

3-5-1995

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Recommended Citation

Soligo, D.; de Harven, E. P.; Quirici, N.; Caneva, L.; and Deliliers, G. Lambertenghi (1995) "Cell Surface Changes of Hemopoietic Cells During Normal and Leukemic Differentiation: An Immuno-Scanning Electron Microscopy Study," *Scanning Microscopy*: Vol. 9 : No. 1 , Article 12.

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CELL SURFACE CHANGES OF HEMOPOIETIC CELLS DURING NORMAL AND LEUKEMIC DIFFERENTIATION: AN IMMUNO-SCANNING ELECTRON MICROSCOPY STUDY

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(Received for publication July 30, 1994 and in revised form March 5, 1995)

Abstract

Hemopoietic cells display a wide range of cell surface antigens which are either lineage specific or acquired during differentiation. Monoclonal antibodies can be used, in conjunction with colloidal gold markers, to identify under the scanning electron microscopy (SEM) at the single cell level, specific lineage or maturation stages in the hemopoietic bone marrow. Normal bone marrow cells, either gradient separated or purified by immuno-magnetic methods and leukemic cell samples, which can be considered as "frozen" stages of hemopoietic differentiation, have been studied with this method. Typical cell surface morphologies, which characterize immature progenitor cells and cells committed or differentiated towards the lymphoid, myeloid, erythroid and megakaryocytic lineage have been identified. Correlations between cell surface features and some hemopoietic cells functions have been attempted on the basis of these findings.

Key Words: Backscattered electron imaging, hemopoietic cells, immuno-gold labeling, immuno-magnetic cell separation, leukemia, leukocyte antigens, lymphoma, monoclonal antibodies, scanning electron microscopy, ultrastructure.

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Introduction

Hemopoietic cells display a wide array of cell surface molecules which represent surface enzymes, cytokine receptors, adhesion molecules, and binding sites for soluble components of the immune system or for bacterial or viral structures (Pinto *et al.*, 1994). Among these molecules, it has also been clearly established that some are lineage restricted while others are modulated during hemopoietic cell differentiation (Leukocyte Typing IV, 1989).

Taking advantage of lineage and/or stage specific monoclonal antibodies (mAbs), a phenotypic scheme of hemopoietic cell differentiation can be drawn. Monoclonal antibodies can be used in conjunction with immuno-gold markers to identify, under the scanning electron microscope (SEM) at the single cell level, specific stages of hemopoietic cell differentiation (de Harven *et al.*, 1984, 1986; Soligo *et al.*, 1988, 1989). This can be achieved either with normal bone marrow, where cells are very heterogeneous and small subpopulations are difficult to identify, or with enriched populations separated with immuno-magnetic, or immuno-affinity columns or cell sorters. An alternative approach is to study leukemia cell samples which can be considered "frozen" stages of hemopoietic differentiation and where the majority of cells homogeneously displays the same pattern of antigenic expression.

The aim of our study was to identify cell surface structures which may be lineage specific and are recognized during hemopoietic cell differentiation. In addition, the precise localization of cell surface antigens with immuno-SEM may provide some insights into the functions of these molecules, which are in many cases still incompletely defined.

Materials and Methods

Bone marrow samples from 5 donors for allogeneic bone marrow transplantation, 24 cases of acute myeloid leukemia (AML) of different FAB subtypes (Bennet *et al.*, 1976), 13 cases of acute lymphoblastic leukemia

(ALL) with various phenotypes, 1 case of γ/δ cutaneous T cell lymphoma, 5 cases of chronic lymphocytic leukemia (CLL) and 14 cases of B cell lymphoma with bone marrow involvement were included in this study. In addition, lymph node suspensions from two cases of centrocytic-centroblastic lymphoma were also studied.

Bone marrow cells were separated by centrifugation on a Ficoll-Hypaque gradient and the low density fraction was rinsed twice with phosphate-buffered saline (PBS). In addition, an aliquot of 1×10^8 bone marrow cells from 2 normal donors was subjected to immunomagnetic separation with the magnetic cell sorting system (MACS; Milteny Biotec GmbH, Bergisch Gladbach, Germany). More precisely, cells were labeled with an anti-CD34 monoclonal antibody (QBEnd10) and subsequently with rat anti-mouse antibodies conjugated with paramagnetic beads; the labeled cells were then placed in steelwool columns under an external magnetic field. Unbound cells were washed out and CD34⁺ cells were collected after the removal of the magnetic field (Milteny *et al.*, 1990). Separated cells were allowed to attach to 0.1% poly-L-lysine pretreated glass coverslips for 30 minutes, and either fixed in 2% glutaraldehyde for 60 minutes for routine SEM or prefixed for 10 minutes with 0.2% glutaraldehyde in 0.1 M PIPES buffer, incubated for 20 minutes in 1% glycine in PBS and twice for 10 minutes in PBS supplemented with 1% bovine serum albumin (BSA), 10% decomplexed antibody (AB) serum and 0.1% sodium azide for subsequent immunostaining.

Mononuclear cells were incubated with a panel of monoclonal antibodies including: -QBEnd10 (Immunotech International, Marseille, France) against the CD34 molecule, -VIPL2 (kindly provided by W. Knapp) and CLB-thromb/1 (kindly provided by A. von dem Borne) against the gpIIIa/CD61 molecule, JC159 (Dakopatt, Glostrup, Denmark) recognizing anti-glycophorin A, -My9 (Coulter Electronics Ltd., Luton, UK) against the CD33 antigen, -LeuM1 (Becton Dickinson, Mountain View, CA, USA) recognizing the CD15 determinant, -OKM1 (Ortho Diagnostic System, Milano, Italy) for the CD11b antigen and -LeuM5 (Becton Dickinson) against the CD11c molecule, -J5 (Coulter) recognizing the CD10/CALLA antigen, -HD37 (Dakopatt) against the CD19 and BA-1 (Hybritech, San Diego, CA) recognizing the CD24 molecule, -DakoT2 (Dakopatt) against CD7, -Leu1 (Becton Dickinson) recognizing the CD5 antigen and -TCRdelta (T Cell Science, Cambridge, MA, USA) recognizing γ/δ T lymphocytes. Following a 30 minute incubation with the primary antibody, cells were rinsed 3 times with buffer and incubated for 60 minutes with goat anti-mouse IgG conjugated with 30 or 40 nm colloidal gold particles (GAM-G-30, GAM-G-40, Janssen Pharmaceutica, Beerse, Belgium) and finally

Figure 1. Immuno-magnetically separated CD34⁺ normal bone marrow cells: (a) CD34⁺ cells show either a merely smooth surface or few micro-ridges and small blebs (upper and left); SEI; bar = 5 μ m. (b) A single CD34⁺ cell showing a smooth surface with a large aggregate of paramagnetic iron beads (arrows); SEI; bar = 2 μ m.

Figure 2. Bone marrow cells from a case of M1 leukemia. The cell on the right is almost completely smooth, while the other on the left starts showing initial features of myeloid differentiation (surface ridges); SEI; bar = 1 μ m.

Figure 3. Long term bone marrow culture at 4 weeks, showing a typical "cobblestone area" consisting of early bone marrow progenitor cells covered by a stromal cell layer (left). At the right of the picture, cells with initial signs of myeloid differentiation are seen in the process of leaving a bone marrow "niche"; SEI; bar = 10 μ m.

Figure 4. Well differentiated myeloid cell from a case of M2 leukemia showing surface ridges; SEI; bar = 1 μ m.

Figure 5. Mature granulocyte showing well developed surface ridges and immuno-gold labeling with CD15 (LeuM1) Mab; SEI/BEI; bar = 1 μ m.

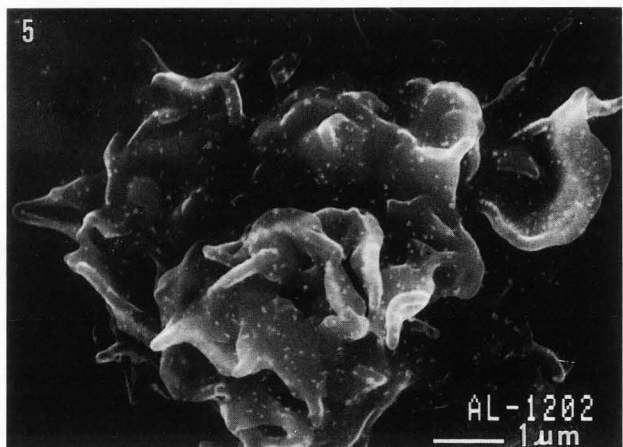
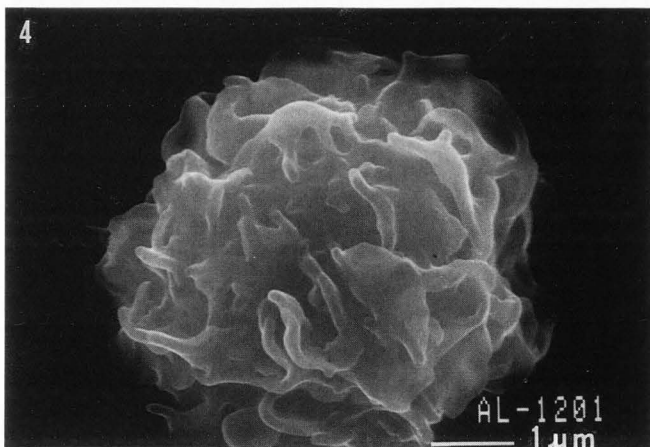
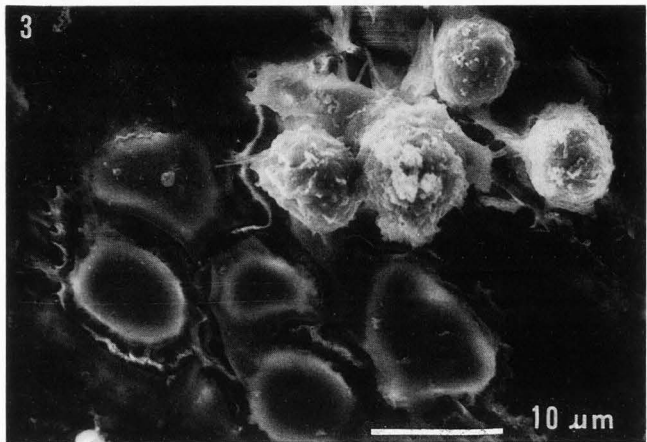
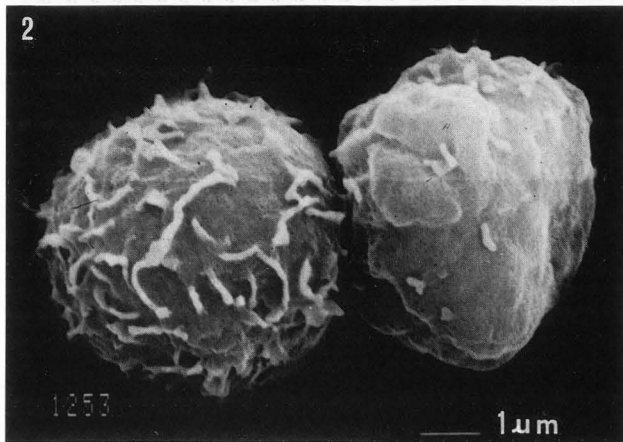
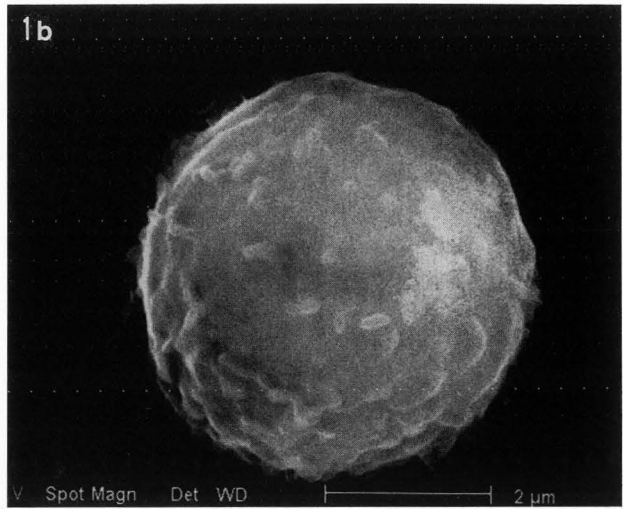
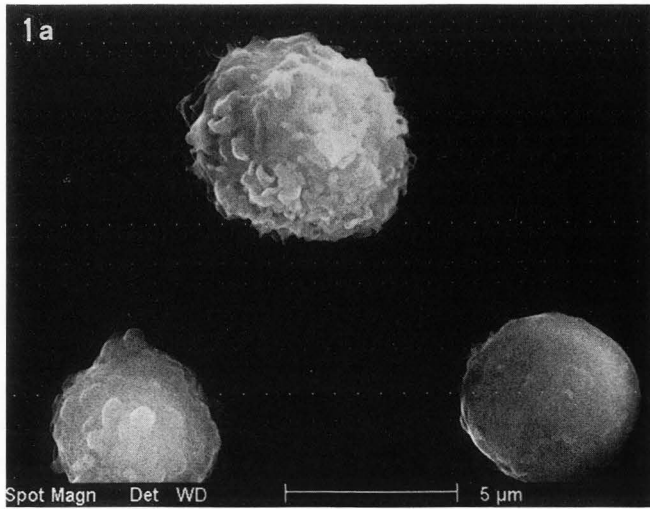
rinsed with PBS, postfixed with 2% glutaraldehyde in PBS. Long-term bone marrow cultures were established on glass coverslips in 24 well culture plates seeding 2×10^6 normal bone marrow mononuclear cells in Iscove modified Dulbecco medium (Gibco BRL, Life Technologies Inc., Gaithersburg, MD, USA) supplemented with 15% fetal calf serum (FCS), 15% horse serum and 10^{-5} M hydrocortisone. At 4 and 8 week intervals, the cultures were rinsed with PBS and fixed in 2.5% buffered glutaraldehyde.

All coverslips were critical point dried from CO₂ following routine procedures (de Harven and Soligo, 1986). Samples were then mounted on aluminum stubs, coated with approximately 20 nm of evaporated carbon and observed either with a Philips XL20 or with a JEOL 840 SEM equipped with a LaB₆ cathode and a solid state BE detector; both SEMs were operated at accelerating voltages of 15 to 30 Kv. Optimum surface morphology is routinely obtained in the secondary electron imaging (SEI) mode, while unambiguous identification of the colloidal gold markers usually requires backscattered electron imaging (BEI).

Results and Discussion

It is well known that the cell surface morphology of blood cells can be altered, to some extent, during all

Cell surface changes of hemopoietic cells



SEM preparation procedures, including Ficoll separation, precursors cells enrichment, adhesion and fixation (Wetzel *et al.*, 1973; Wetzel, 1976). In addition, labeling procedures have been shown to completely destroy the delicate surface morphology of blood cells, unless they are prefixed. The technique we used for the SEM

examination of blood cells has been found to be extremely useful during more than 10 years experience and showed the following advantages: 1) it can be consistently reproduced with very little sample to sample variability; 2) it does not produce cell loss due to variable attachment; and 3) it allows to best preservation both

some cell surface features and antigenicity of the sample (de Harven and Soligo, 1986).

When we applied this technique to the examination of the normal bone marrow, a very heterogeneous cell population was evident with a predominance of relative mature myeloid and erythroid cells. The study with immuno-gold techniques of small cell populations is therefore difficult and time consuming. An easier identification can be achieved only with pre-enriched cells or on leukemic cell samples.

Early progenitor cells

The CD34 antigen is present in about 1% of normal bone marrow cells: CD34⁺ cells have a higher clonogenic potential than whole bone marrow cells but still include cells already committed to different hemopoietic lineages and primitive stem cells (Civin *et al.*, 1984). These latter should represent from 0.1 to 1% of the CD34⁺ bone marrow cells. They should also have a typical immuno-phenotype (CD34⁺, HLA-DR⁻, CD38⁻, Thyl⁺) and high proliferative and repopulation capability (Katz *et al.*, 1985).

The SEM examination of CD34⁺ magnetically separated cells, with a purity ranging from 93% to 96%, showed that more than 50% of the cells are small and round with a completely smooth surface (Figs. 1a and 1b), while the remaining cells display rare microvilli and small blebs. This finding, which was confirmed on M₀-M₁ myeloid leukemias (Fig. 2) and on pre-B and T lymphoblastic leukemias (shown later in Figs. 8 and 11), clearly indicates that early progenitor cells have an almost featureless surface and that surface projections are produced only at a relatively late stage of differentiation. In the normal bone marrow, stem cells are known to display on their surface a wide array of growth factor receptors and of adhesion molecules, and are completely surrounded by stromal cell dendrites (Cattoretti *et al.*, 1993) (Fig. 3). The surface of the stem cells is coated with extracellular matrix proteins which both serve for growth factor presentation and for anchoring to the bone marrow "niches". The round, smooth surface morphology of stem cells is probably the most adequate in this setting: as soon as they progress toward commitment and differentiation, they start moving out of the hemopoietic "niches" (Fig. 3) and finally leave the bone marrow environment through the endothelial sinuses wall to enter blood circulation. Some cell surface projections may be related to cell movements or may be necessary for cells to loose their initial cohesiveness and escape stromal cell anchorage.

Early myeloid cells

Early myeloid cells are easily identified in the whole bone marrow with immuno-gold labeling even if precise differentiation stages, as defined by light microscopy,

Figure 6. Cell from a case of myelo-monocytic leukemia (M4) showing the different spectra of surface morphologies seen in myeloid differentiation: (a) Small ridges and ruffles are present in this cell coexpressing granulocytic and monocytic cell surface features; SEI; bar = 1 μ m. (b) Typical spread monocyte cell with large ruffles; SEI; bar = 1 μ m. (c) Typical myeloid cell with surface ridges; SEI; bar = 1 μ m.

Figure 7. Langerhan's cells from a normal epidermal cell suspension after labeling with CD11c (LeuM5) and 30 nm colloidal gold particles; SEI; bar = 1 μ m.

Figure 8. Early lymphoid cell from a common ALL showing a completely smooth surface; SEI; bar = 1 μ m.

Figure 9. Centrocitic-centroblastic B-cell lymphoma cell infiltrating the bone marrow. Elongated microvilli are evident on the surface of this cell; SEI; bar = 1 μ m.

Figure 10. A typical lymphoid cell from a bone marrow infiltrate from a case of splenic villous lymphoma, showing elongated broad-based microvilli and ruffles; SEI; bar = 1 μ m.

need either double labeling studies or examination of leukemic cell samples representing "frozen" stages of differentiation.

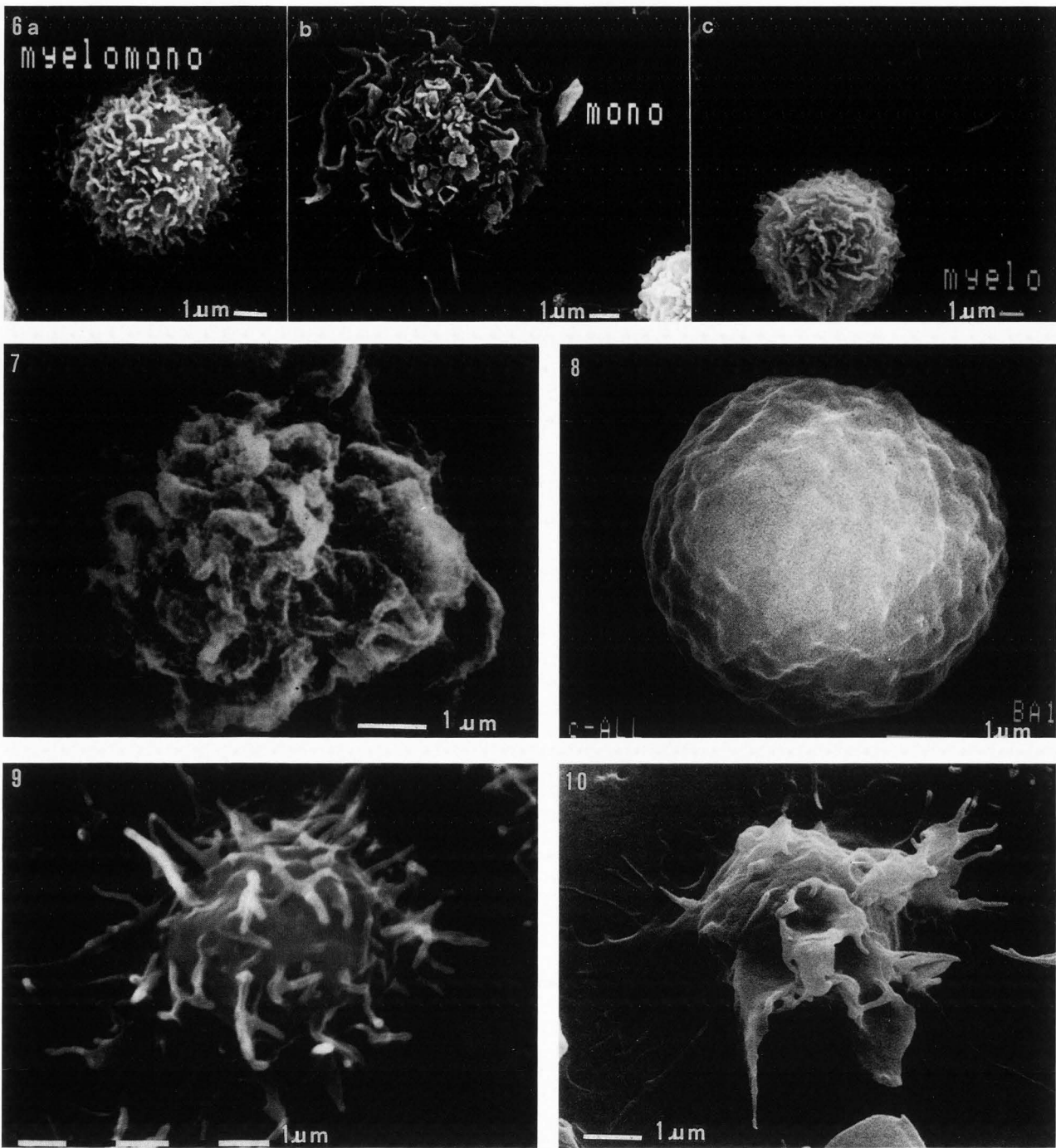
Myeloblasts start showing surface ridges, initially small and rare (Fig. 2), and subsequently, large and well developed (Fig. 4, and also shown later in Fig. 6c). A typical morphology is seen in promyelocytic cells: these cells are large with a surface packed with ridges of different shape and some microvilli. This cell surface morphology of myeloblasts and promyelocytes can be further confirmed by cytochemical techniques in the BEI of the SEI mode (Soligo *et al.*, 1985).

Late myeloid cells

The cell surface morphology of mature granulocytes, both in the bone marrow and in peripheral blood, is invariably characterized by surface ridges (Fig. 5).

Once myeloid cells start differentiating toward the monocytic lineage (Fig. 6a), as indicated by the expression of CD11b and CD11c both in normal and leukemic bone marrow, surface ruffles, sometimes extremely well developed, appear (Fig. 6b). These cell surface projections are then invariably present both in circulating (Fig. 6b) and in resident monocytic cells such as tissue and bone marrow macrophages and in Langerhans cells (Fig. 7) (De Panfilis *et al.*, 1989).

Well developed ridges and ruffles seem, therefore, to represent the morphological counterpart of phagocytic activity which is maximally present in monocytic cells.



Lymphoid cells

Early lymphoid bone marrow cells, of both T and B lineages, are almost invariably displaying smooth surfaces (Fig. 8, and also shown later in Fig. 11). The appearance of surface projections seems to be a relatively late event in lymphoid ontogeny (Soligo *et al.*, 1989).

Pre-B cells start showing small ridges and pleomorphic microvilli, which are then predominant in mature B lymphocytes. The same cell surface morphology is also

seen in lymph node cell suspensions or in bone marrow infiltrates of B cell lymphomas (Fig. 9). The cell surface morphology of B cells of secondary lymphoid organs seems nevertheless more complex and difficult to define. Nodal B-cells are morphologically and phenotypically heterogeneous, reflecting differentiation, functional and activation steps taking place during antigen processing and initiation of the immune response. Moreover, lymph node or spleen cell suspensions can be

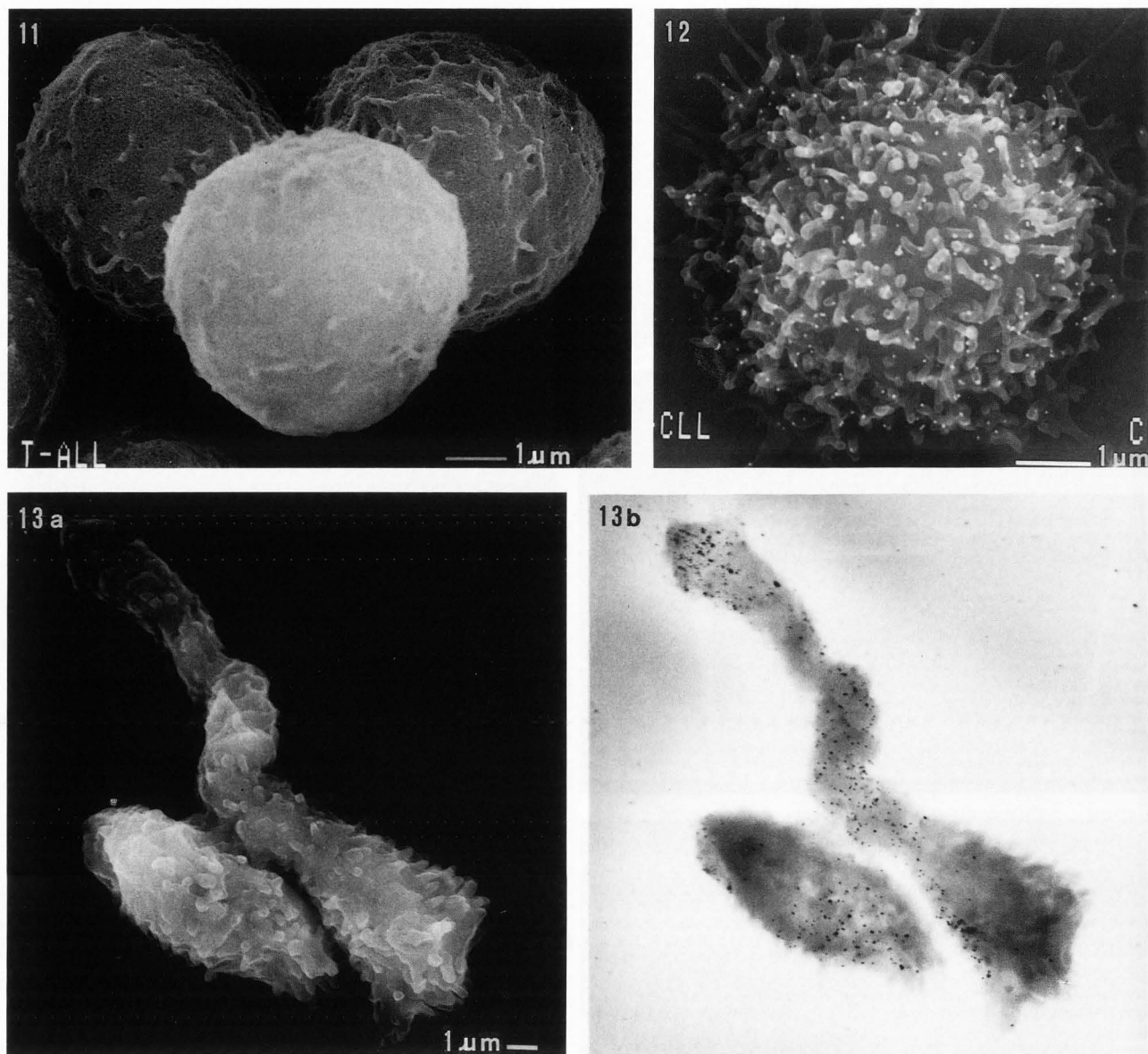


Figure 11. Acute T lymphoblastic leukemia cell with an almost smooth cell surface morphology; SEI; bar = 1 μ m.

Figure 12. T chronic lymphatic leukemia cell showing abundant stub-like microvilli and immuno-gold labeling with an anti CD3 Mab; SEI/BEI mixed image; bar = 1 μ m.

Figure 13. Lymphoid cells from an epidermal cell suspension in a case of γ/δ T cell lymphoma. The two cells show an elongated shape, short microvilli and ridges and are labeled with a TCR delta 1 Mab and 30 nm gold particles: (a) SEI; (b) BEI; bar = 1 μ m.

obtained only after mechanical or enzymatic manipulation of the tissue which can alter the delicate surface morphology of the cells.

We examined by SEM the bone marrow and the infiltrating cells of precisely phenotyped B lymphomas arising from the marginal zone, follicle mantle and inter-follicular tissue. In the majority of cases, the surface

features of these cells was rather heterogeneous but characterized by a variable number of long microvilli (Soligo *et al.*, 1994). On the other hand, a very typical surface morphology was observed in spleen lymphomas with villous lymphocytes (SLVL), a subset of non-Hodgkin's lymphomas already well characterized by light microscopy (LM) morphology and immuno-phenotype.

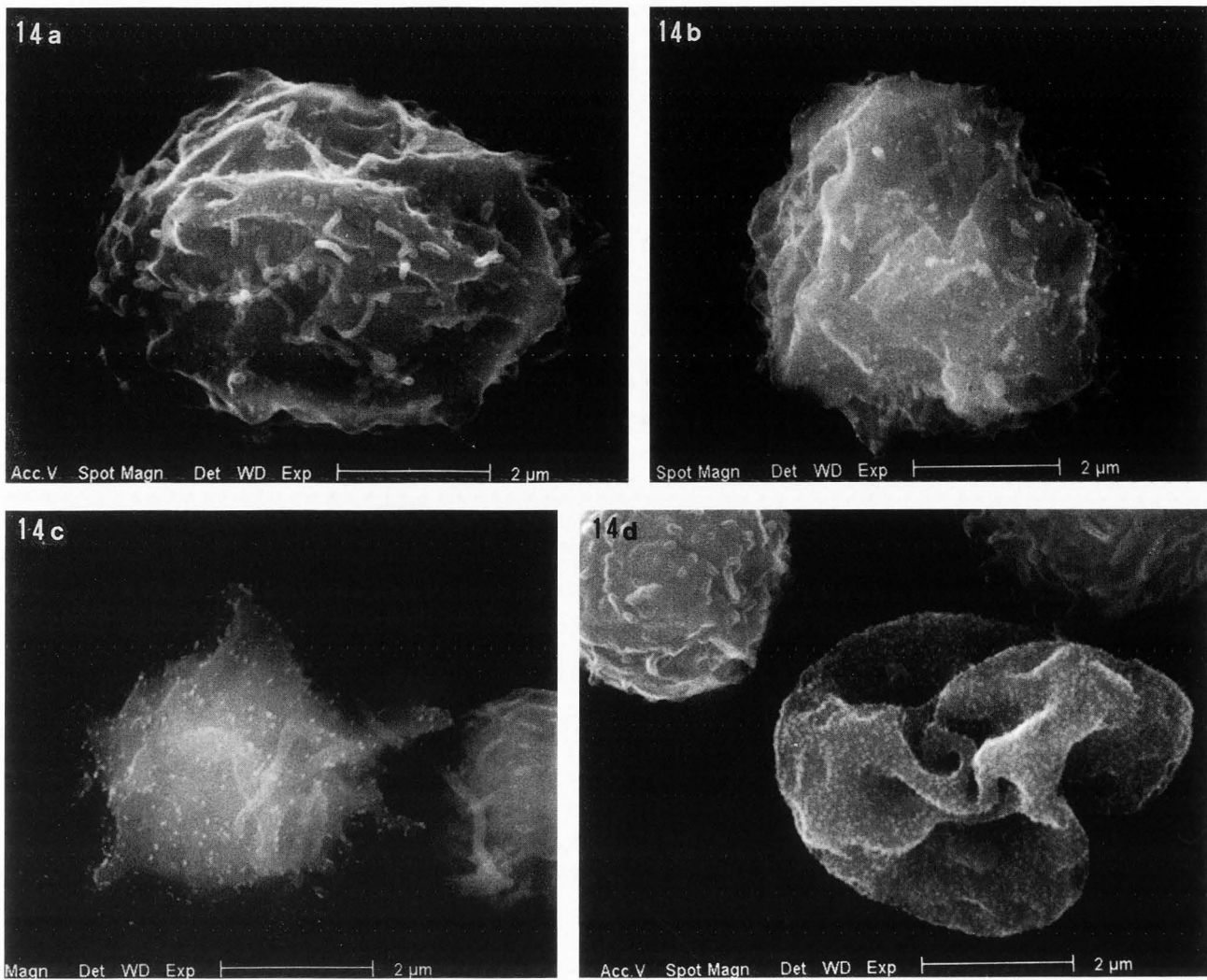


Figure 14. Normal bone marrow cells labeled with anti-glycophorin-A and 30 nm colloidal gold particles: (a) Early erythroblasts showing micro-ridges and rare microvilli; SEI; bar = 2 μ m. (b and c) In later stages of erythroid differentiation cells show an irregular shape with pointed ruffles; (b) SEI; (c) BEI; bar = 2 μ m. (d) Bone marrow reticulocytes showing a typical convoluted shape. SEI; bar = 2 μ m.

In these cases, SEM revealed the presence of elongated, broad-based microvilli, small ruffles and blebs (Fig. 10); this surface morphology seems rather pathognomic and, as in the case of hairy cell leukemia, it could be of diagnostic relevance (Soligo *et al.*, 1994).

The same differentiation scheme holds true in T cell ontogeny: T cells are initially smooth (Fig. 11) and start showing "stub-like" microvilli (Fig. 12) at a stage when the full repertoire of T cell antigens is present including the α/β TCR, the CD3 complex and when the CD4/CD8 antigens are co-expressed. Large numbers of uniform, stub-like microvilli are then invariably present on terminally differentiated T cells and in peripheral blood T lymphocytes (de Harven *et al.*, 1986).

The cell surface morphology of human γ/δ T lymphocytes is on the other hand difficult to define; in one case of γ/δ T cell cutaneous lymphoma (Berti *et al.*, 1991), neoplastic T cells infiltrating the skin, under the SEM, showed an elongated shape, generally with a long uropod, small surface ridges and isolated microvilli (Figs. 13a and 13b). Whether this cell surface morphology is consistently present on this subset of T lymphocytes remains to be definitely confirmed, although a similar morphology was described for normal T cell clones with γ/δ receptors by two different authors (Grossi *et al.*, 1989; Arancia *et al.*, 1991).

The consistency of some surface features does not seem to be fortuitous but rather suggests involvement in

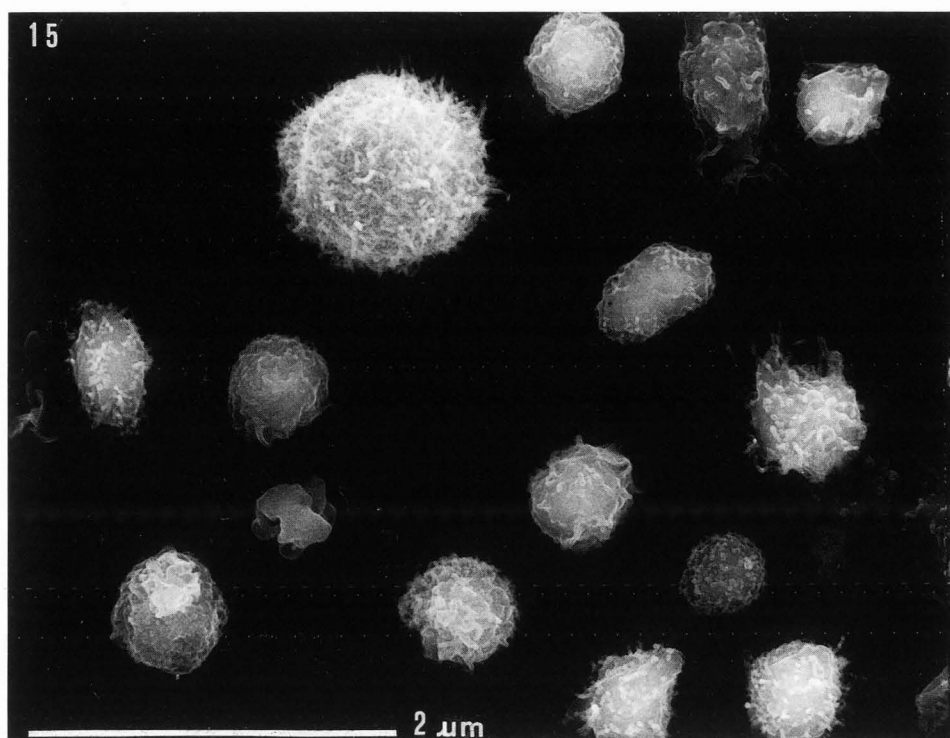


Figure 15. Normal bone marrow cells labeled with an anti-CD61 Mab and 30 nm colloidal gold particles. A large labeled megakaryocyte, which has a highly villous surface, is visible on the upper left of the picture; SEI; bar = 2 μm .

some specific functions. To elucidate the role of cell surface projections seen by SEM, a better understanding of some surface antigen functions would be required. A more refined immuno-SEM technology, hopefully reaching one to one antigen-antibody labeling ratio, as well as *in vitro* systems in which the interactions between lymphoid cells, antigen presenting cells and their microenvironment can be analyzed, will also be necessary.

Erythroid cells

Typical cell surface features of round erythroid cells at the late pro-erythroblast stage, are regular, inconspicuous micro-ridges (Fig. 14a). In later stages of erythroblastic differentiation, cell surfaces become extremely irregular, with smooth, short and pointed ruffles (Fig. 14b). Subsequently, cell outlines become even more convoluted (Fig. 14c) and the protrusion of a condensed, pycnotic nucleus can be easily seen by SEM, especially with silver staining of the chromatin in the BEI mode (Soligo *et al.*, 1985). These cell surface changes lead to the typical reticulocyte morphology (Fig. 14d) as already described in early studies, (Bessis, 1973).

Megakaryocytic (MK) cells

Early megakaryocytic cells show smooth surfaces with isolated large blebs, as indicated by CD61 labeling both in normal bone marrows and in M7 leukemias. Large, hyperdiploid MK cells are then characterized by a surface completely covered with large microvilli (Fig. 15) among which, in later stages of differentiation, round and pedunculated protrusions can be seen, pos-

sibly representing different stages of platelet formation.

Conclusions

In conclusion, a cell surface scheme of hemopoietic cell differentiation is apparently now available.

Some typical cell surface features are consistently observed in the different lineages at different maturation stages. These cell surface projections probably represent morphological counterparts of specific leukocyte functions.

Many leukocyte surface antigens are now biochemically, molecularly and functionally well characterized (Pinto *et al.*, 1994) and, taking advantage of immuno-gold techniques, especially when coupled with high resolution SEM in the backscattered electron imaging mode, the functional role of leukocyte surface projections will be soon clarified.

Acknowledgments

We are grateful to Mario Azzini (Future Image, Milan, Italy) for the photographic art-work.

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Discussion with Reviewers

A. Campos: The cells were deposited on poly-l-lysine coated coverslips and fixed with glutaraldehyde. Adhesion can modify the surface feature of some cells, especially those of myelo-monocytic lineage. Because the authors base their conclusions on cell surface projections, how might adhesion might have affected the appearance of these cells?

Authors: The problem of the leukemic cell surface modification induced by poly-l-lysine attachment has been debated for over a decade and, of course, we are aware of these problems. Having seen cells fixed prior to and after the adhesion to coverslips, we concluded that surface ruffles and ridges are generally retained by cells of granulocytic and monocytic lineage independently of the preparation procedure and the activation state of these cells.

A. Campos: The authors show mature granulocytes with well developed ridges. It was recently shown (Haslett *et al.*, *Am. J. Pathol.* **119**: 101-110, 1985;

Natts *et al.*, Cell Motil. Cytoskel. **19**: 159-168, 1991) that this type of morphological feature is related with cellular activation in the process of separation and isolation with Ficoll-gradients. How can the authors be sure that the ridges are not due to some technical factors?

Authors: The same cell surface morphology of granulocytes cells (both unlabeled and labeled with a D1 or CD15 MoAbs and colloidal gold) was observed on buffy coat preparations of normal peripheral blood and in Ficoll separated mononuclear cell fractions in numerous experiments performed in our laboratory. This seems to indicate that the effect of the Ficoll by itself is negligible. Nevertheless, we cannot exclude that other factors such as the shear-stress during venipuncture, the temperature shifts occurring from the "*in vivo*" to the "*ex vivo*" state, the anticoagulant used (we generally utilize sodium citrate) and all the process of sedimentation and/or centrifugation may be responsible of some degree of cell activation and consequence of some alteration in the cell surface morphology.

A. Campos: The authors show T-cells with stub-like microvilli. Using cytochemical techniques with SEM, we have identified a subpopulation of smooth cells with a pattern of acid hydrolase expression that characterizes differentiated T-cells (Fernandez-Segura *et al.*, Histochemistry **94**: 315-321, 1990; Campos *et al.*, Scanning Microscopy **5**: 175-185, 1991). Do you have evidence of a subpopulation of CD3⁺ mature T-cells that lack stub projection?

Authors: We see a very small percentage of CD3⁺ smooth lymphocytes also in our normal PBL preparations. It could be interesting to correlate the expression of acid hydrolase and CD3⁺ with double immuno-cytochemical/immuno-gold BEI techniques to further characterize this T-cell subset. We do not think this kind of experiment would be appropriate to this paper.

R.M. Albrecht: The use of specific antibodies has been extremely useful in positive identification of individual cells as to type and subtype. However, over the years, questions have arisen with respect to the effects of preparative procedures on living cell morphology. Could the authors comment on controls used to insure that the observed morphology is relatively similar to that of the cells in the "*in vivo*" state prior to exposure to cell separation, cell enrichment, labelling, adhesion (to poly-L-lysine), and fixation procedures?

Authors: Some of the your concerns have been discussed both in the text and in the answers to Prof. Campos above. As concerns the labeling procedures, the artifacts are very likely because the cells were prefixed with 0.1% glutaraldehyde.

R.M. Albrecht: One or two cells of each type are shown to demonstrate clearly and at sufficient magnification the characteristic features and labeling patterns. It would be helpful if the authors could comment on the morphologic variability seen within each particular cell type.

Authors: In our interpretation, the presented micrographs are representative of each group even if there is a little variability within each cell type.

R.M. Albrecht: Have cell sorting procedures such as FACS been employed to select particular cell populations which are then observed by SEM? Are such sorting procedures compatible with retention of characteristics structural features?

Authors: Cell sorting procedures, such as FACS (fluorescent activated cell sorting), have not been used since in preliminary experiments with sorting procedures we had evidence of very poor cell surface morphology preservation. An attempt could be made by sorting mildly glutaraldehyde prefixed cells (0.1%) labeled with fluorochrome conjugated Mabs. This could be potentially very useful to confirm our data and we plan to do it in the future.