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# IMMUNO-SCANNING ELECTRON MICROSCOPY OF NORMAL AND LEUKEMIC LEUKOCYTES LABELED WITH COLLOIDAL GOLD

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## Abstract

The immunogold method, utilizing 40 nm colloidal gold particles which can be selectively visualized with the scanning electron microscope (SEM) in the backscattered electron imaging mode was used for the study of blood cells incubated with various monoclonal antibodies. Numerous anti-leukocyte monoclonal antibodies still recognize lightly glutaraldehyde prefixed antigens and can be used to identify various blood cell types and even to recognize their different maturation stages.

Clearcut differences in surface morphology exist among peripheral blood normal leukocytes and even among the principal lymphocyte subclasses. Marked heterogeneity in surface morphology is, on the other hand, evident when studying precursors or leukemic cells. Immature cells show, nevertheless, relatively smooth surfaces while some distinct surface features appear on cells already committed toward a specific differentiation lineage. Hairy cells can also be precisely identified, especially when in small number in heterogeneous populations, combining their typical surface morphology with their positivity for B1 and Leu M5 monoclonal antibodies.

Key Words: Scanning Electron Microscopy, Immunogold, Monoclonal antibodies, Leukemia.

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## Introduction

The surface morphology of blood cells as seen with the scanning electron microscope (SEM), has raised initial controversies (8,30,31). Although the main classes of normal leukocytes generally show distinct surface architectures, it remains very difficult to differentiate among their different subpopulations (8). When leukemic cells are studied, cell surface morphology appears even more variable from case to case (8). In addition, the role of so called extrinsic factors, among which preparation procedures will always have to be classified, probably requires further studies.

Some early attempts to characterize the surface morphology of blood cells under the SEM, after positive identification with the light microscope (3), are time consuming and of debatable value (31). More recently cytochemical reactions for the SEM in the backscattered electron imaging (BEI) mode were developed (22). They allowed a more precise cell type identification (22,23). These techniques present, however, several limitations resulting from: 1) the small number of cytochemical reactions available, 2) their restriction to the identification of early precursors, 3) the variable expression of enzymes in leukemic cells, 4) the sophistication of the technique, 5) the poor compatibility, in some cases, with a good preservation of surface morphology.

Immuno-SEM, in which specific antibodies conjugated with a visible marker label intact cell surfaces, should be, at least theoretically, the best method to clearly define the surface characteristics of blood cells. Until recently, however, 3 types of problems were affecting immuno-SEM methods.

The first was the lack of satisfactory antisera (7,13). This problem is now completely overcome since a large number of monoclonal antibodies specifically recognizing both "differentiation" or "lineage-specific" antigens is available, thus, allowing the recognition of many steps of blood cell maturation.

The second problem refers to the marker itself. When too big, the marker covers the cell surface, when too small it is not visible under the SEM and is difficult to distinguish from natural surface projections (7,13). This problem was also recently overcome with the use of colloidal gold particles (14). The distinct size and shape, the stability when coupled with proteins, but especially the selective visualization under the SEM in the BEI

mode make this marker ideal for SEM (9,17).

The third problem deals with the preservation of a satisfactory cell surface morphology which is generally lost after the long and repeated incubations of unfixed cells. This problem was also overcome when it was observed that mild glutaraldehyde prefixation (0.2%, 10 min) does not preclude specific recognition of many antigens by their corresponding antibodies. As it will be shown, a large number of anti-leukocyte antibodies share these characteristics, opening the way to precise surface characterization of blood cells.

## Materials and Methods

Peripheral blood and bone marrow cells from 5 healthy donors, from 5 leukemic patients and from 1 patient with idiopatic thrombocytopenic purpura were separated by gradient centrifugation with Ficoll-Hypaque or by gravity sedimentation after the addition of 3% dextran. $10^6$ separated cells were then resuspended in 1 ml of 0.1 M phosphate buffered saline (PBS) pH= 7.3 and one drop of the cell suspension was placed on small, poly-l-lysine pretreated glass coverslips. After 30 min. the coverslips were rinsed with PIPES buffer, fixed for 5-10 min. with 0.2% buffered glutaraldehyde (pH 7.2), and then extensively rinsed in a 0.1% solution of glycine in PIPES buffer. Cells were then rinsed twice for 5 min. in 0.1 M PBS containing 1% BSA, 0.2% sodium azide and 1% decomplemented AB serum and incubated for 30 min. with the relevant monoclonal antibody at the appropriate dilution. Finally, the cells were rinsed twice in PBS/BSA/NaN3, incubated for 60 min. with 40 nm colloidal gold particles (GAM G-40, Janssen Pharmaceutica, Beerse, Belgium), rinsed twice in PBS and postfixed with 2% buffered glutaraldehyde. After dehydration in graded ethanols, critical point drying from $CO_2$, coating by evaporation of 10-20 nm of carbon, samples were observed with a JEOL JSM 840 instrument, or with a Philips SEM 505.

## Results and Discussion

The immunogold method described presents numerous advantages for the study of the surface characteristics of blood cells. Cell surface morphology after mild glutaraldehyde fixation is closely resembling that of unlabeled cells, the gold-marker is easily and selectively visible when the BEI mode is employed, and the specificity of the labeling is expected from the use of well characterized monoclonal antibodies (9).

As indicated in Table 1, the different leukocyte classes and subtypes as well as their different maturation stages can be identified with a panel of specific monoclonal antibodies which are still reactive after mild glutaraldehyde prefixation.

This will allow, with a systematic study, to clearly define the cell surface characteristics of blood cells, and to better understand the role of "extrinsic factors" and of preparation procedures. The different surface characteristics of leukemic cells and their diagnostic usefulness will emerge from such SEM studies. Our preliminary observations with this technique in the study of normal pheripheral blood lymphocytes suggest the existence of two distinct morphologies in T and B-derived cells: T cells showing short and monomorphic microvilli while B lymphocytes show generally elongated and pleomorphic microvilli and sometimes short ruffles (Fig. 1).

The surface morphology of NK lymphocytes seems, on the other hand, more hard to define. This is not surprising since the only monoclonal antibody available for the SEM of this subclass (Leu 7) is known to label also other lymphocyte subpopulations (1).

Unfortunately our efforts to find monoclonal antibodies specific for other subclasses like T-helper or suppressor did not meet with success so far. It is nevertheless possible that new monoclonal antibodies suited for this purpose will soon be available.

Our labeling experiments with MoAbs recognizing mature granulocytes and monocytes confirm that the former show surface "ridges" (Fig. 2) while the latter are generally characterized by more developed "ruffles".

MoAbs recognizing antigens involved in leukocyte functions (LFA-1) (25) like chemotaxis and phagocytosis when labeled with the immunogold method at the SEM can also be used to study the surface changes during these events (Fig. 2).

The cell surface of leukemic blood cells is more difficult to characterize than that of their normal counterpart (7,19,20), and, therefore, the need for immunological identification is even greater. Our observations so far indicate that very immature cells, both lymphoid and myeloid, have smooth surfaces with sparse and flat microridges (Figs. 3, 4) while differentiated myeloid and monocytic cells present more developed surfaces with ruffles and ridges (Fig. 5). A great heterogeneity of surface morphologies seems to exist among leukemic cells: this may reflect the fact that any single case may represent a different maturation stage, that cells from different lines can be simultaneously present or that a variability of cell surface projections, intrinsic to the leukemic process, may exist as part of the phenotypic heterogeneity of the disease. The immunogold method has been, however, successfully employed in the study of hairy cell leukemia where the leukemic cells show a typical surface appearance under SEM (11,24). Labeling with a panel of monoclonal antibodies we were able to confirm the existence of a typical surface morphology (Fig. 6) and the usefulness of the method as an adjunct diagnostic procedure (24); in fact few hairy cells admixed with other normal cells were precisely indentified for their typical morphology and reactivity for B1 and Leu M5 MoAb, which respectively recognize B-cells and monocytes in addition to hairy cells (21).

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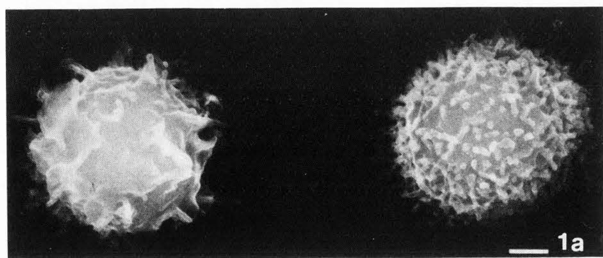


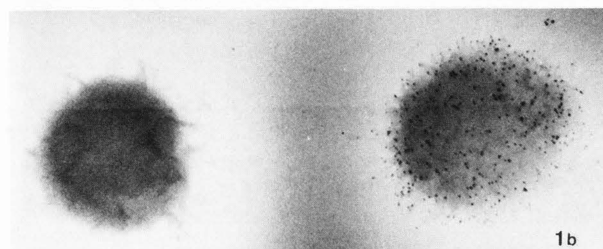


**Figure 1**: Peripheral blood mononuclear cells incubated with Leul MoAb and GAM G 40.

**Figure 1a)** Secondary electron imaging. Two distinct surface morphologies are present. The cell at the left shows short ridges and microvilli while the one at the right bears stub-like microvilli. The labeling with colloidal gold is extremely difficult to evaluate on this picture.

**Figure 1b)** Backscattered electron imaging. The cell at the right, showing numerous colloidal gold particles on its surface, can be correctly identified as a T-cell. 30 kV. Bar = 1 μm.

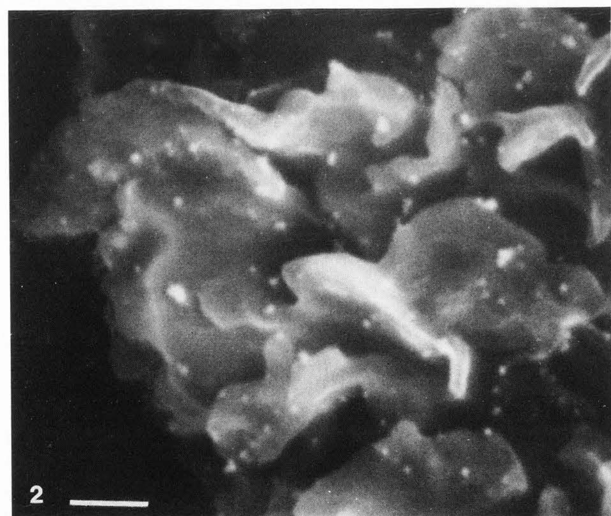


**Figure 2**: Peripheral blood granulocyte incubated with an anti-LFA-1 MoAb and GAM G 40. Mixed second-ary and backscattered electron imaging. An intense colloidal gold labeling is visible on well developed ridges: this surface appearance is typical of granulocytes. 30 kV. Bar = 0.5 μm.

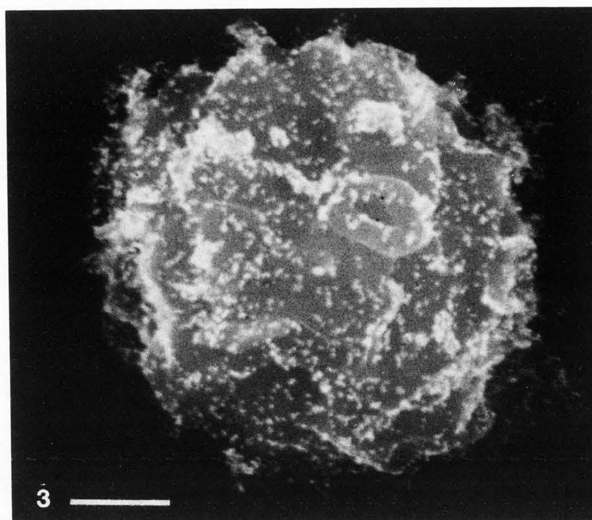


**Figure 3**: Bone marrow cell from a case of myeloblastic leukemia, incubated with 3C5 MoAb and colloidal gold. Mixed secondary and backscattered image. Few ridges are the only surface features visible on this cell heavily labeled with this MoAb which recognizes very early stages of myeloid differentiation. 30 kV. Bar = 1 μm.

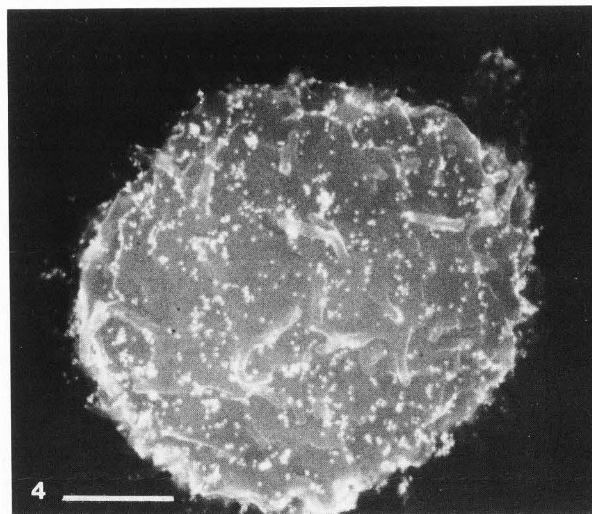


**Figure 4**: Bone marrow cell from a case of lymphoblastic leukemia incubated with J5 MoAb and GAM G-40. Mixed secondary and backscattered electron imaging. The surface morphology of this cell is very similar to that of the cell in Fig. 3, although the positive labeling with J5 indicates its belonging to an early phase of lymphoid differentiation. 30 kV. Bar = 1 μm.

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Table 1: Monoclonal antibodies for the SEM characterization of blood cells.

| Blood Cells | Differentiation stage/sub-population | MoAb | Source | Ref. |
|---|---|---|---|---|
| Lymphocytes | Immature | Ia | BD* | 18 |
| | | J5 | BD | 15 |
| | T-cells | Leu1 | BD | 10 |
| | NK-cells | Leu7 | BD | 1 |
| | B-cells | B1 | Coulter Electr. | 26 |
| | | BA1** | Hybritech | 2 |
| | Activated-T-cells | anti TAC | Ueda R. | 28 |
| Myeloid cells | Immature | Ia | BD | 18 |
| | | 3C5 | Tindle R | 27 |
| | | OKM1 | Orthomune | 5 |
| | All stages | Leu M1 | BD | 12 |
| | Mature | D2 | Shumak KH | 21 |
| | anti-leukocyte-function | Tsi/18.11 | Springer T | 25 |
| Monocytic cells | All stages | OKM1 | Orthomune | 5 |
| | | Leu M5 | BD | 4 |
| | | FMC 17 | Brooks | 6 |
| Megakaryocytes | gp Ib | AN51 | Mc Michael | 16 |
| | gp IIb - IIIa | J15 | Coulter Electr. | 29 |

*BD = Becton Dickinson  ** reactive also with mature granulocytes

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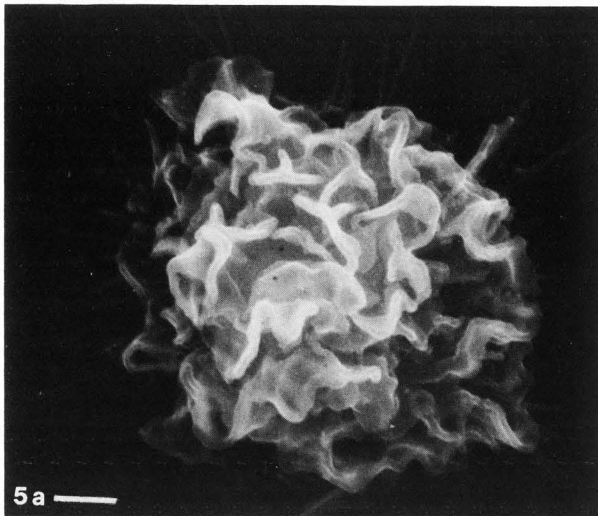


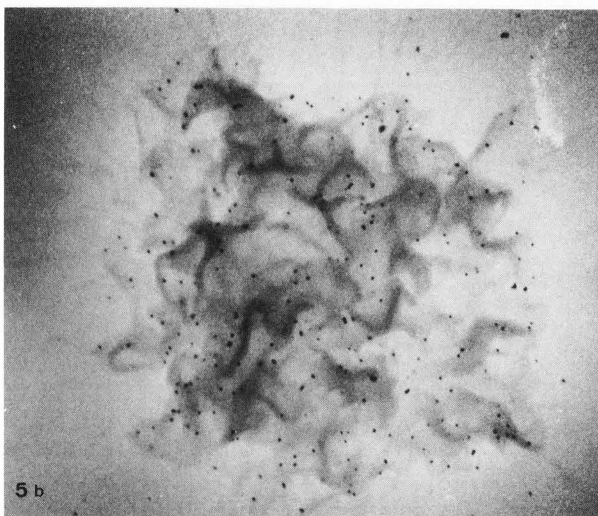


**Figure 5**: Bone marrow cell from a case of monoblastic leukemia incubated with Leu M5 MoAb and GAM G 40.

**Figure 5a)** Mixed secondary and backscattered electron imaging. Heavy labeling with this antibody, specific for the monocytic lineage, is visible on this cell with large surface ruffles.

**Figure 5b)** Backscattered electron imaging; reverse signal polarity. Single gold particles are visible and could eventually be counted. 30 kV. Bar = 1 μm.

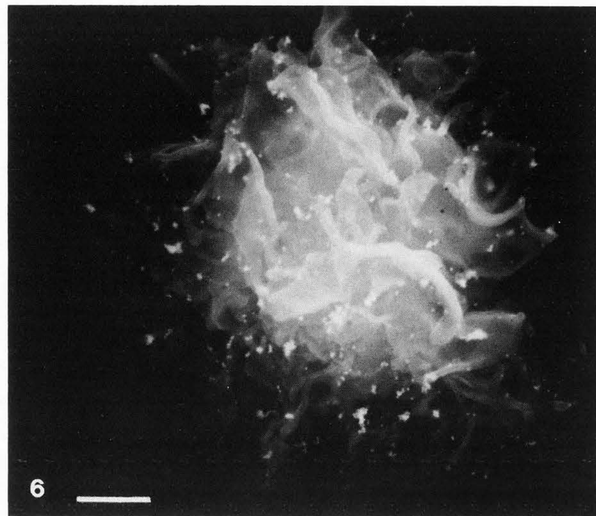


**Figure 6**: Peripheral blood mononuclear cell from a patient with hairy cell leukemia labeled with Bl MoAb and GAM G 40. Mixed image (SE and BE). Note the typical surface morphology of hairy cells with large ruffles and microvilli combined with the labeling with Bl, MoAb generally restricted to B-cells. 30 kV. Bar = 1 μm.

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## Discussion with Reviewers

Reviewer I: Normal and leukemic cells were labeled by immunogold conjugates after intitial reaction with specific monoclonal antibodies (MoAb). While it is claimed that "numerous antileukocyte MoAb still recognize lightly glutaraldehyde-prefixed antigens" no sufficient data is brought to enable the readers to assess the specificity of labeling of pre-fixed cells with the MoAb listed in the Table. The issue is very important in cases of acute leukemias, since in most of the immature cells several kinds of antigenic determinants are reactive only to a limited extent. It is well established that even a mild fixation alters the reactivity of antigens and receptors, which in the case of undifferentiated leukemia cells might also abolish the tendency of the cells to react even with specific MoAb.

Authors: The monoclonal antibodies used in this work were chosen for the following characteristics:

- intense labeling under SEM;
- TEM controls (labeling with and without prefixation) showing limited loss of bound gold particles;
- more recently we are using epipolarization light microscopy to quickly evaluate the labeling and thus to decide if the antigen we want to label will "resist" fixation.

We are aware that a reduction of labeling density can occur and has to be considered especially when trying to do quantitation or with antigens poorly expressed on the surface of leukocytes.

Reviewer I: What are the actual percentages of cells (from diverse normal and leukemic donors) which were positively labeled in the present immuno-SEM study by the different types of MoAb listed in Table 1?

Authors: Actual quantitation of the labeling is not presented in this paper but is currently being calculated in our laboratory based on direct comparison of the percentage of labeled cells under the SEM in the BEI mode and in light microscopy with epipolarization.

Reviewer I: HCs can very easily be recognized by routine SEM especially when admixed with other cells. I would expect the authors to discuss the fact that the "other normal cells" in HCL are either B-lymphocytes or monocytes and using B1 and Leu M5 can only lead to falsely identifying these "normal cells" as hairy cells. The authors do not discuss nor mention the fact that highly specific MoAb for HCs were and are currently introduced in the literature.

Authors: In our experience HCs cannot be so easily recognized under the SEM, especially when admixed with other cells. The other normal cells in a Ficoll-Hypaque preparation of pheripheral blood or bone marrow are lymphocytes (but only a minority with a B phenotype), monocytes and a variety of bone marrow precursors. B-lymphocytes (small round cells with microvilli and sometimes small ruffles) are never Leu M5 (+), a monclonal of the CD 18 group recognizing a Mac-1-like antigen expressed by monocytes but also by hairy cells; on the other hand monocytes (large cells with round ruffles) are never labled with B1, a monoclonal recognizing a 2 chain 32-34 KD molecule exclusively present on B-lymphocytes. As discussed in a previous paper (24) more "specific" MoAbs for HCs like HC-1, HC-2 (Posnett et al (1982). J. Clin. Invest. 70, 254) do not label prefixed cells.

Reviewer I: High-power photographs of selected cells are unacceptable in immuno-SEM studies, unless accompanied by parallel low-power figures of the same specimens showing several cells together (5-10 cells in each frame). It seems unjustified to mention all the kinds of normal and pathological cell types (M&M) if they are not properly listed in a table that details the labeling-index in each case, or if they are not illustrated as indicated above (5-10 cells per frame). Figure 6 might serve as an excellent example for the "selected" way of displaying the results: it shows a ruffled cell that is positively labeled for B1, "a MoAb generally restricted to B-cells". Since you claim that B cells are also ruffled, how do you know for sure that this labeled cell is a hairy cell but not a B-cell? By a subjective interpretation of the size of the ruffles in each case?

Authors: Unfortunately to resolve gold particles in the BEI mode usually requires relatively high magnification imaging and therefore low magnification pictures do not permit one to distinguish between the specific elemental contrast of the gold marker and the diffuse nonspecific signal of unlabeled cells.

As far as Fig. 6 we hope that double labeling experiments, currently in progress (32) will help clarify cell type identification in such cases.

Reviewer II: Have you studied quantitatively the effect of glutaraldehyde fixation conditions on the extent of labeling for the various monoclonal antibodies used in this study?
Authors: Since the paper was submitted for publication the quantitative evaluation of the effect of prefixation on the labeling density has been performed by the study of the same preparation in epipolarization illumination. It indicates that, at least in the case of Leu 1 and B1, there is no significant loss of labeling that can be attributed to prefixation.

Reviewer III: What is the concentration of the gold particles used in the labelling?
Authors: Unfortunately we were not able to do it systematically but when tested, optical density of the 40nm gold particles solution was of 7.0 at 520 nm.

Reviewer III: As described in the procedure, the cells were in contact with a polylysine coated surface for 30 min prior to fixing. Is the cell morphology affected by this exposure? I realize that the polylysine is used to adhere the cells to the substrate.
Authors: We are aware of the potential changes introduced by the attachment of the live cells onto the poly-l-lysine treated substrate. However we feel it is more important to minimize the risk of cell loss by insuring stable attachment of the cells under highly standardized conditions.

## Additional Reference

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