11-11-1995

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Etienne de Harven
University of Toronto

Davide Soligo
IRCCS

Hilary Christensen
University of Toronto

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IMMUNOGOLD LABELING FOR THE DIAGNOSIS OF LEUKEMIA BY TRANSMISSION AND SCANNING ELECTRON MICROSCOPY

Etienne de Harven[1*], Davide Soligo[2] and Hilary Christensen[1]

[1]Department of Pathology, University of Toronto, Toronto, Ont., Canada
[2]Ospedale Maggiore, IRCCS, Milano, Italy.

Abstract

For the cell type diagnosis of leukemia in adult patients, particularly when the sampling of bone marrow is difficult, the study of peripheral blood leukocytes (PBLs) by immuno-electron microscopy provides significant information, as illustrated here in two cases of hairy cell leukemia and seven cases tentatively identified as megakaryoblastic leukemia (M7). Indirect immunogold labeling with the B-ly7 monoclonal antibody (CD103) proved valuable in confirming the diagnosis of hairy cell leukemia. Immunogold labeling for the GpIIIa platelet glycoprotein (CD61) was used in cases where the light microscopy of blood films revealed possible megakaryoblastic leukemia. Under the electron microscope, however, the CD61 positive cells showed, in almost all cases, a wide spectrum of megakaryopoietic differentiation which made the diagnosis of M7 questionable. Most of the CD61 positive cells featured cytoplasmic differentiation markers such as alpha granules and demarcation membranes, further confirming the presence of circulating megakaryocythemia, a phenomenon described many years ago in various myeloproliferative disorders. It is suggested, therefore, that many of these cases should not be identified as true megakaryoblastic leukemias.

*Address for correspondence and current address:
Etienne de Harven,
Le Mas Pitou - La Calanquette,
2879 Route de Grasse,
06530 Saint Cézaire sur Siagne,
France
　　Telephone and FAX number: (33) 93 60 28 39

Introduction

In adult patients, leukemias are classified in distinct subtypes which are routinely diagnosed by examination, under the light microscope, of bone marrow aspirates or biopsies, and of peripheral blood films. The FAB classification (Bennett et al., 1976, 1985) recognizes seven myeloid (M1 to M7) and three lymphoblastic (L1, L2 and L3) forms of the disease.

Thirty years ago, recognizing the various leukemic subtypes was of questionable redeeming value since prognosis was invariably fatal. Although precise cell typing has sometimes been regarded as an academic exercise, it provides important prognostic information, since myeloid leukemias follow a more aggressive course than lymphoblastic disease.

Fortunately, the prognosis of acute leukemias has been significantly improved over the past decades by: (1) the progress of chemotherapy, and (2) the refinement of methods for bone marrow transplantation. However, the most encouraging results of several therapeutic protocols are, as reported worldwide, cell-type restricted. For example, alpha-interferon, deoxycoformycin and 2-chlorodeoxyadenosine induce very prolonged remissions in almost all cases of hairy cell leukemia (HCL), while the same treatments are apparently of no therapeutic value in other forms of leukemia. In promyelocytic leukemia (PML, FAB: M3), all-trans retinoic acid alone results in significant remissions, while the same therapy has no effect in other types of leukemias. Obviously, precise cell typing of acute leukemias is no longer an academic exercise. It has become an absolute necessity for the selection of the most appropriate therapy for each individual case of leukemia.

Precise cell typing can be achieved by several methods: (1) routine hematology, (2) flow cytometry, (3) cytogenetics, (4) molecular diagnosis, and (5) ultrastructural characterization of the leukemic cells by electron microscopy. A combination of these methods is probably the most efficient approach to correct diagnosis, in spite of their relatively poor concordance reported recently (Choi et al., 1994).

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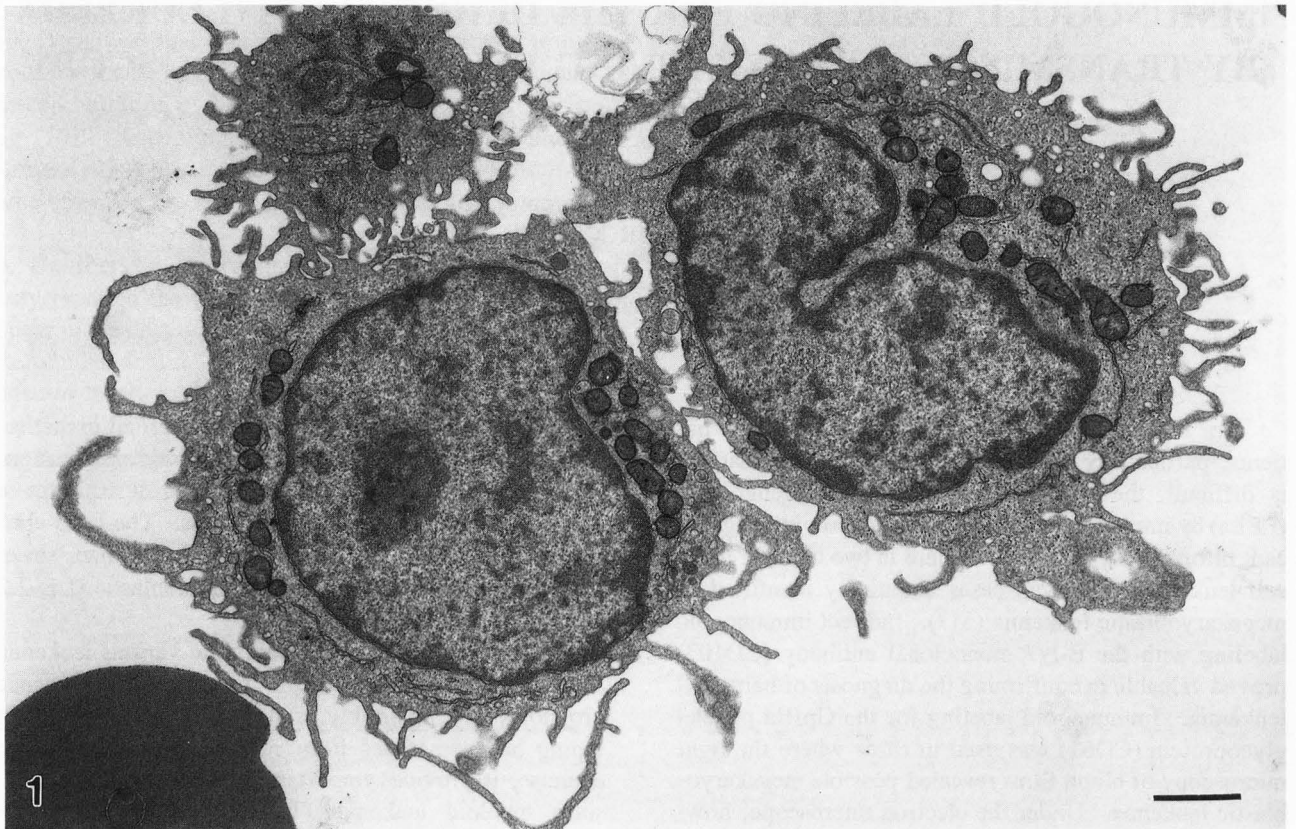
Figure 1. Two typical hairy cells featuring the characteristic broad based surface projections and several short microvilli. Their cytoplasm is moderately rich in organelle (mitochondria and rough ER). Nuclei have some margination of heterochromatin and inconspicuous nucleoli.

The purpose of this paper is to demonstrate that transmission and scanning immuno-electron microscopy of peripheral blood leukocytes (PBLs) of leukemic patients provide valuable diagnostic information not readily obtainable by other methods, particularly when bone marrow aspiration is difficult or non-diagnostic. This is frequently the case in hairy cell leukemias (HCL) and in megakaryoblastic leukemias (M7) with myelofibrosis (Bennett *et al.*, 1985). The use of immuno-electron microscopy in the study of hairy cell and of megakaryoblastic leukemia is particularly indicated in view of the absence of cytogenetic markers and the inaccuracy of flow cytometry for the study of these two diseases. Indeed, flow cytometry can hardly distinguish between megakaryocyte precursors, giant platelets, and platelet aggregates.

Materials and Methods

Peripheral blood leukocytes

A close collaboration with the Hematology/Oncolo-

gy clinic at the Toronto General Hospital allowed us to select cases in which a diagnosis of hairy cell leukemia (HCL) or of megakaryoblastic leukemia (M7) was suspected by light microscopy of blood films.

Citrated peripheral blood was collected from these patients prior to initiation of chemotherapy. Mononuclear cells were separated by centrifugation on Ficoll-Hypaque gradients, and prefixed for 10 minutes (not any longer!) with a solution of phosphate buffered 0.1% glutaraldehyde which will prevent internalization of the antigen-antibody-gold complexes. All preparatory steps were at room temperature. When a diagnosis of HCL was anticipated (2 cases), the mononuclear cells were incubated for 60 minutes with an undiluted preparation (110 μg/ml) of the B-ly7 murine monoclonal antibody (Poppema *et al.*, 1989; Visser *et al.*, 1989; de Harven *et al.*, 1994). When a diagnosis of megakaryoblastic leukemia was suspected (7 cases), the cells were incubated with a 1:50 dilution of the anti-GpIIIa (CD61) murine monoclonal antibody (Y2/51, Dakopatts, Glostrup, Denmark). For scanning electron microscopy
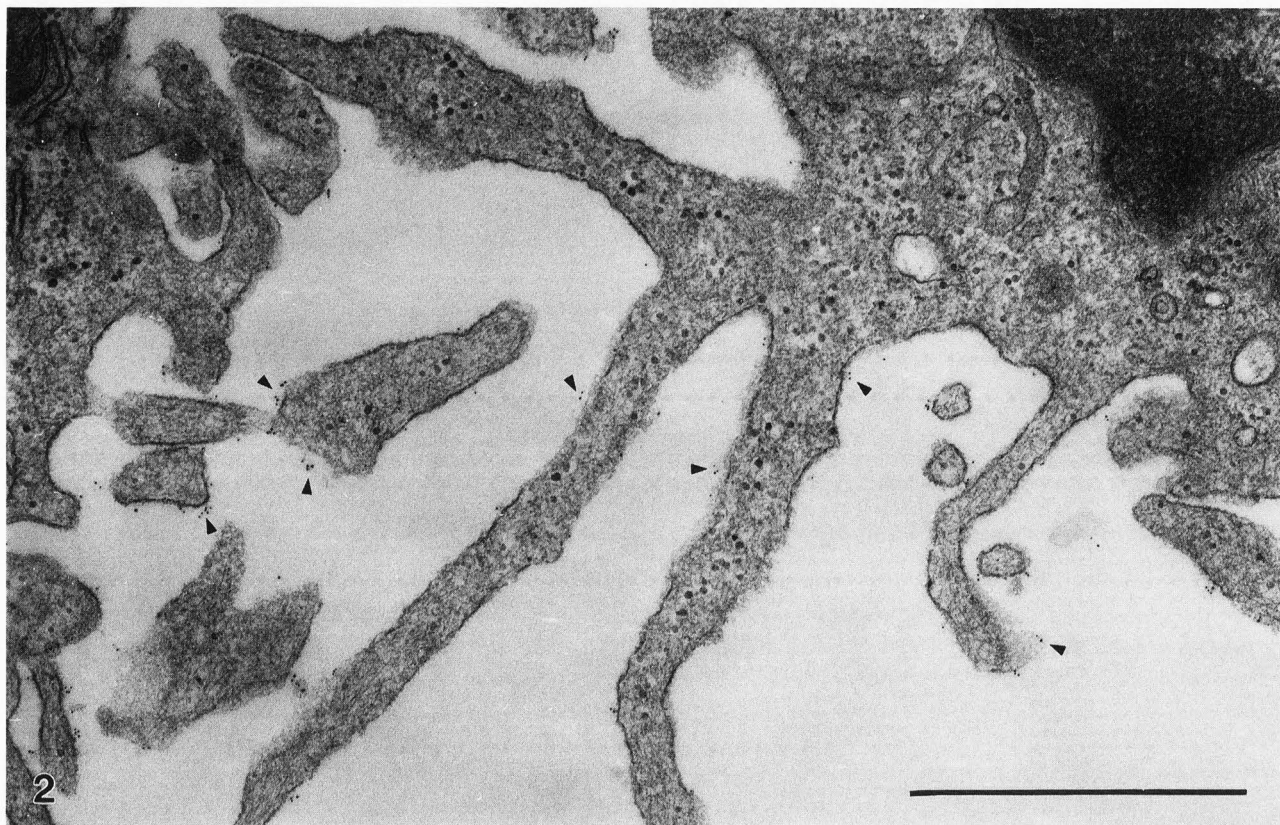
Figure 2. Indirect immunogold labeling of another hairy cell incubated with the B-ly7 antibody and studied at high magnification to facilitate the observation of the numerous 5 nm gold markers, frequently seen in small clusters on cell surface (arrows).

(SEM), cells were attached on poly-l-lysine coated glass coverslips and dip-rinsed for all preparatory steps, strictly avoiding any risk of air drying. For transmission electron microscopy (TEM), the cells were incubated in suspension and centrifuged at low speed for each step. Since indirect immunogold labeling was used, secondary incubation was with goat anti-murine IgG1 adsorbed on 5 nm colloidal gold particles for TEM and 30 nm colloidal gold particles for SEM (GAM-G5 and GAM-G30, respectively, from Janssen Pharmaceutica, Beerse, Belgium). Dilution of the antibody conjugates depended on gold particle size: 1:20 for the 5 nm, and 1:10 for the 30 nm size particles. Final fixation was with 2.5% glutaraldehyde followed by osmium tetroxide for TEM, and with glutaraldehyde alone for SEM. Fragments of cell pellet were embedded in Epon/Araldite for TEM; SEM coverslips were dried at the critical point of CO_2 and conductive coated with evaporated carbon only. For TEM, a JEOL 1200-EXII (JEOL-USA, Inc., Peabody, Mass.) was used, at original magnification ranging from 4,000 to 25,000 X. For

SEM, a JEOL 840 instrument was used, fitted with the standard solid state annular backscattered electron detector and with a lanthanum hexaboride cathode (Denka, Mitsui and Co., Tokyo, Japan). Imaging was at 15 kV accelerating voltage for both secondary (SE) and backscattered electron (BE) signals.

More details on the procedures used for immunogold labeling have been previously published (de Harven and Soligo, 1986, 1989; de Harven et al., 1984).

Results

Hairy cell leukemias

Under the TEM, hairy cells are primarily identified by their long, "dagger-shaped" surface projections. These projections are, in most cells, easily distinguished from surface microvilli by their greater length and by their diameter which is characteristically larger near the cell surface than at the tip of the projection. In nearly 50% of the cases (Daniel and Flandrin, 1974), the cytoplasm features a very distinct structure classically
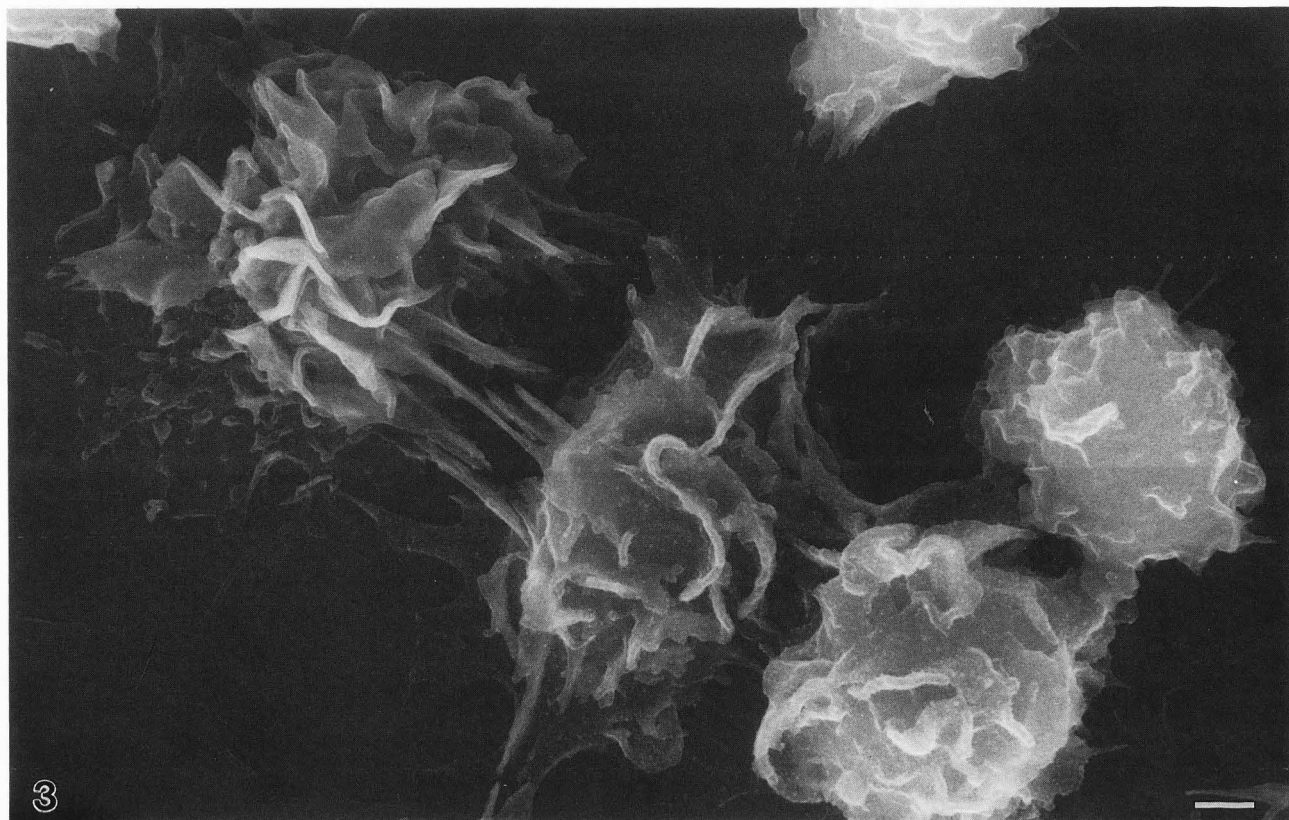
**Figure 3**. Scanning electron micrograph of the hairy cells illustrating the typical ruffling surface morphology. The gold markers are only faintly recognized in this secondary electron image.

described as "ribosome-lamellae complex" or RLC (Katayama *et al.*, 1972). The presence of these structures strongly supports a diagnosis of HCL, although their absence does not rule it out. Moreover, it should be emphasized that RLC have been described in non-hairy cell diseases. RLC were observed in only one of the two cases studied here. Other cytoplasmic organelles (mitochondria, Golgi, RER) were unremarkable, although their large number suggested well differentiated cells, not primitive blasts. With a significant amount of marginated heterochromatin and small, inconspicuous nucleoli (Fig. 1), the ultrastructure of the nuclei of hairy cells did not resemble that of progenitor cells or blasts.

The B-ly7 antibody recognizes an antigen present on the surface of almost all hairy cells; it has recently been enclosed in the CD103 cluster (Cepek *et al.*, 1995). The expression of this antigen as demonstrated by immunolabeling with 5 nm colloidal gold markers was relatively intense, unquestionably above non-specific background. Admittedly, counting gold markers present on cell surfaces is of limited biological value (de Harven *et al.*, 1987). Nevertheless, 100-200 gold markers were frequently counted on one thin-section of a single cell, sometime as single markers or as small clusters (Fig. 2). It is important to note that a few cells with the typical morphology of hairy cells were clearly unlabeled and therefore considered as B-ly7 negative. The percentage of hairy cells varied considerably from one patient to another. The non-hairy cells were mostly lymphocytes. Typical monocytes were practically never observed in the peripheral blood of these patients, confirming a previous report by Seshardi *et al.* (1976). An extensive TEM search for B-ly7 positivity in non-hairy cells of these two patients remained entirely negative. In one control TEM experiment with mononuclear cells from a normal donor, rare lymphocytes were found B-ly7 positive and all monocytes were found negative.

Under the SEM, the florid surface morphology of hairy cells is striking (Fig. 3). The abundance of ruffles and long surface projections is diagnostic at first glance. The clinical importance of this observation has long been noticed (de Harven and Lampen, 1978). Since the time interval between collection of the patient's blood and SEM observation can be as short as three to four hours,
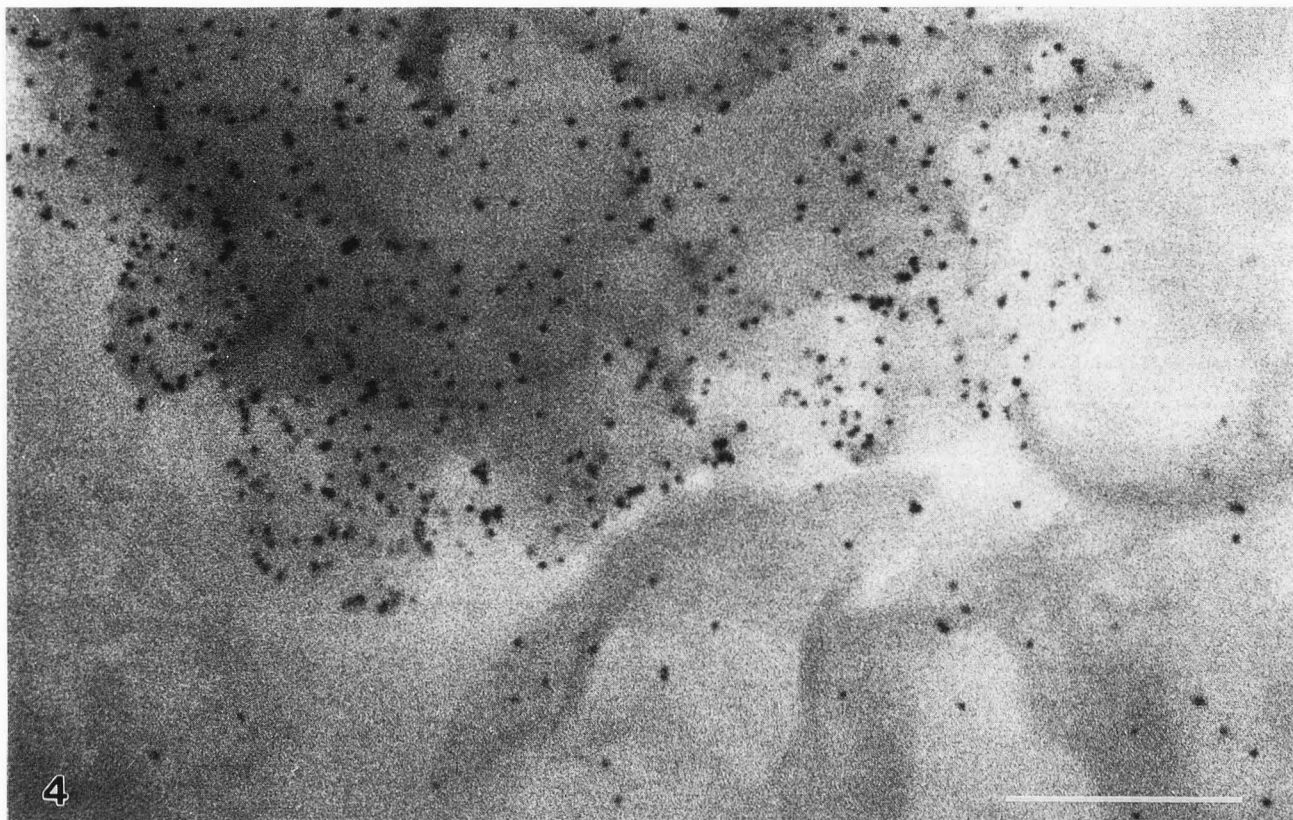
Figure 4. In the backscattered electron imaging mode (reverse polarity), the 30 nm gold markers are unambiguously identified throughout the cell surface of the same hairy cell as that shown on Figure 3.

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reports can reach the clinical hematologists prior to therapeutic decisions. Of course, processing time is longer if immunogold labeling is performed. Using the B-ly7 antibody, most hairy cells were heavily labeled (Fig. 4) in spite of the low labeling efficiency of 30 nm gold markers (de Harven and Soligo, 1989). According to earlier reports (de Harven et al., 1984), and once more confirmed here, the backscattered electron (BE) imaging mode of the SEM is, by far, the most efficient approach to an unambiguous recognition of positively labeled cell surfaces. Unquestionably, the search for weakly labeled non-hairy cells would have been very difficult if this investigation had been limited to the use of the secondary electron (SE) imaging mode. Using the BE mode, one can clearly state that a few hairy cells with the typical florid surface morphology were entirely B-ly7 negative, as already seen in TEM. At variance with the TEM observations, rare B-ly7 positive cells with the surface morphology of lymphocytes were observed. This subset of B-ly7 positive cells, obviously lymphocytes (de Harven et al., 1986), represented about 1% of the total lymphocyte population in this sample.

However, an extensive search, in the BE imaging mode, for B-ly7 positive lymphocytes in one control sample from a normal donor remained entirely negative (230 cells counted).

Megakaryoblastic leukemias (M7)

The study of megakaryoblastic disease has been performed by immuno-TEM, all cells being primed with the anti-GpIIIa (CD61) monoclonal antibody. GpIIIa, a surface glycoprotein known as one portion of the GpIIb/IIIa complex, was found expressed on all platelets, all megakaryocytes and many megakaryoblasts. Seven cases in which the diagnosis of M7 was suspected by light microscopy of peripheral blood films, have been studied and the observations summarized as follows.

All platelets were heavily labeled and served as a convenient internal control, ensuring that the GpIIIa glycoprotein had been effectively labeled with the immunogold marker in each sample. The labeling density, expressed as the number of 5 nm colloidal gold markers per 1 μm of cell contour (de Harven and Christensen, 1992), was apparently maximum on platelets and served
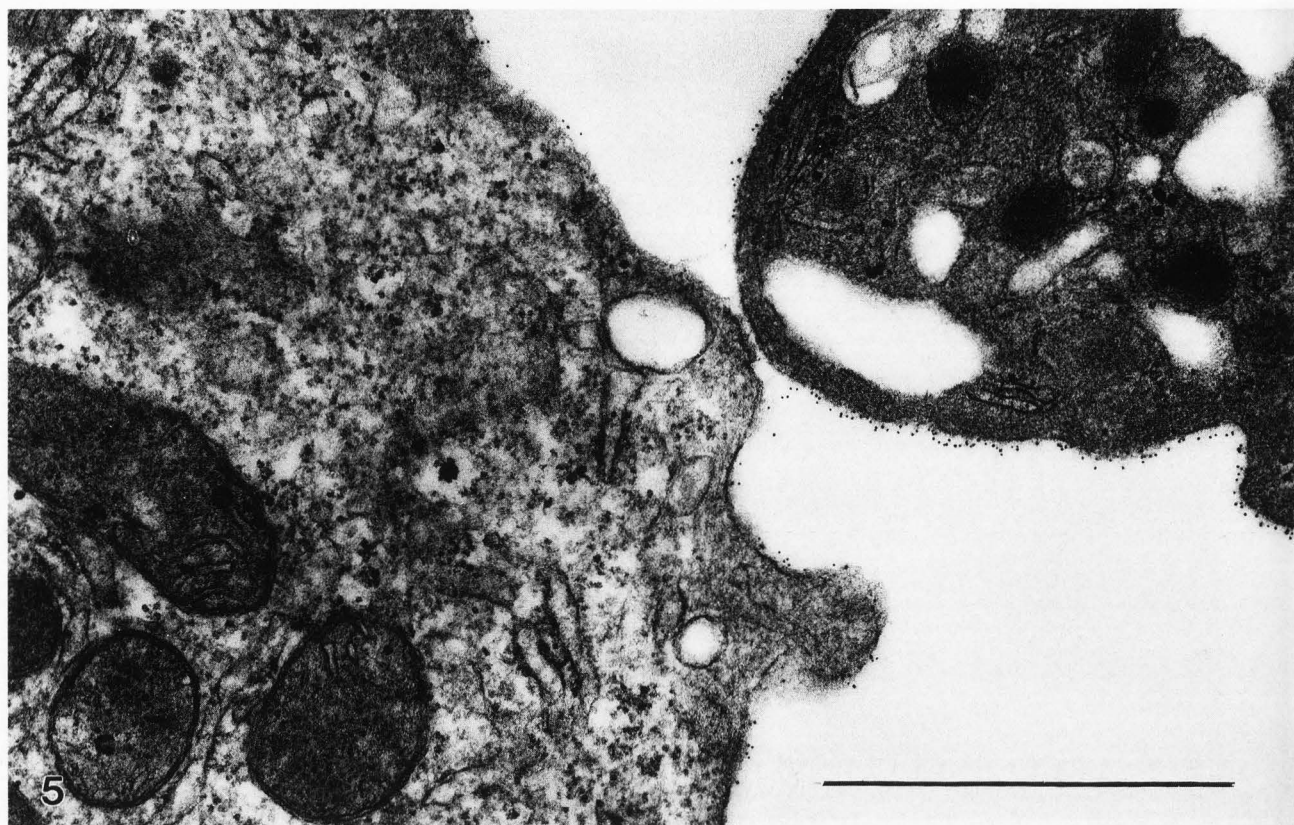
Figure 5. High magnification of a megakaryocyte precursor cell showing intense labeling for the CD61 antigen. Note the higher labeling density on the surface of the adjacent platelet (upper right).

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as a reference to evaluate the level of GpIIIa glyco-protein expression on the surface of megakaryocyte precursors. Giant platelets, equally heavily labeled, were frequently observed. They were somewhat difficult to distinguish from circulating megakaryocytes, since the apparent absence of any nuclei could be due to thin-sectioning artifacts.

Megakaryocyte precursors were identified by the presence of either one or more ultrastructural features, i.e., (1) alpha granules, (2) demarcation membranes, and (3) large surface blebs. More primitive cells of that lineage featured a high nucleo/cytoplasmic ratio and a predominantly euchromatic nucleus with a large nucleolus. All megakaryocyte precursor cells were found CD61 positive (Fig. 5), with various degrees of labeling density. A quantitative analysis was performed in an attempt to establish whether or not the cytoplasmic level of differentiation correlated with the level of expression of the glycoprotein on the cell surface. The results of this analysis were completely negative, indicating that the presence and/or the number of cytoplasmic differentiation organelles appears independent from the level of

expression of GpIIIa on cell surfaces, as previously reported (de Harven and Christensen, 1992), although platelets and megakaryocytes were always heavily labeled.

Primitive blasts with a very high N/C ratio and practically no evidence of cytoplasmic differentiation were frequently GpIIIa positive (Fig. 6). The gold markers on these cells were evenly distributed along the whole contour of these cells and, therefore, do not correspond to focal absorption of platelet membrane fragments as demonstrated by Breton-Gorius *et al.* (1987) on the surface of monocytes. These cells would not have been identified as primitive megakaryoblasts without immunolabeling for GpIIIa. Moreover, since the IIIa portion of the glycoprotein complex is expressed early in megakaryocytic differentiation (Koller *et al.*, 1991), we probably would have missed identifying these cells had we used an antibody directed against the entire complex (CD41 and CD61).

Finally, primitive blasts, very similar to the one shown in Figure 6, were found unlabeled. One can only hypothesize that some of these cells probably represent
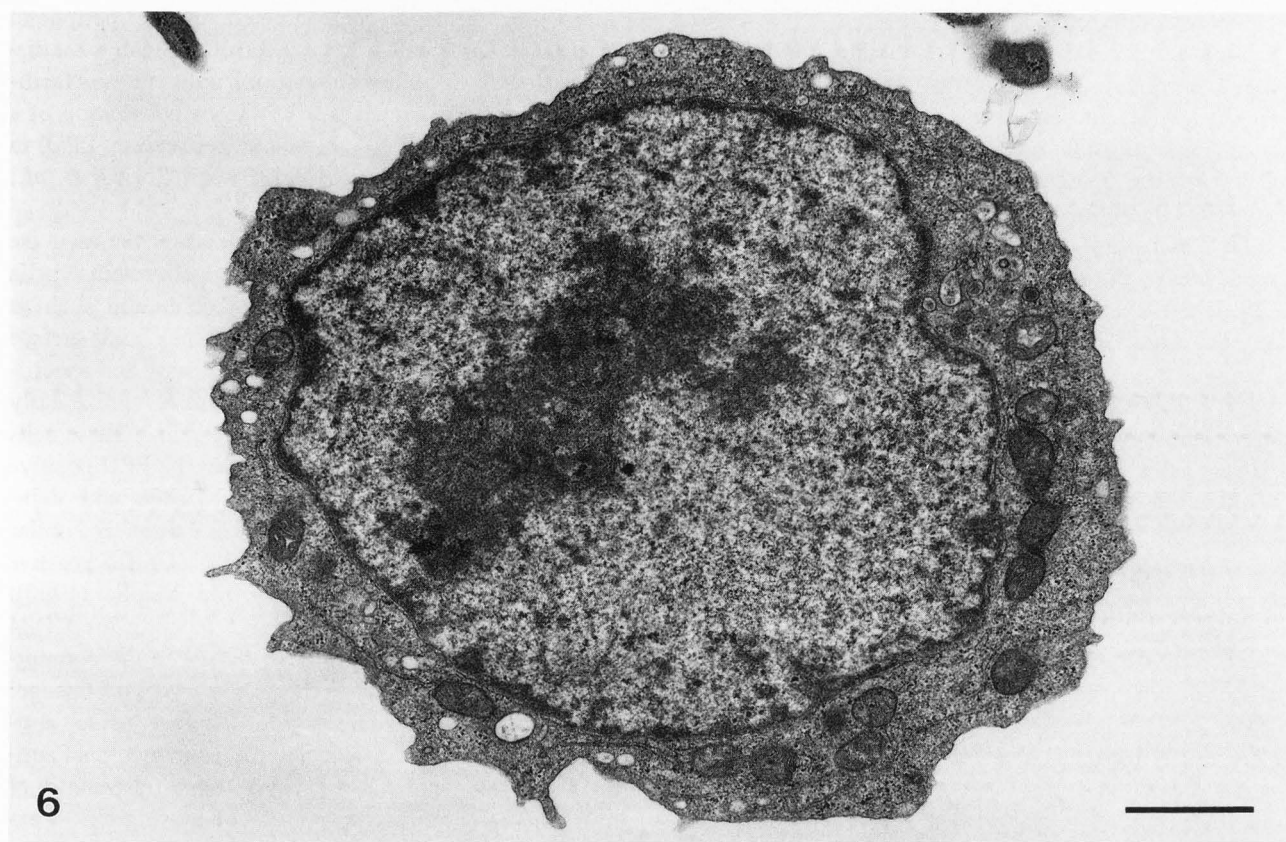
Figure 6. A primitive megakaryoblast shows weak CD61 labeling (only 9 gold markers can be counted over the entire cell margin on this figure, a labeling density which is, however, definitely regarded as above non-specific background). Note the large size of the nucleolus, the predominantly euchromatic nucleus, and the high nucleo-cytoplasmic ratio.

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primitive megakaryoblasts. Methods for double immunolabeling (de Harven *et al.*, 1990) will be used in further studies to find out whether such cells are CD34 positive and to precisely characterize the ultrastructural correlations between the (CD34+/CD61-) and the (CD34+/CD61+) and (CD34-/CD61+) phenotypic differentiation steps.

Discussion

Hairy cell leukemia remains an intriguing disease for several reasons. The normal counterpart of hairy cells have not, to our knowledge, been identified ultrastructurally. Hairy cells have never been seen undergoing mitotic division and do not incorporate tritiated thymidine (Gee T, personal communication, 1992). They share surface features (Polliack *et al.*, 1974) and surface markers (Soligo *et al.*, 1985; de Harven *et al.*, 1992) of lymphocytes and monocytes, and respond well to several therapeutic protocols which are without benefit in other forms of leukemia. After the characteriza-

tion of the B-ly7 monoclonal antibody (Poppema *et al.*, 1989), one would have expected ultrastructural identification of the normal counterpart of hairy cells; yet, to our knowledge, this has not so far been reported. Our observation of B-ly7 positive lymphocytes in one case of HCL is intriguing because, in one sample from a normal donor, we did find B-ly7 positive lymphocytes by TEM and could never confirm the finding by SEM. This apparent discrepancy between TEM and SEM observations is probably due to the small number of B-ly7 positive lymphocytes. Of course, these studies should be repeated on larger numbers of normal control samples for more precise ultrastructural characterization of cells which are likely to represent the normal counterparts of hairy cells. Further studies with double labeling for B-ly7 and B- or T-lymphocyte surface markers will help to identify the nature of the B-ly7 positive lymphocytes. Still, if such studies do confirm a lymphocytic derivation of hairy cells as frequently claimed in the past, we would still have to understand why HCLs do not respond to therapeutic protocols that induce remissions in

lymphoid malignancies.

From the point of view of the ultrastructural hematologist, hairy cells are interesting for two reasons: (1) their surface morphology is the best target for rapid diagnostic identification under the SEM, and (2) they frequently feature cytoplasmic ribosome-lamellae complexes (RLC). Admittedly, RLC have been described in non-HCL hematological disorders (see, Ghadially, 1982, for a review). However, over the past ten years one of us (de Harven) has been studying, with the TEM, more than 500 cases of leukemias and associated disorders without having ever encountered RLC in non-hairy cell diseases. This negative report is probably of some consequence in view of the long standing research interest in HCL of this investigator.

Other remarks concern our GpIIIa labeling studies on megakaryoblastic leukemia (M7). The seven patients studied had advanced, rapidly progressing disease and represented a heterogeneous group in which three were described as chronic myelogenous leukemia (CML) in blast crisis. Could we confirm, by GpIIIa labeling of PBLs, the existence of a true megakaryoblastic leukemia in all cases? The answers are mostly negative, because we frequently observed a spectrum of differentiation of megakaryocytic elements which corresponds likely to some form of extra-medullary megakaryocytopoiesis or megakaryocythemia in patients with bone marrow extensively invaded by another neoplastic clone.

The presence of megakaryocytes in the peripheral blood of normal individuals has long been reported (Efrati and Rozenszajn, 1960; Herbeuval et al., 1962a). Furthermore, large numbers of megakaryocytes were found in the peripheral blood in cases of hematopoietic malignancies, particularly in chronic myelogenous leukemia (CML) as reported by Herbeuval et al. (1962b) using immuno-fluorescence with platelet anti-serum (Duheille et al., 1962). These observations confirmed an earlier report by Minot (1922) who had already observed that, in some cases of myelogenous leukemia, many megakaryocytes appear in the peripheral blood. Circulating megakaryocyte progenitors (CFU-MK) were described in 9 out of 10 cases of myelofibrosis (Han et al., 1988). Moreover, circulating megakaryoblasts were characterized by immuno-electron microscopy in ten cases of myeloproliferative diseases (Matolcsy and Majdic, 1990).

The identification of megakaryocytes and their precursors was a matter of great interest in the 1960's when many investigators applied the newly discovered methods of diagnostic cytology in attempts to demonstrate metastasizing cells in the peripheral blood of cancer patients. Hume et al. (1964), for example, using Millipore filtration and Papanicolaou staining, failed to demonstrate embolic tumor cells but recognized the number of mega-

karyocytes frequently encountered in the peripheral blood of patients with a large spectrum of malignant diseases. Identifying circulating megakaryocytes was facilitated to an important degree by the demonstration of a distinct peroxidase activity (platelet peroxidase, PPO) in platelets and megakaryocytes (Breton-Gorius et al., 1972, 1984).

In addition, we found no correlation between the level of expression of the surface glycoprotein GpIIIa and ultrastructural markers of differentiation such as alpha granules, demarcation membranes and surface blebs. However, because of the sensitivity and specificity of GpIIIa labeling we were able to recognize early megakaryocyte progenitor circulating cells. These cells appeared much less differentiated than the PPO positive MK precursors described by Breton-Gorius and Vainchenker (1986). They are, however, extremely similar to the "lymphocyte-like" mononuclear GpIIIa positive cells demonstrated by Matolcsy and Majdic (1990). Further studies will include double labeling for CD34 and GpIIIa to recognize the early steps in the commitment of primitive blasts to the megakaryocytic lineage. Hopefully, double immunogold labeling using anti-GpIIIa IgG and biotinylated anti-CD34 monoclonal antibody will soon enable us to analyze the ultrastructure of primitive cells during the process of loosing CD34 expression and acquiring that of GpIIIa. This critical step in the commitment to megakaryocytic differentiation, modulated by the synergistic effect of several growth factors, is under active investigation in other laboratories (Debili et al., 1993).

In **conclusion**, two different forms of leukemia in adult patients, i.e., hairy cell leukemia (HCL) and megakaryocytic leukemia (M7) have been studied by immuno-electron microscopy of peripheral blood leukocytes. In HCL, electron microscopy provided an important diagnostic confirmation, necessary in view of the very specific response of this disease to certain therapeutic protocols.

In so called "M7", electron microscopy of PBLs demonstrated, in several cases, a spectrum of differentiation probably similar to the megakaryocythemia described many years ago by several authors studying the peripheral blood of patients with a variety of malignant conditions, including CML. Therefore, some of these cases should probably not be diagnosed as "megakaryoblastic" leukemia (M7). Similarly, when the bone marrow is packed with proliferating lymphoblasts, observing circulating myelocytes or erythroblasts is never interpreted as a need to modify the diagnosis nor to consider a possible biphenotypic disease. The high degree of sensitivity and specificity of immunogold labeling for the identification of CD61 on primitive megakaryoblasts plays a major role in current studies of in vitro and in

vivo megakaryocytic differentiation (Zipursky *et al.*, 1995).

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Discussion with Reviewers

Reviewer I: It would be of interest to document the precise subcellular distribution of the B-ly7 antibody on the hairy cell surface which could add to the understanding of the function of the corresponding antigen. For example, adhesion molecules are often concentrated on the tip of microvilli.

Authors: We are familiar with the frequent concentration of many antigens on surface microvilli. However, this does not seem to be the case for the antigenic sites recognized by the B-ly7 or the anti-GpIIIa antibodies.

Reviewer I: Could you give more information on the patients studied, particularly, the bone marrow and blood counts?

Authors: A paper is in preparation which will be submitted to a hematology journal and in which additional clinical data concerning the "Query M7" patients will be provided. More details on the clinical data of the HCL patients have already been published (see de Harven *et al.*, 1994).

Reviewer I: Megakaryocyte precursors that display α-granules and demarcation membranes are not any more precursors but simply megakaryocytes. The number of cytoplasmic organelles cannot be correlated to cell differentiation; more important is the nucleus-cytoplasmic ratio, texture of the chromatin, and shape of the nucleus. Please comment.

Authors: We do not agree with the reviewer's remarks on terminology: we have seen α-granules and/or demarcation membranes in apparently diploid cells with a relatively high nucleo/cytoplasmic ratio and which should definitely be classified as megakaryocyte precursors.

J.E. Beesley: In diagnostic testing, control samples are obviously of great importance. Would the authors please give details of their positive and negative controls for their experiments.

Authors: We have never found any CD61+ cells in any other case of leukemia, not considering platelets, of course.

J.E. Beesley: Would the authors please give an indication of the immunolabeling normally expected with GPIIIa?

Authors: We have found a considerable variation in the level of GpIIIa labeling in MK precursor cells, and we could not, therefore, illustrate what should be regarded as the "normal" level expected in these cells.

A. Liepins: Is there a particular reason why the B-ly7 antibody was selected in these studies instead of HML 1, Ber-Act 8 and/or LFG1?

Authors: The reason we have worked with the B-ly7 antibody is simply that it was kindly made available to us by Dr. S. Poppema. We would be most interested if the reactivity of hairy cells for HML 1, Ber-Act 8 and/or LFG 1 would receive similar attention in the future, in view of the fact that they all belong to the CD103 cluster, as recently published (Cepek *et al.*, 1995). Blocking experiments would be useful in ascertaining whether or not these various antibodies compete indeed for the same or closely adjacent antigenic sites.

A. Liepins: In the megakaryoblastic leukaemia (M7) cases, labelling was carried out with anti-GPIIIa (CD-61) which recognizes fibronectin and vibronectin receptors on megakaryocytes and platelets. How do these results contribute to the clinical diagnosis of this type of leukaemia?

Authors: Labelling with anti-GPIIIa did not contribute significantly to the diagnosis of M7 leukemia primarily because, in most of our cases, a broad spectrum of differentiation of CD61+ cells were recognized by TEM, which is hardly compatible with the identification of leukemic clones. Anti-CD61 labelling made it possible to recognize megakaryocyte precursor cells which would have been, otherwise, left unidentified. It helped to recognize the spectrum of differentiation which frequently characterizes the megakaryocythemia accompanying some cases of CML and other myeloproliferative disorders. But it did not permit to identify these CD61+ cells as actually leukemic. In short, our study indicated that it is far from enough to recognize megakaryocyte precursor cells on blood films by light microscopy to consolidate a diagnosis of M7 leukemia and that immunogold-TEM with anti-CD61 can only confirm megakaryocythemia, not the megakaryocytic nature of the disease or of the blast crisis observed in some cases of CML.

R.M. Albrecht: The authors note cells, as the one shown in Figure 6, can be found either labeled or unlabeled. They note at higher magnification 9 gold particles are seen on the cell. The authors should make clear that this is over the entire cell margin, not just the portion seen at higher magnification? It might be useful to note what 9 particles in one section relate to, roughly, with respect to the total number of labels over the entire cell surface, i.e., what would be seen by SEM. What is the normal "background"?

Authors: First, the question of "normal background". One of the most significant advantages of immunogold labeling is its remarkably low non-specific background, as compared, for example, with techniques of immunoperoxidase. Still in all our immunogold labeling work, we had to set a threshold of significance. We have always set this, arbitrarily, at 5 gold particles (5 nm) for the entire cell contour. In the case illustrated in Figure 6, we counted 9 gold markers on the entire cell contour and, therefore, regarded that cell as B-ly7 positive. We wish we could be more explicit in terms of these numbers counted with EM as they equate to the entire cell surface as seen by SEM. Simple extrapolations are impossible, mainly because we used 5 nm gold for TEM and 30 nm gold for SEM, these two markers having, as is well-known, very different labeling efficiency.