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Bone marrow monocytes in histiocytosis X acquire some phenotypic features of Langerhans cells in long term bone marrow cultures*

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Summary. Bone marrow cells of a patient with Letterer-Siwe disease were cultured for three weeks in long-term bone marrow culture (LTBMC) conditions and examined at one-week intervals with a large panel of monoclonal antibodies by immunohistochemistry and by the immunogold transmission electron microscopy (immunoTEM) technique. Although at diagnosis the bone marrow showed a slight increase of monocytes with a normal phenotype, a rapid expansion of cells expressing CD1a and CD1c was observed already after 1 week of culture. A progressive increase in CD4, CD11b and CD11c expression was also observed. ImmunoTEM of cultured cells demonstrated that $CD1a^+$ cells had macrophage-like morphology, and did not contain Birbeck granules. These findings indicate that bone marrow monocytes acquire some phenotypical features of Langerhans cells in LTBMC and support the hypothesis that these cells may derive directly from a bone marrow monocytic precursor.

Key words: Histiocytosis X – Langerhans cell – Bone marrow culture

Introduction

Macrophages and dendritic cells are two distinct groups of non-lymphoid cells involved in the immune response (Unanue et al. 1984; Tew et al. 1982). Dendritic cells exhibit several features that distinguish them from macrophages: the characteristic dendritic morphology, a more potent accessory cell function, lower levels of lysosomal enzymes and, more importantly, high expression of class II major histocompatibility complex and CD1a antigens (Franklin et al. 1986; Van Vaorhis et al. 1983). At ultrastructural analysis dendritic cells typically contain relatively few cytoplasmic organelles correlated with phagocytic functions, whereas macrophages are rich in lysosomes and endocytic vacuoles (Schmitt et al. 1983). Langerhans cells (LCs), a type of dendritic cell, seem to be of bone marrow origin (Frelinger et al. 1979; Katz et al. 1979) and are widely distributed in the skin and other stratified squamous epithelia (Hammar et al. 1986). A proliferative disorder of LCs is considered to be at least partly responsible for histiocytosis X (HX) based on the observation that the proliferating cells (HX cells) in patients with HX display the essential ultrastructural and immunophenotypic features of LCs, including an abundance of Birbeck granules and surface expression of class II and CD1 antigens (Thomas et al. 1982; Schuler et al. 1983).

Assuming that HX is a proliferative disorder of LCs and that LCs are dendritic cells of bone marrow origin, we studied a patient with "adult" Letterer-Siwe disease (Caputo et al. 1984), the disseminated form of HX, in whom 14% of bone marrow monocytes and monocytosis in peripheral blood were observed at diagnosis. Our aim was to investigate if, in long-term culture conditions, bone marrow cells may acquire the phenotypic features of LCs.

Materials and methods

Case report

The patient we studied was an 80 year old woman with Letterer-Siwe disease; HX lesions were present in the skin as cutaneous eruptions; a skin biopsy showed massive band-like infiltration of HX cells ($CD1a^+$ and $S100^+$ by indirect immunofluorescence

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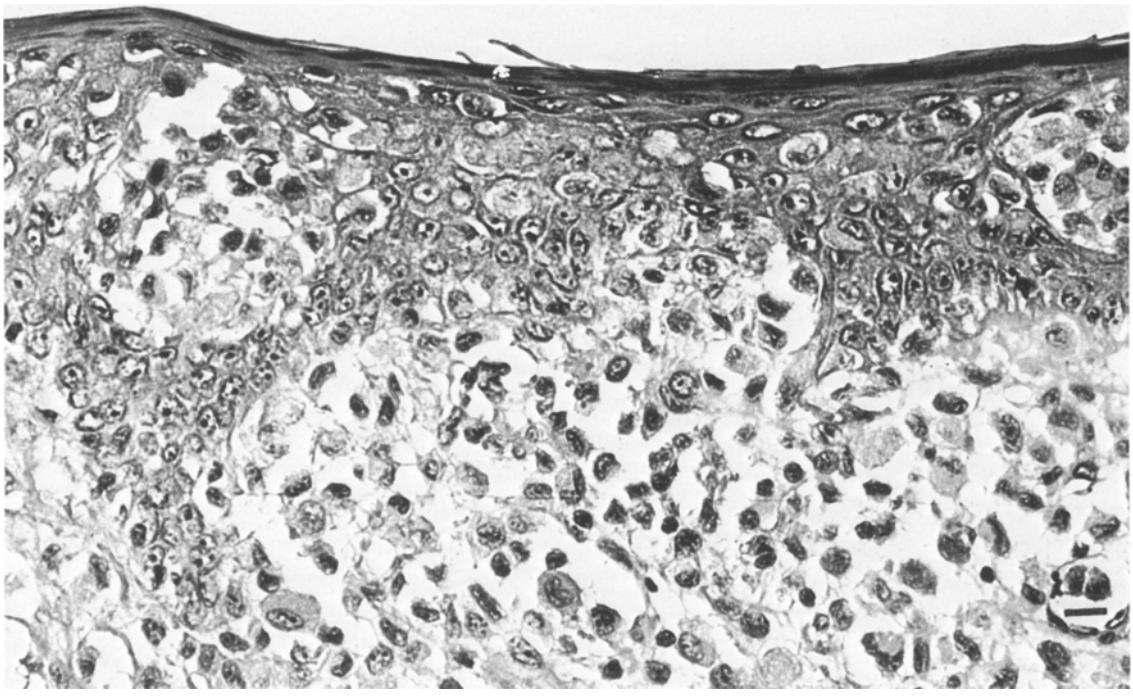


Fig. 1. Skin biopsy showing massive infiltration of the epidermis by HX cells. Haematoxylin-eosin stain. ×800 (*Bar* = 10 μm)

on cryostat sections) with epidermotropism and intraepidermal nests (Fig. 1). Organ and bone involvement was excluded by clinical and radiological investigations, including CT scans. A myelogram and bone marrow biopsy showed normal morphology with a moderate increase in the percentage of normal monocytes (14%), and a mild monocytosis was present also in peripheral blood.

Human bone marrow long term cultures were established as described by Dexter et al. (1984). Bone marrow cells from the patient were separated by Ficoll-Hypaque gradient centrifugation and were plated in 9 ml/25 cm^2 culture flasks (Corning) at a final viable cell count (20×10^6) which is considered optimal for growth of cultures. McCoy's 5A medium was supplemented with 12.5% fetal calf serum, 12.5% horse serum and 1×10^{-5} M hydrocortisone, 10^{-4} M mercaptoethanol, 40 mg/100 ml glutamine, 1 mg/100 ml folic acid and 1% penicillin/streptomicin, (Greenberg et al. 1981). Cultures were incubated at 37° C with 5% CO_2 and maintained by replacing 5 ml of spent medium with fresh medium twice weekly. They were stopped at one, two and three weeks by rinsing repeatedly the flasks to remove all non-adherent cells. Adherent cells were recovered at confluency on two week flasks after treatment with 0.1% trypsin for 10 min at 37° C.

Cells were then examined with cytofluorography, immunocytochemical and immunotransmission electron microscopy (immunoTEM) techniques.

An immunohistochemical study in situ of the adherent layer was also performed weekly after dessication of the culture flasks.

A large panel of Moabs was used, whose characteristics are listed in Table 1.

Labelling of bone marrow culture cells was carried out by standard indirect immunofluorescence (IF). Briefly, 100000 cells in 45 μl RPMI 1640 (Flow Laboratories, Irvine, UK) were incubated first with 5 μl of Moab at the appropriate dilution (or of normal mouse serum for the negative control) for 30 min on ice, and then with 5 μl of fluorescein-conjugated goat $F(ab)_2$ anti-mouse IgG (Dako, Glostrup, Denmark) for an additional 30 min. After washing in RPMI, cells were transferred into microtest tubes and analyzed by flow cytometry with a Becton-Dickinson FACS IV equipped with an argon ion laser.

Immunocytochemical analysis was performed with the alkaline phosphatase anti-alkaline phosphatase (APAAP) technique (Cordell et al. 1984) on cytospin preparations (Cytospin 2, Shandon Elliot, UK) of the adherent and non-adherent cells; after fixation in buffered formol-acetate (BFA) and rinsing in TRIS buffered saline (TBS) pH 7.6, samples were incubated for 20 min with TBS containing 10% human decomplemented AB serum and subsequently reacted for 30 min at room temperature with the primary Moabs described in Table 1. Normal mouse serum (MsIgG, Coulter Electr. Ltd, Luton, UK) and Control Ascites Fluid (BioMakor bm, Rehovot, Israel) were used as controls. After rinsing with TBS, cytospins were incubated for 30 min with rabbit anti-mouse immunoglobulins (Dakopatt, Glostrup, Denmark) and for 15 min with APAAP complexes (Dakopatt). This step was repeated at least twice. Slides were then rinsed in TBS and incubated for 15 min with the alkaline phosphatase substrate containing naphthol-AS-Bi-phosphate (Sigma, St. Louis, USA, Cat. # 2250), neofuchsin (Merck, FRG), sodium nitrite and 1 mM levamisole; preparations were then counterstained with haematoxylin, air dried and observed with a Leitz Laborlux K light microscope.

The same APAAP technique was used for the immunohistochemical study of the adherent layer after desiccation of the plastic bottom of the flasks and mild pre-fixation with glutaraldehyde 0.2%.

A routine ultrastructural analysis of bone marrow cells was performed at diagnosis. Immuno-transmission electron microscopy was also performed on the cells of the supernatant after one week of culture as previously described (Polli et al. 1987). In particular, cells were prefixed with glutaraldehyde 0.1% in

Table 1. Monoclonal antibodies used in this study

Name	CD	Target cell	Source/Ref.
TEC-T6	1a	Cortical thymocytes, LCs	Techno Genetics[a]
L 161	1c	Cortical thymocytes, LCs	Bernard, Boumsell (1986)
OKT11	2	T-cell lineage	Ortho Diagnostic[b]
anti-Leu4	3	Mature T-lymphocytes	Becton Dickinson[c]
anti-Leu3	4	T-cell lineage, monocytes, macrophages, some dermal dentritic cells	Becton Dickinson
anti-Leu9	7	T cells and NK cells	Becton Dickinson
OKM1	11b	Metamyelocytes, granulocytes, monocytes	Ortho Diagnostic
anti-LeuM5	11c	Granulocytes, monocytes, macrophages	Becton Dickinson
anti-LeuM1	15	Myeloid cells, some monocytes, activated T-cells	Becton Dickinson
B4	19	B cells and B precursors	Coulter Electr. Ltd.[d]
anti-Leu14	22	B cells	Becton Dickinson
My9	33	Myeloid precursors	Coulter
TEC-gp120	34	Early myeloid precursors	Techno Genetics

[a] Techno Genetics, Cernusco S/N (MI), Italy
[b] Ortho Diagnostic System, Milano, Italy
[c] Becton Dickinson, Mountain View, CA, USA
[d] Coulter Electr. Ltd, Northwell Drive, Luton, England, UK

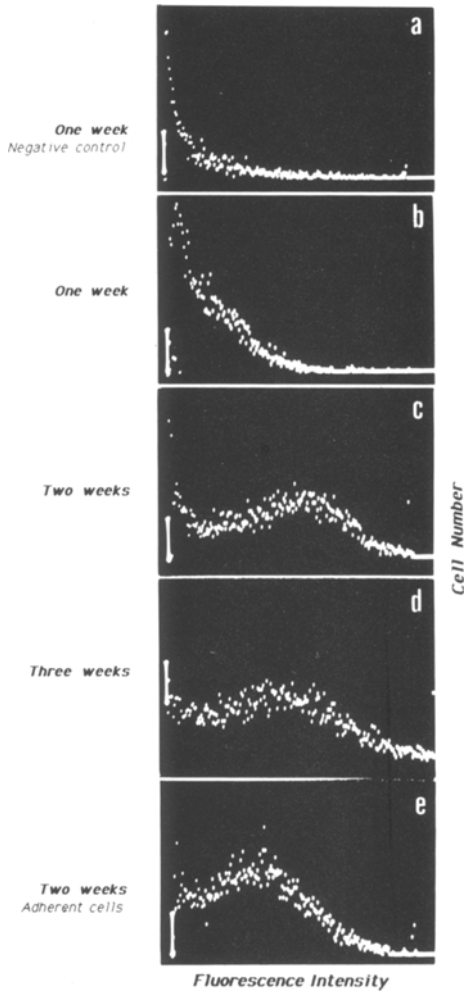


Fig. 2a–e. Flow cytofluorimetric analysis of CD1a expression by bone marrow cells in long term cultures. **a** Cells of the supernatant after one week of culture, stained with normal mouse serum (negative control). **b** Same as in **a**, but stained with CD1a MAb. **c** Same as in **b**, but after two weeks of culture. **d** Same as in **b**, but after three weeks of culture. **e** Cells from the adherent layer after trypsinization at two weeks of culture, CD1a staining. Data are presented as single parameter histograms. The x-axis represents relative fluorescence intensity (Log scale), and the y-axis the relative cell number (10000 cells per histogram)

Table 2. Immunophenotypic analysis of bone marrow cells* in LTBMC at different time intervals

	Monoclonal antibodies (CD)							
	CD1a	CD1c	CD2	CD4	CD11b	CD11c	CD15	CD19
Basal	0	0	5	3	35	30	10	3
1 week	27	20	3	5	50	45	21	4
2 weeks	50	20	3	25	60	55	40	3
2 weeks**	34	20	nd	0	nd	50	20	3
3 weeks	60	35	nd	40	50	60	15	nd

* Numbers refer to the percentage of positive non-adherent cells evaluated with a fluorescence activated cell sorter
** Percentage of positive cells in the adherent layer after trypsinization

PBS 0.1 M for 5 min, rinsed in PBS containing 1% glycine and with PBS containing 1% bovine serum albumin (Sigma cat. a-4378) and 1% human decomplemented AB serum (PBS/BSA/AB serum); subsequently cells were incubated for 30 min with the TEC-T6 Moab (CD1a), rinsed twice with PBS/BSA, incubated for 30 min with goat anti-mouse immunoglobulins conjugated with 15 nm colloidal gold particles (GAM-G40, Janssen Pharmaceutica, Beerse, Belgium), rinsed twice in PBS and postfixed for 30 min in glutaraldehyde 2% in PBS and for 30 min in 1% osmium tetroxide in PBS. After dehydration in graded alcohols and propylene oxide, samples were embedded in an Araldite mixture, and ultrathin sections, obtained with a LKB Ultrotome IV ultramicrotome, were observed with a Philips EM410 transmission electron microscope at 80 kV.

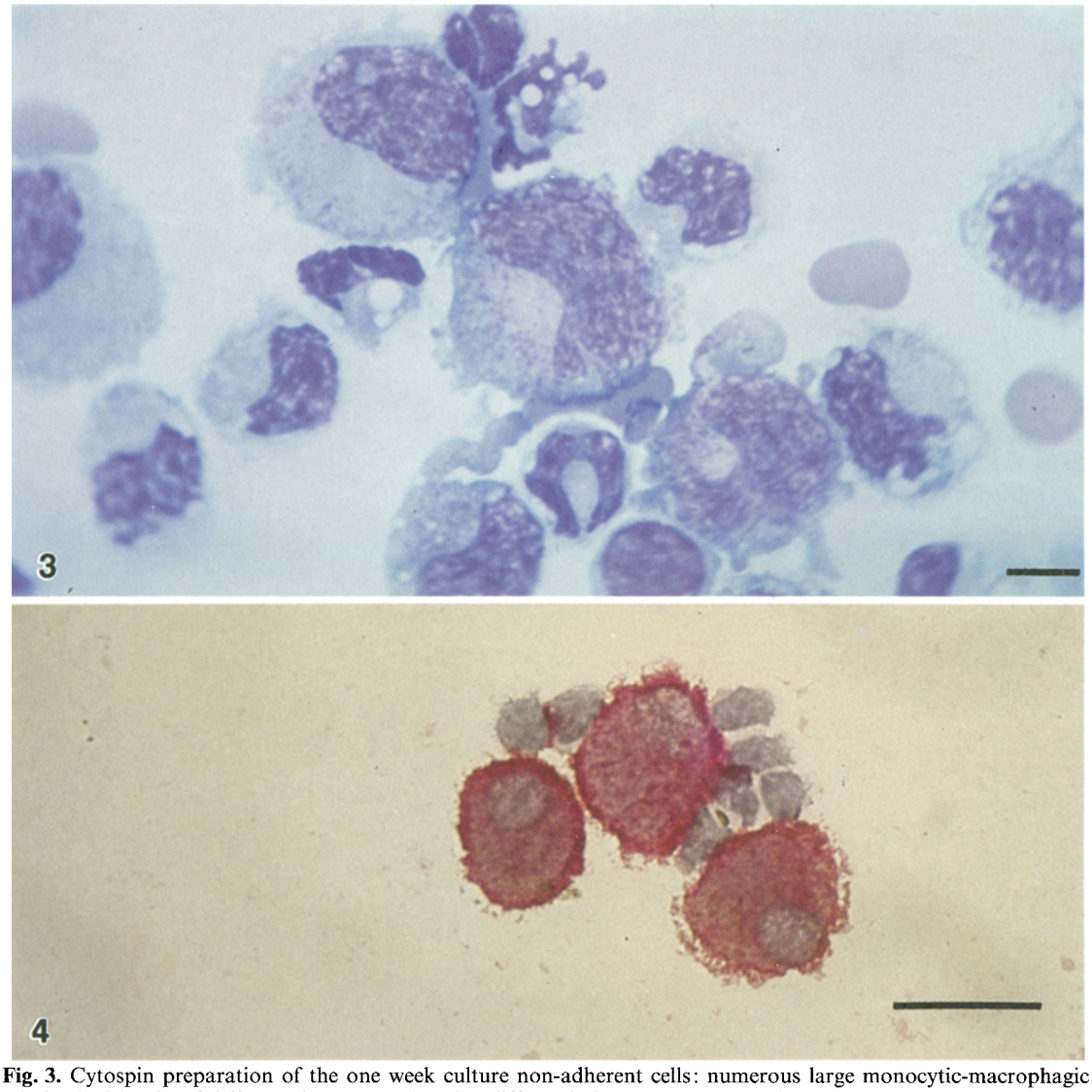


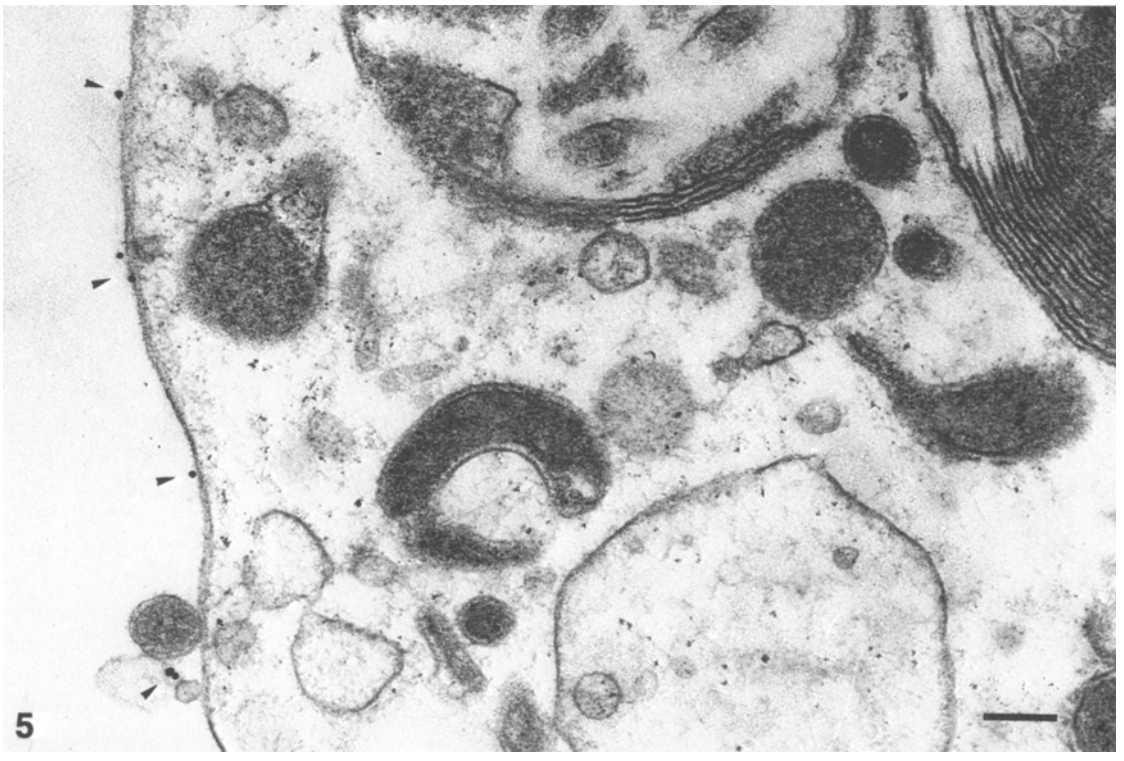
Fig. 3. Cytospin preparation of the one week culture non-adherent cells: numerous large monocytic-macrophagic cells are visible admixed with cells of different lineages undergoing differentiation. May-Grunwald Giemsa stain. × 5500 (*Bar* = 1.8 μm)

Fig. 4. Same sample as in Fig. 2, cells were labelled with CD1a Moab stained with the APAAP technique and counterstained with haematoxylin. × 200 (*Bar* = 100 μm)

Results

The bone marrow sample obtained at diagnosis showed a slight increase in monocytes (14%) with normal morphology also at TEM. FACS analysis of the supernatant (Table 2 and Fig. 2) showed the appearance of a large number of $CD1a^+$ and $CD1c^+$ cells after one week of culture; an increase of $CD11b^+$ and $CD11c^+$ cells was also observed. In the following weeks a progressive increase in CD1a expression was again seen both in adherent and non-adherent cells, but not of CD1c, CD11b and CD11c antigens; an increase of $CD4^+$ cells also occurred after the first two weeks of culture but only in the non-adherent population.

Immunocytochemical studies on the supernatant demonstrated that $CD1a^+$, $CD1c^+$ and some of $CD11b^+$ and $CD11c^+$ cells had macrophage-like features (Figs. 3–4). Ultrastructural studies using CD1a and colloidal gold showed 30% of cells labelled with colloidal gold at one week of culture. Positive cells had macrophage-like morphology, with round nucleus and abundant cytoplasm containing numerous lysosomes and phagolysosomes, mitochondria and myelin figures (Fig. 5); typical Birbeck granules were not observed in these sam-



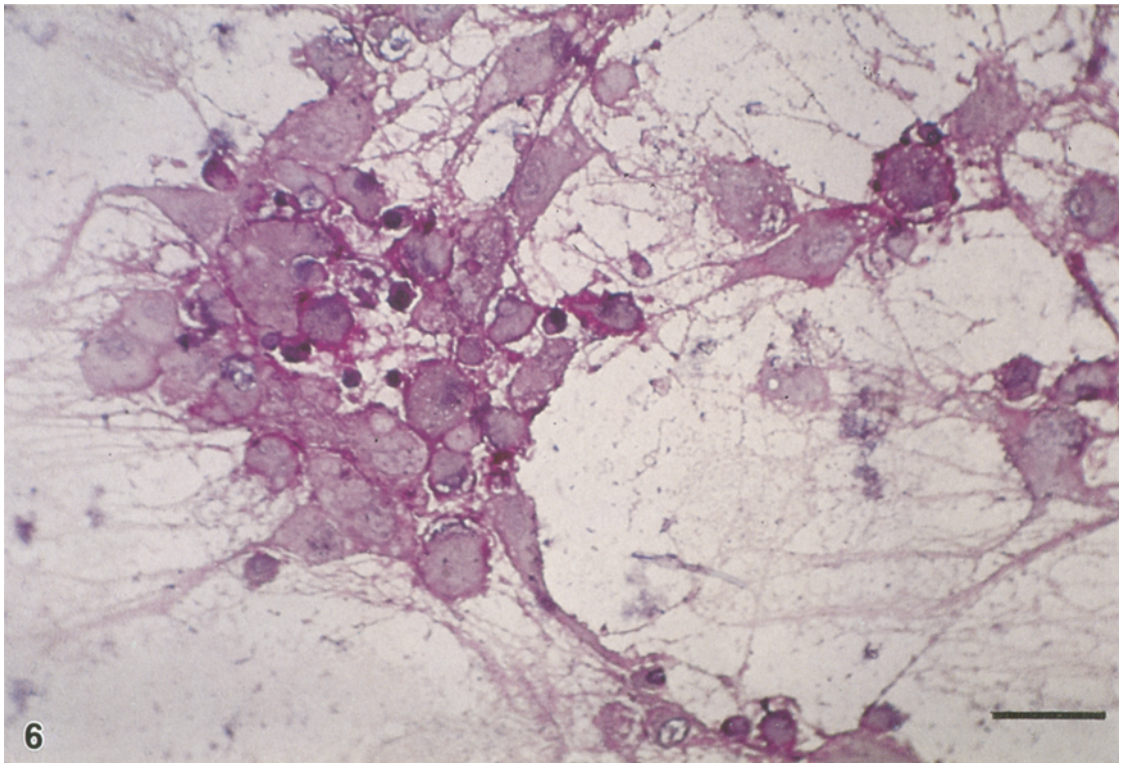


Fig. 5. Detail of a cell in the supernatant of the culture after one week showing numerous lysosomes and phagolysosomes in the cytoplasm and several gold particles (*arrows*) indicating labeling with CD1a Moab. Uranyl-acetate and lead nitrate staining; × 5500 (*Bar* = 0.18 μm)

Fig. 6. Adherent layer of the culture after two weeks: numerous adherent cells forming a network show a membrane staining with CD1a Moab. APAAP stain. Haematoxylin counterstain; × 1500 (*Bar* = 10 μm)

ples. Higher expression of CD15, probably due to the normal differentiation of myeloid cells in culture, was also present in the first two weeks.

A significant increase of $CD1a^+$, $CD11b^+$, $CD11c^+$ cells was also demonstrated in the adherent layer, after the first two weeks, using an "in situ" characterization of the culture flask bottom. The latter, when observed after "in situ" immunocytochemistry, showed a network of $CD1a^+$ dendritic cells forming a continuous adherent layer (Fig. 6). Numerous round cells were present on top of this layer, of which only a few scattered ones were also $CD1a^+$.

Moabs CD1b, CD2, CD7, CD19, CD22, CD33, CD34 were also tested on cells of the supernatant, but a significant increase in the number of positive cells during the culture time was never seen (Table 2).

Discussion

The ontogeny of Langerhans cells has been the subject of debate since their discovery about twenty years ago (Langerhans 1968). Ten years ago two different experiments performed by Frelinger et al. (1979) and Katz et al. (1979) demonstrated that, in the mouse, LCs have a bone marrow origin; studies on the skin of radiation-induced bone marrow chimeric mice showed that a high percentage of epidermal LCs derives from the bone marrow of donor animals.

Evidence for the bone marrow origin of LCs also in humans was provided by Pelletier et al. (1984) with a similar experiment on sex-mismatched bone marrow transplants. The monocytic derivation of LCs was more recently suggested by Gothelf et al. (1986) who identified an $HLA\text{-}DR^+$, $T6^+$, $Mo1^+$, $My4^+$ subpopulation in bone marrow peanut agglutinin (PNA)-separated mononuclear cells. Subsequently de Fraissinette et al. (1987) observed that, after one week of culture of PNA-separated normal bone marrow mononuclear cells, the percentage of $CD1a^+$ cells changed from 0.1% to 6%; ultrastructural studies indicated that $CD1a^+$ cells had monocytic features but lacked Birbeck granules. Similar results were obtained more recently on normal unseparated bone marrow after culture in methyl cellulose (de Fraissinette et al. 1988).

In our case a marked expansion of $CD1a^+$ cells was observed in LTBMC from unseparated, apparently normal bone marrow. These $CD1a^+$ cells, which after three weeks constituted up to 60% of all cells in the culture supernatant, showed in addition other phenotypic markers that are weakly expressed on LCs like CD4, CD11b and CD11c. As in the de Fraissinette (1987 and 1988) experiments, monocytic/macrophagic ultrastructural features were also present in the $CD1a^+$ cells. The LTBMC adherent layer, which is normally composed of different cell types like macrophages, fibroblasts, endothelial cells and so-called blanket cells (Dexter et al. 1984), consisted almost exclusively of $CD1a^+$ cells after only one week of culture.

All these data, taken together, provide further evidence for a bone marrow origin of LCs. In our case, transformed cells were probably already present in the bone marrow representing an expanded marrow compartment of neoplastic LCs precursors. The particular culture conditions of LTBMC may induce a subpopulation of monocytic cells (PNA^+, $CD1a^+$, $HLA\text{-}DR^+$) or their transformed counterparts (as in our case) to acquire some characteristics of LCs and possibly give them some proliferative advantage. It is evident from other studies that all dendritic cells and macrophages are very sensitive to local factors and that the expression of some surface antigens depends upon the state of differentiation or the influence of different cytokines (Walsh et al. 1985, 1986). The final differentiation step to a full Langerhans cell repertoire which includes Birbeck granules, may require however still unknown cutaneous factors, as proposed by Murphy (1986).

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