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IMMUNO-CYTOCHEMISTRY WITH BACKSCATTERED ELECTRONS

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Abstract

Some cytochemical reaction products are visible inside the cytoplasm of cells observed with the scanning electron microscope (SEM) using the backscattered electron imaging (BEI) mode. Methods can be utilized whenever they result in the deposition of heavy metal, like silver, lead or osmium at the sites of the enzymatic reaction.

More recently the BEI mode of the SEM has been demonstrated to improve the detection of immunogold labeled cell surface antigens. Colloidal gold particles, 40 to 15 nm in diameter can be efficiently used for immuno-specific labeling. Moreover, cytochemical reactions can be applied to previously immunogold labeled cells, therefore combining the results of enzyme cytochemistry and of surface labeling at the level of each individual cell. The choice of fixative, incubation media, dehydration and drying methods should be guided by considerations on the sample characteristics for optimal electron scattering. Cytochemical as well as immuno-labeling reactions are not used "per se" but in combination with the study of cell surface morphology which needs, therefore, to be sufficiently well preserved. Coating should provide good conductivity and secondary electron emission, while emitting a minimal number of backscattered electrons. The application of these methods considerably enhances our capacity to characterize with the SEM the surface morphology of precisely identified subpopulations of many cell types.

KEYWORDS: Scanning Electron Microscopy, Backscattered electron imaging, cytochemistry, surface labeling, colloidal gold.

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Introduction

The fact that cytochemical methods for both light and transmission electron microscopy can also be applied to scanning electron microscopy in the backscattered electron imaging (BEI) mode was first demonstrated by Watanabe (40), Abraham and DeNee (1,2) and by Carr and McGadey (9). Improved resolution at the single cell level, as well as visualization of intracytoplasmic organelles was demonstrated by Becker and de Bruyn (4) and Becker and Sogard (5). The possibility to apply cytochemistry in the BEI mode to clinical pathology was initially shown by Carter (10) whose work stimulated us to adapt different cytochemical stains to the diagnostic study of hematological diseases (33,34,35). Bowen et al. (7) should also be mentioned for their use of a bromine-labelled azo dye seen both in the backscattered electron and in the X-Ray imaging modes, and Le Furgey for the first BE image of lead-copper stained mitochondria (19). Finally the application of image analysis to the study of BEI micrographs was recently shown by Thiebaut et al. (36) (Table 1.)

Backscattered electron imaging was also applied in immunocytochemistry to visualize surface markers susceptible to provide adequate atomic number contrast. Hartman and Nakane (16) labeled sections of fresh frozen tissue with peroxidase/osmium labelled antibodies, selectively visualized due to their intense electron backscattering. However, colloidal gold markers soon attracted the interest of many immuno-electron microscopists (18). Efficient visualization of these markers in the BEI mode was initially reported by Trejdosiewicz (37). Imaging colloidal gold particles in the BEI mode appears as an interesting method in immuno-SEM since it combines the numerous advantages of colloidal gold (12) with optimum visualization in the SEM (11, 17). The efficiency of immunogold labeling being considerably

dependent upon the small size of the colloidal particles (12), advantage of the high resolution of currently available SEM in the BEI mode must be taken. BEI resolution of 20 nm and 15 nm gold particles has been demonstrated by de Harven et al. (11) and Walther et al. (39) respectively (Table 2). Finally, it has been shown that enzyme cytochemistry can be applied simultaneously with immunogold labeling of human leukocytes prepared for TEM (30), opening the way to the use of such combined methods in SEM.

Materials and Methods

Chemical Fixation

Chemical fixation must preserve

Table 1 Cytochemistry in the BEI mode

Authors	Year	Ref.#	Cell/ Tissue	Stained structures	Enzyme	Metal	Z
Watanabe	1972	40	Lymphnode	lysosomes	Acid phosphatase	Pb	82
Watanabe & Ohishi	1972	41			Alkaline phosph.	Pb	82
					Endogenous perox.	OsO_4	76
Abraham & DeNee	1973/	1	Liver	nuclei	Silver stain	Ag	47
	1974	2	Lung	basement mbs			
DeNee et al.	1974	13	Kidney	reticulin collagen			
Carr & Mc Gadey	1974	9	Pancreas		Glucose-6-phosph.	Pb	82
			cerebellum		Thiamine-pyrophos.	Pb	82
			Epydidimus Golgi		Acid Phosphatase	Pb	82
Becker & de Bruyn	1976	4	Leukocyte Bone Marrow	lysosomes	Endogenous perox.	OsO_4	76
Becker & Sogard	1979	5	Fibroblasts	nuclei	Silver stain	Ag	47
			Tumor cells	accessory	Endogenous perox	OsO_4	76
			Sperms	fibers and mitochondria	Cytochrom. c oxidase	OsO_4	76
Carter	1980	10	Macrophages	lysosomes	Acid phosphatase	Pb	82
Newcombe & Boyde	1980	27	Langerhans cells		ATP-ase	Pb	82
Soligo & de Harven	1981	33	blood cells	nuclei	Silver stain	Ag	47
		34		lysosomes	Endogenous perox.	OsO_4	76
					Esterase	OsO_4	76
					Acid phosphatase	Pb	82
				phagosomes	Alkaline phosph.	Pb	82
Bowen et al.	1983	7	Thymus	lysosomes	Acid phosphatase	Br	35
LeFurgey et al.	1983	19	Heart cells	mitochondria	Lead-copper stain	Pb	82
						Cu	29
Thiebaut et al.	1984	36	Transformed cells	NOR	Silver stain	Ag	47

Table 2 Immunocytochemistry in the BEI mode

Authors	Year	Ref.#	Marker	Size	Tissue/cell
Hartman and Nakane	1981	16	peroxidase-osmium complexes	----	Mouse kidney
Tredjdosiewicz et al.	1981	37	colloidal gold particles	45 nm	Fibroblasts and bladder epithelium
de Harven et al.	1984	11	PA-gold	45-20 nm	Leukocytes
Hicks and Molday	1983	17	dextran-gold	30 nm	Fibroblasts
Nava et al.	1984	26	PA-gold	40 nm	Red blood cells
Walther et al.	1984	39	PA-gold	15 nm	Red blood cells

Table 3 Cytochemical methods for the BEI

Method	Ref.#	Incubation medium	Post stain	Comments
Endogenous peroxidase	4,5,6,15,33 34,35	DAB and H_2O_2	OsO_4 $CuSO_4$ $Co(NO_3)_2$ $HAuCl_4$	Copper or cobalt post stain do not provide adequate contrast. Gold chloride unpredictably precipitates
Esterase	35	naphthyl-thiol-acetate and fast blue BB	OsO_4	Staining generally weak
Acid phosphatase	3,5,7,9,10 14,32,34,35	Na-β-glycero-phosphate and lead nitrate; naphtylthiol AS-BI phosphoric and 2,5-dibromoaniline	----	Widely used; high contrast despositions
Alkaline phosphatase	24,29,35	Na-β-glycero-phosphate and lead nitrate	----	Diffuse electron scattering
ATPase	27	ATP and lead nitrate	----	Diffuse electron scattering
Silver methenamine	1,2,5,10 16,32,33 34,34	silver nitrate sodium borate methenamine	----	Very high contrast and resolution in flat cells.
Cytochrome c oxidase	5	DAB	OsO_4	limited to mitochondria

cytochemical activity as well as cell surface morphology. In fact, cytochemical SEM studies in the BEI mode, if not correlated with cell surface morphology, are generally useless. In most applications, the best fixative seems to be glutaraldehyde, while fixation with paraformaldehyde, osmium tetroxide, potassium permanganate and picric acid has been, in our experience, always unsatisfactory as far as cell surface preservation is concerned. In addition, glutaraldehyde has always been known to be compatible with the majority of cytochemical reactions. (31).

A particular problem relative to fixation is encountered in immunocytochemistry, where coverslip attached cells undergo numerous, long incubations with media which poorly support cell viability and which are not strictly isotonic or of constant ionic composition. If cells are not prefixed, their surface morphology will, inevitably, be very poorly preserved; in addition, fixation will consolidate cell attachment to the coverslip, thus preventing undesirable cell loss which would be incompatible with any quantitative evaluation of the samples. Finally, clustering, capping and/or endocytosis of surface antigens will occur in cells labeled, at room temperature, prior to fixation (Fig 1). This can be useful to visualize, in the BEI mode at low magnification, aggregates of marker particles. However, antigen redistribution should be prevented if a correlation between surface morphology and antigenic expression is to be established. This can easily be achieved with a brief fixation with glutaraldehyde at low concentration (Fig 2). It should not be obtained with incubation at 4°C or in presence of sodium azide which are both known to alter cell surface structure (21,22). Of course, the structure of certain antigens can be altered by fixation to the point of being no longer recognized by their specific antibodies; it became recently apparent, however, that many monoclonal antibodies can still efficiently recognize their glutaraldehyde prefixed antigens (23).

Incubation Media For Cytochemistry

The various incubation media which we have used, so far, in studies on human leukocytes are listed in Table 3. None was found to be noticeably damaging to the cell surface architecture. All of them resulted in a specific deposition of the electron backscattering reaction product, without significant

diffusion away from the site of the reaction. Specific remarks concerning the various cytochemical markers under study are as follows.

Lead containing reaction products. Many cytochemical reactions end up with the deposition of lead salts which are visible, with high contrast, in the BEI mode of the SEM, even when the reaction product is localized deep inside the cytoplasm (33). TEM controls should always be run in parallel, however, to insure that non-specific staining, and/or diffusion artefacts have been kept to a minimum. Sometimes lead deposits are very small or dispersed in the cytoplasm and a sufficient BE signal cannot be obtained. In the case of alkaline phosphatase, for example, the enzyme is diffusely distributed and can, therefore, hardly be visualized unless it is concentrated in the phagocytic vacuoles of human leukocytes (29).

DAB-osmium methods have been utilized for the demonstration of different enzymatic reactions (Tables 1,3). The affinity of osmium for diaminobenzidine (DAB) and its high atomic number (Z=76) have been exploited for both TEM and SEM in the BEI mode. Limitations of the method reside in the osmium deposition in all phospholipid containing structures. The diffuse, non specific BE signal emitted by osmium stained structures can, however, be minimized by working at low accelerating voltages (33,34). Other metals like copper, nickel, cobalt or gold which also bind to DAB without interacting with other cell components have been tested but the results obtained were never completely satisfactory (35). These methods have, however, the potential advantage of forming stable reaction products, at variance from osmium which sublimates away in samples kept under vacuum. (Personal observation).

Silver stain methods were initially utilized in the BEI mode of SEM for collagen, reticulin fibers, basement membranes and nuclear structures (1,2,13) (table 1). Silver stain is very good for visualization of tissue components at low magnification but sometimes gives poor results at high magnification. In flat or spread cells the nucleus is close to the cell surface and nuclear details can be visualized with such good resolution and contrast as to allow morphometric studies, as shown by Thiebaut (36). In these conditions, a thin layer of gold coating still permits the collection of a satisfactory BE signal (32). Although samples undergo long incubations at 60 ^{o}C and at high pH, cell surface preservation is generally satisfactory.

Incubation Media For Immunocytochemistry

Surface antigens can be identified by polyclonal or monoclonal antibodies. In the case of murine monoclonals of the IgG type, labeling can be obtained with an appropriate dilution of goat anti-murine Ig/colloidal gold conjugate (Fig 2), available commercially (GAM-G40 or GAM-G20, Janssen Pharmaceutica, Beerse, Belgium). When monoclonal antibodies of the IgM type are used, efficient labeling has been demonstrated with a more indirect technique which involves a secondary incubation with a goat anti-murine IgM antibody, followed by a protein A/colloidal gold conjugate (10). In either method, colloidal gold particles, from 40 to 15 nm in diameter (Fig 3), appear as excellent markers for SEM immunocytochemistry in the BEI mode, due primarily to the high atomic number of gold (Z=79), and the high stability and uniform shape of the particles (Table 2). In addition, visualization of the gold marker in the BEI mode will reveal particles "hidden" behind surface microvilli, or ruffles, or inside coated pits, making possible the quantitative evaluation of the labeling which could not be attained with the incomplete viewing of the marker given by the SE observation of the same sample. Finally gold markers appear to be ideal for combined immunocytochemical studies. Gold particles of different sizes can be recognized in the BEI mode (Fig 4), thus opening the way to double labeling studies with the SEM. In addition gold particles labeling surface antigens can be easily seen in the BEI mode simultaneously with intracytoplasmic enzymatic localizations (Figs 5a,6b).

Dehydration and Drying

Dehydration and drying are done according to routine procedures (33). Shrinkage of cells, is always expected to occur (8), but, in some way can be regarded as advantageous for BEI observation since internal structures become closer to the surface and therefore generate a more intense BE signal. However, if shrinkage represents too much of a problem and seems to make quantitative interpretation impossible, then alternate methods of drying will have to be used (20).

Mounting

Optimum imaging of cells in the BEI mode will only be achieved if a minimum of electrons are backscattered from the surface on which the cells are attached (substrate). The substrate should not interact with any of the incubation media (metal coverslips, for instance, should not be used for silver stains) and should be resistant to the solvents used in sample processing. The substrate should also resist beam damage (working with large beam spot size will cause plastic coverslips to buckle, especially if a high intensity

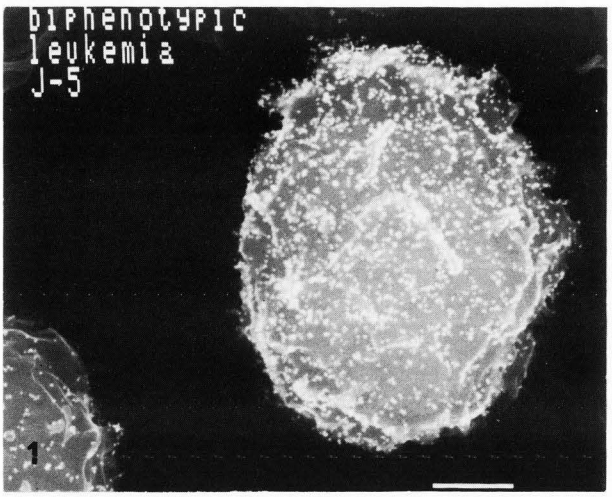


Fig. 1. A 25 kV mixed SE/BE image of a leukemic cell, labeled after incubation with the J5 monoclonal antibody (Becton-Dickson) and a goat-anti-murine IgG antibody conjugated with 40 nm colloidal gold particles (GAM-G40, Janssen Pharmaceutica, Beerse, Belgium) without prefixation. Note the clustering of the gold particles and the complete loss of surface detail. (bar = 1 µm)

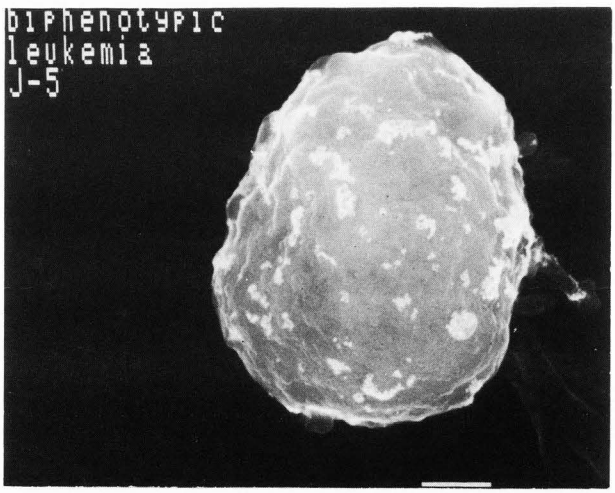


Fig. 2. A 20 kV mixed SE/BE image of a similarly labeled leukemic cell, but after mild glutaraldehyde prefixation. Note the absence of clustering, therefore the relatively even distribution of the marker and the preservation of surface details. (bar = 1 µm)

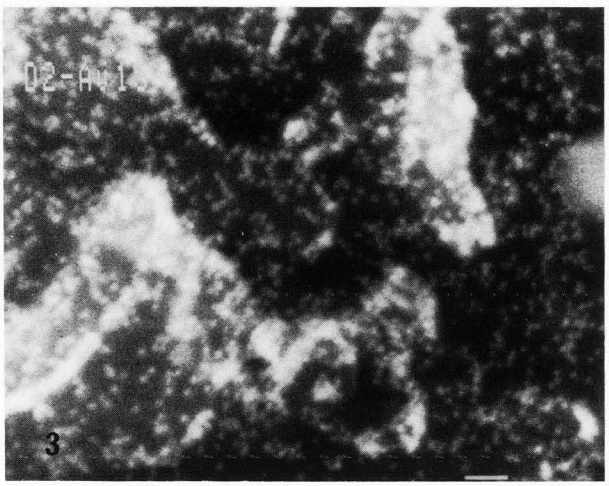


Fig. 3. A 38 kV mixed SE/BE image of a normal human granulocyte incubated after prefixation with the monoclonal antibody D2 (from Dr. K. Shumak, Toronto, Canada) and with a 13 nm colloidal gold conjugate. Note the resolution of single gold particles. (bar = 100 nm)

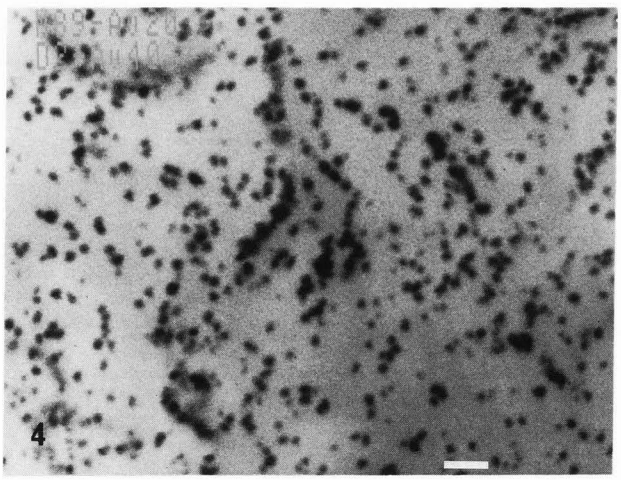


Fig. 4. A 30 kV BE (reverse polarity) image of a prefixed human granulocyte incubated with a mixture of two monoclonal antibodies (one IgG and the other IgM) recognizing different surface antigens. The IgG was labeled with GAM-G20, and the IgM with a 40 nm colloidal gold conjugate.
Note that the marker particles fall into two distinct size classes, indicating that double labeling with 40 and 20 nm gold particles is possible. (bar = 100nm)

electron source (LaB_6) is used). Glass coverslips best fulfill these prerequisites. For samples prepared for BEI study, electrical conductivity is generally insured by carbon coating. A more conductive material, however, would reduce charging of the samples and permit one to decrease the thickness of the coating, ultimately improving resolution both in the BEI and in the SEI modes. To improve conductivity different substrates such as metal coverslips, silicone wafers, and gold or copper carbon coated grids have been used (25). In general the drawbacks of these substrates reside in their cost and/or in their fragility which has to be considered in samples exposed to extensive handling. However the best BEI resolution was shown only when such highly conductive substrates have been utilized for cell mounting (11,39).

Coating

Coating the cell samples with a thin layer of a conductive substance is always necessary for cytochemistry in the BEI mode since samples quickly deteriorate at the relatively large beam spot- size and high accelerating voltages used; in addition, coating is necessary for optimal secondary electron emission from the sample surface. Carbon coating is still unsurpassed for BEI since it makes the sample conductive while allowing for some penetration of the beam inside the cells and the reemergence of backscattered electrons. In some cytochemical applications, when intensity of the BE signal is very high, it has been demonstrated that a thin layer of gold is compatible with a sufficient recovery of backscattered electrons (32). For immunocytochemistry in the BEI mode, problems are slightly different, since backscattered electrons originate from the cell surface and a greater resolution is generally needed. Again, the optimum coating is obtained with a thin film of carbon which mainly acts as a conductive layer and does not increase the size of a backscattering marker particle. Ultrathin films of low atomic number metals (28) should also be considered however, for the BE imaging of the immunogold markers.

Viewing of the samples

To obtain an optimal signal and the maximum amount of information from the observation of samples in the BEI mode, the following conditions must be met. The first deals with beam spot size which is directly proportional to the signal obtained and inversely proportional to the resolution of the image. The accelerating voltage is another very important variable, since penetration of the beam increases with the energy of the incident electrons (5). With higher beam energy, an increased contrast is expected. In addition, different accelerating voltages can be used to explore cells at different depths, i.e. subsurface structures with low energy (5kV) and more deeply located structures with progressively increasing energies (30kV, for example). Optimal working distance should be adjusted to use the best collecting geometry of the detector.

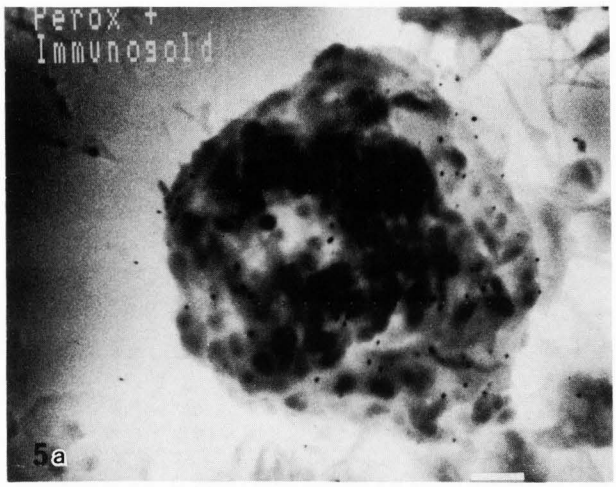
Fig. 5. A 10 kV BE (reverse polarity) image of a prefixed human granulocyte surface labeled for D2 specificities with GAM-G40 and subsequentially treated for the cytochemical detection of endogenous peroxidase (33). A combined imaging of the surface labeling and of the cytochemical reaction products is demonstrated. (bar = 1 μm)

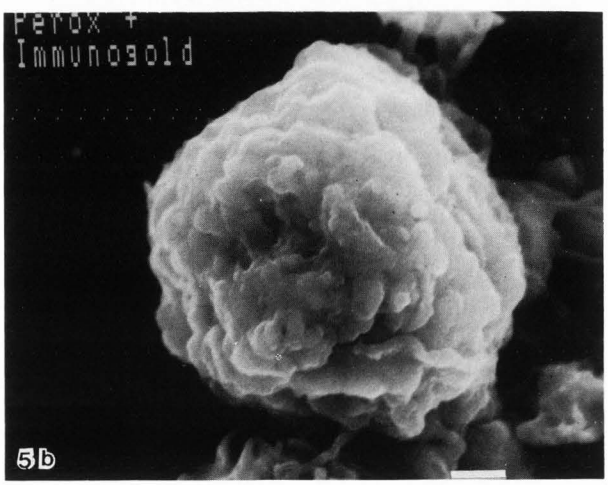
Fig. 5b. Same cell as in Fig 5a, but in the SEI mode.

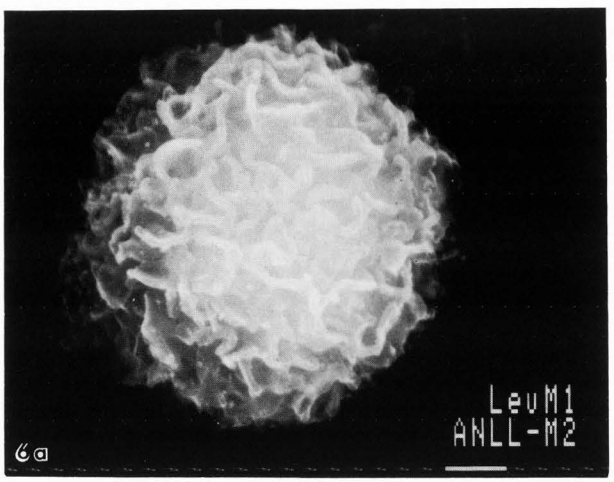
Electrons from the primary beam are backscattered by the cell surface as well as by internal structures: this surface scattering is generally utilized to give a faint image of the cell surface. SE images should, however, always be analyzed to secure a correct interpretations of BEI observations.

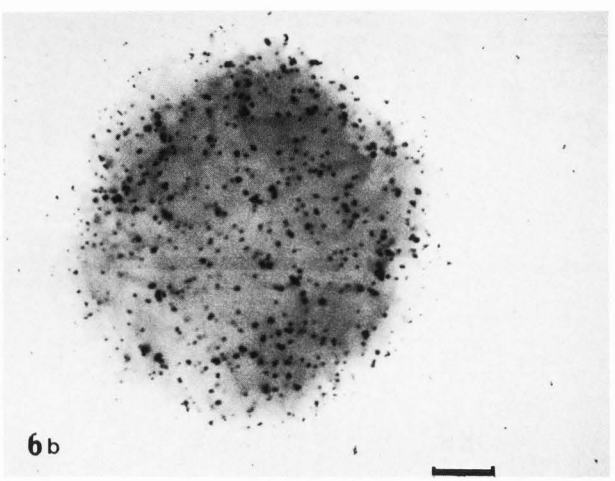
Electronic processing of the signal can significantly alter interpretation of the results. For instance, the image of some backscattering structures can be lost while some others can be enhanced in pictures taken at different contrast. Mixing secondary and backscattered electron signals, as described by Volbert (38), offers a possibility to improve the results of BEI cytochemistry. This can be particularly useful in order to subtract from the SE image the signal contribution from SE-II (28) or to provide a pure BE image of internal structures. More importantly, signal mixing permits one to display in a single image both the SE and BE signals. This has been shown advantageous in the interpretation of immunogold labeled cell surfaces (Figs 6a,6b,6c) (11). An additional possibility to manipulate the BE signal is to invert its polarity, therefore observing "black" backscattering structures and thus providing images of internal structures similar to those we are used to seeing in TEM. This can be useful, although some contrast and image depth may be lost in the process.

In conclusion, it appears more and more clearly that the phenomenon of electron backscattering, which was until a few years ago regarded as an unhelpful interference in SE imaging, can now be utilized to add important analytical capabilities to the SEM, especially in the areas of cytochemistry









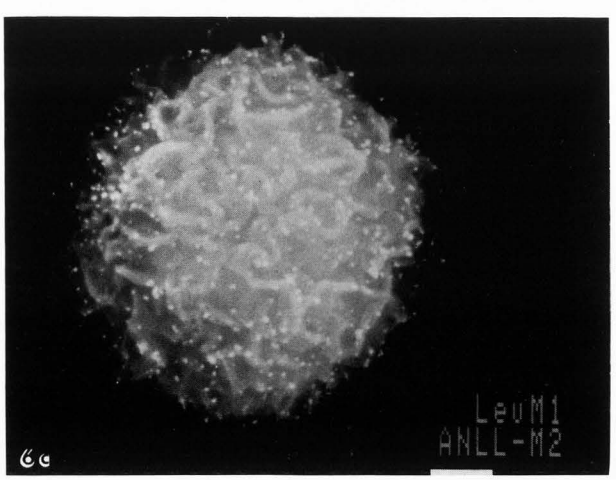


Fig. 6a. A human leukemic cell, incubated with the Leu-M1 monoclonal antibody (Becton-Dickinson) followed by GAM-G40, seen in the SEI mode. The gold particles can occasionally be seen.
(20 kV. bar = 1 μm)

Fig. 6b. The same cell, seen in the BEI mode, reverse polarity. Apparently all the gold particles are visualized in high contrast. In spite of the fact that some particles have formed small aggregates, most of them are seen as single particles. (20 kV. bar = 1 μm)

Fig. 6c. Mixing the SE and the BE signals from the same cell, a combined image is obtained in which both surface morphology and gold labeling are simultaneously demonstrated (BE signal is now in normal polarity).
(20 kV. bar = 1 μm)

and of immunogold labeling of cell surfaces.

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