fig. 2. Acute myeloblastic leukemia. BEI mode of SEM (reverse polarity). Numerous peroxidase positive granules are visible inside three cells. Bar = 3 µm

Fig. 3. T-cell acute lymphoblastic leukemia. BEI mode of SEM (reverse polarity). Cells incubated for the demonstration of acid phosphatase activity clearly observed in two of them. Bar = 3 µm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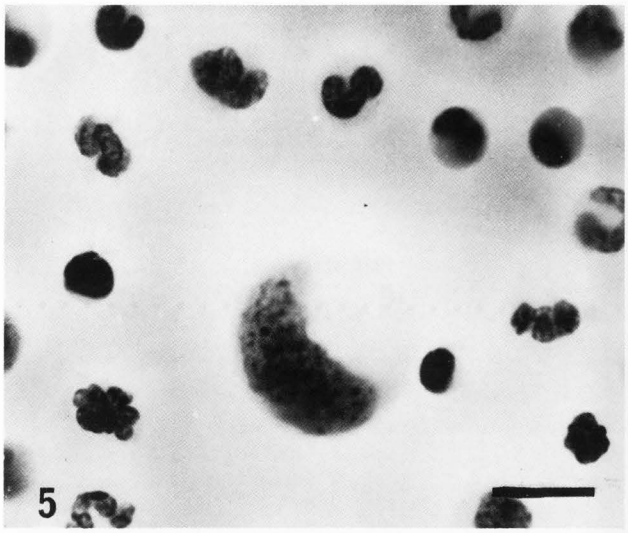
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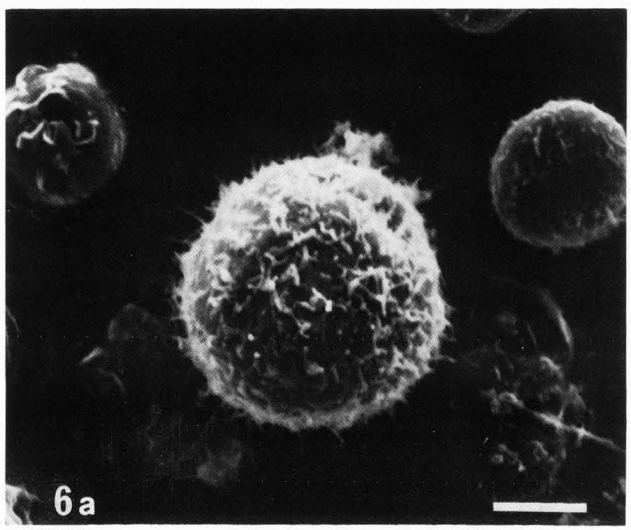
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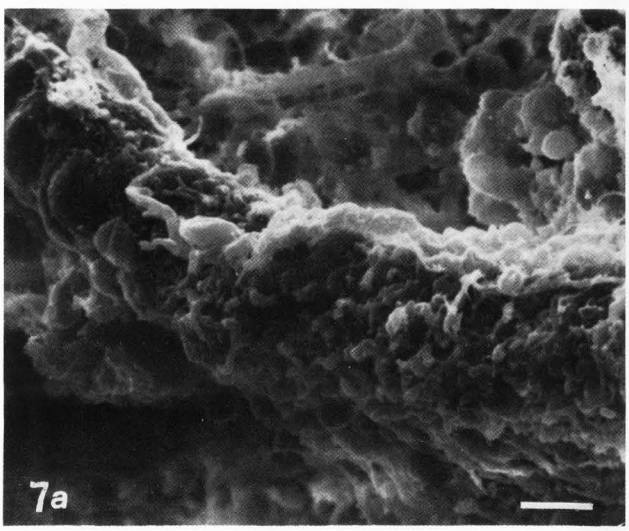
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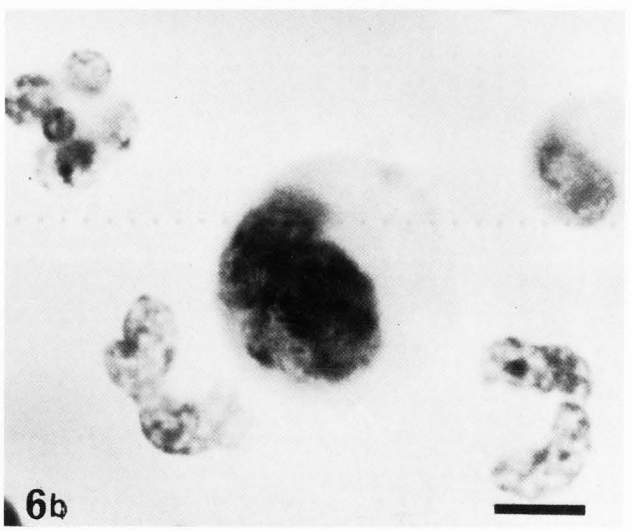
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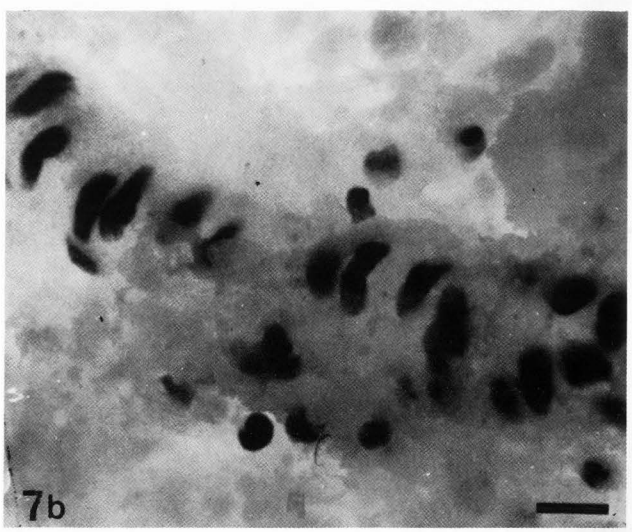
7b

Fig. 4: Peripheral blood from a thalassemic patient. Silver methenamine stain. BEI mode of SEM (reverse polarity). A late erythroblast in the process of extruding a condensed nucleus as observed next to a normal discoid shaped erythrocyte.
Bar = 3 µm

Fig. 5: Normal bone marrow leukocytes stained with a silver methenamine nuclear stain. BEI mode of SEM (reverse polarity). The cell in the middle of the micrograph can be identified as belonging to the megakaryocytic lineage on the basis of its dimension and typical nuclear morphology.
Bar = 1 µm

Fig. 6: Bone marrow cells from a patient affected by idiopathic thrombocytopenic purpura. Silver methenamine nuclear stains. a) Secondary electron imaging (SEI) mode of SEM, b) BEI mode of SEM (reverse polarity). The large cell in the middle of the micrograph shows a typical megakaryocytic bilobated nucleus.
Bar = 2 µm

Fig. 7: Normal human spleen incubated in a silver methenamine medium. a) SEI mode of SEM b) BEI mode of SEM. A large blood vessel is recognized in the spleen parenchyma only when utilizing the BEI mode (b) which shows numerous concentrically arranged nuclei belonging to subendothelial muscle cells.
Bar = 1 µm

Cell Type Identification

Cytochemical methods applicable to the BEI mode of SEM have been developed with the aim of obtaining more information from cell or tissue samples. This was much needed in hematology since leukocytes are difficult to recognize on the basis of cell surface morphology alone (23,31,32). The possibility to correlate cell surface morphology with enzymatic pattern and/or intracytoplasmic structures permits cell type identification under the SEM in mixed or heterogeneous cell populations. This technique, even if it did not put an end to the debate on cell surface characteristics of lymphocyte subsets (5,13), is nevertheless very useful to identify other normal and leukemic blood cells (4,8,26,27,28). The myeloperoxidase reaction was particularly adequate for the recognition of myeloid precursors both in normal and in leukemic patients (Fig. 2) (6,7,26,27,28). The BEI mode of the SEM was used in particular to make evident barely myeloperoxidase positive granules which are not visible with the light microscope and can only be identified in thin sections prepared for TEM (27). Acid phosphatase reaction permitted us to identify early monoblasts and T-lymphoblasts under the SEM (28) (Fig.3). In the BEI mode of the SEM, small cytochemical reaction sites at various depths and on a large number of cells are visualized simultaneously, and a rapid cytochemical identification of leukemic cells in combination with immunological markers both at the light and transmission electron microscope levels is possible (27,28).

The cell surface morphology of blood cells such as erythroblasts and megakaryoblasts has not been described with the SEM. This could be due to the extreme difficulty in recognizing them among the very heterogeneous bone marrow immature cells. The SEM of erythroid precursors was described by Bessis only in peripheral blood and at the reticulocyte stage (9). These cells can, however, be easily identified in the BEI mode after silver staining of their nuclei. The latter are typically round and progressively pyknotic (Fig. 4). The surface changes which accompany erythroid cell maturation can therefore easily be followed both in normal and in pathological conditions.

Megakaryocytes have been only tentatively described with the SEM in the lung (30), after elaborate separation with velocity sedimentation techniques (12), or in culture (11).
The BEI mode of SEM permits the easy recognition of megakaryocytes by their typical nuclear morphology (Fig. 5, 6a,b). The technique offers a unique opportunity to study the surface morphology of these cells and the sequential steps of platelet formation.

While our experience has been mainly limited to the study of cells from peripheral blood or bone marrow the need for cell type identification by appropriate cytochemical methods is even more pressing in the SEM study of solid tissues. In hematopoietic tissues, in fact, cells are generally heterogeneous. Moreover, extracellular matrix, suboptimal cell surface preservation as well as charging artefacts can all affect cell type identification. Among the different available cytochemical methods, silver staining of the nucleus is no doubt the most useful and has been applied to the study of many different tissues with the SEM (1,2,7,15). In

our limited experience with spleen and bone marrow, this method revealed structures which had previously been almost impossible to recognize. In particular, lymphatic follicles with different degrees of lymphocyte activation, as well as macrophages, reticular, muscle and endothelial cells were immediately identified (Figs. 7a and b). The amount of information which can easily be obtained is considerable, and should result in important advances in the study of hematopoietic tissues.

Cell Surface Characteristics

Since the first applications of SEM to the study of blood cells, the question was raised whether some cell surface projections were typical of a certain cell type or due to the expression of different metabolic, cell cycle or maturation stages. Conversely, did they merely result from artefactual changes induced by preparative procedures (13,31,32)? Some of these questions can, in part, be answered now utilizing BEI cytochemical methods which permit a correlation between the degree of nuclear and cytoplasmic maturation and the cell surface morphology of leukocytes (27). From the study of a large number of normal and leukemic cells it appears that: very immature cells have generally smooth surfaces, whatever their lineage; mature lymphocytes display a variable number of microvilli; ruffles are almost invariably observed on monocytes and macrophages; myeloid precursor cells have surface ridges, even if not constantly.
These surface features, the interpretation of which is now being further confirmed with immuno-SEM methods (13,14), can be observed both in cells attached to a substrate shortly after separation and subsequently fixed, as well as in tissue cells. Cells fixed in suspension or fixed a long time after attachment to a substrate, or cells in culture show a variable surface morphology. More frequently, however, they display an irregular but smooth surface. Provided standardized preparative procedures are followed, some surface features may be used safely to identify several blood cell types under the SEM in the secondary electron imaging mode.

Conclusions

The contributions of BEI cytochemistry to the study of blood cells have been, up to now, rather limited although this technique seems to offer interesting possibilities. The development of more specific stains is needed. The application of existing methods to the study of multicellular structures, where the three-dimensional viewing of SEM micrographs can provide unique morphological information, can be foreseen to contribute important data in the near future. Other promising applications are anticipated in the field of combined SEM methods. One of the advantages of BEI cytochemistry is that it permits the simultaneous visualization of intracellular cytochemical reaction products together with the labeling of the intact cell surface with immunological markers. The combination of acid phosphatase cytochemistry with a colloidal gold immuno-SEM method is now being explored in our laboratory with interesting preliminary results (Fig. 1a,b). Moreover, the classification of normal and leukemic cells as well as the study of surface antigens in various differentiation stages is expected to benefit from such combined cytochemical and immunological analysis with the SEM.

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Discussion with Reviewers

J.L. Abraham: I have no specific questions except for a general one to ask the authors to elaborate on specifics of diagnostic problems which were resolved by their techniques.

Authors: We routinely utilize myeloperoxidase and acid phosphatase BEI cytochemistry in the diagnosis of non-lymphoblastic leukemias. Cases of hypogranular myeloid leukemia or of scarcely differentiated monoblastic leukemia can have few or sparse granules, respectively MPO or APase positive, which can be seen by the BEI but not by LM. Positive identification of these cells can be reached with the BEI in few hours while the final ultrastructural diagnosis will be obtained only days after when TEM cytochemical controls will be available.

R.P. Becker: What developments do you foresee in the field of blood cell SEM cytochemistry in the next five years?

Authors: The more interesting developments in blood cell cytochemistry are now in the combination of cytochemical and immunological techniques both for LM and TEM. The same combined techniques should prove to be feasible and useful also for the SEM in the near future. Systematic study of hematopoietic tissues with cell type recognition by SEM cytochemistry or immunolabeling is another field of great interest, scarcely studied up to now.

R. Albrecht: How would you evaluate the limit of detection, localization, and pattern of deposition of BEI cytochemistry over light cytochemistry?

Authors: While with LM we can examine only sections of blood cell films, with the BEI we can examine whole cells, particularly the reaction products in the cytoplasmic rim just underneath the plasmalemma at depth depending on many variables (specimen composition, characteristic of the reaction product, energy of the incident electron beam etc). Limitations, however, derive from the sensitivity of the BEI detector since, at least theoretically, low Z elements or sparse depositions (scattering only few electrons) should also be seen with an optimal detector.

Although it is well recognized that most of the cytochemical reactions studied here were originally described under the light microscope, it is clear that only the SEM in the BEI mode permits to correlate the cytochemical make-up of the cells with their surface structures.

R. Albrecht: The use of BEI cytochemistry as an improved alternative to light microscopy cytochemistry does not preclude the use of light cytochemistry on the same samples. The use of light microscopy to view the electron (and photon) dense deposits permits "checking" of samples prior to SEM evaluation. The more classic dye-type reaction products can also be viewed allowing the determination of several cytochemical parameters on a single specimen. Two dark heavy metal deposits can, unless the distribution patterns are non-overlapping or very distinctive, mask each other. However light microscopic determination of one marker followed by BEI of another is possible and can significantly increase the information obtained. The same is true with respect to immunolabelling; gold bead labelling, if dense enough, can be seen using the light microscope,

and fluorescent antibodies can be attached to gold beads (the label is then visible by UV light; SEM; TEM; HVEM; and, if dense enough, by visible light). Fluorescent second antibodies can also be used to label the ligand or antibody attached to the gold bead. These procedures again permit "checking" of samples prior to SEM observation and bridges the gap between fluorescent antibody studies and SEM studies using the gold bead procedure. An additional advantage is that fluorescent activated cell sorting procedures can be used to separate cell populations which can then be viewed by SEM and the surface antigen distribution determined.
Authors: Thank you for the interesting remarks.

J.S. Hanker: Are you aware of any publications, or do you have any experience, suggesting the discrimination of platelet subtypes, or of true platelets from particles (e.g., pseudoplatelets) produced by fragmentation of blood cells other than megakaryocytes, by a combination of BEI cytochemistry and SEI?
Authors: No, we are not aware of any publication of BEI/SEM of pseudoplatelets. If they contain myeloperoxidase (MPO) positive granules pseudoplatelets could be easily distinguished from true platelets which of course lack MPO activity.

J.S. Hanker: The authors have missed an opportunity to indicate some other possible uses of, and important points about, the combination of BEI cytochemistry and SEI in pathology. It was pointed out in this journal last year (Berman EL et al., Scanning Electron Microscopy /1983/Pages 311-321), and earlier by Hayes (SEM/1972/57), that granules and nuclear lobes of granulocytes, which enable their discrimination, are much more readily observed in flattened air dried, than in rounded freeze-dried or critical point dried cells. Perhaps the most important contribution of the paper by Berman et al., is the demonstration that routine blood films and preparations of patients on microscope slides, if cytochemically stained with a procedure where a metal end-product is deposited, can be used for BEI-SEI studies, even years after preparation?
Authors: Although it is true that nuclear and granular details are sometimes visible in air dried specimens, we think that the loss of surface details makes the SEM study of such samples almost useless. The BEI methods described in our paper permit on the other hand both the visualization of internal structures and the preservation of good surface morphology. The copper nitrate method described by Hanker has been utilized also by us to counterstain DAB treated leukocytes (ref. 28). The application of BEI to metal stained blood films is of course very interesting and worth trying.

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