Identification of Hematopoietic Progenitors of Macrophages and Dendritic Langerhans Cells (DL-CFU) in Human Bone Marrow and Peripheral Blood

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Colonies of cells with distinctive dendritic appearance were observed in methylcellulose cultures of human bone marrow and peripheral blood mononuclear cells (PBMC). Such cells appeared alone in colonies of less than 50 cells, together with macrophages in mixed colonies and also within clusters of T lymphocytes at high culture cell numbers. The morphologic resemblance to lymphoid dendritic cells was confirmed by electron microscopy and the cells were distinguished from macrophages by immunoenzymatic and immunogold labeling with monoclonal antibodies (MoAbs). Like macrophages they were HLA-DR* and CD4+. However, they lacked nonspecific esterase and the macrophage cytoplasmic marker Y1/B2A. Most strikingly, cells were strongly HLA-DQ* and expressed CD1a (T6), which is characteristic of skin Langerhans cells. Their functional similarity to lymphoid dendritic cells was demonstrated by their ability to stimulate allogeneic mixed leukocyte reactions. Dendritic cell colony numbers were estimated in both bone marrow and peripheral blood of controls and in leukemia and lymphoma patients before and after chemotherapy. Colony numbers were low in control blood and in patients before treatment (<1.0 to 3.7/10⁶ cells). However, during hematopoietic recovery the mean value increased to 37.5/10⁶ cells and this increase correlated closely with the observed increase in circulating colony forming unit-granulocyte macrophage (CFU-GM) in individual patients. Autoradiographic studies demonstrated mitotic activity within CD1a+ colonies and a linear relationship between cultured cells and both pure and mixed colonies was consistent with their derivation from a single precursor. These data indicate that a novel hematopoietic progenitor of dendritic/Langerhans cells (DL-CFU) may now be identified in a clonal assay system and suggest a probable common progenitor for these cells and macrophages.

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Myeloid colonies (CFU-GM) were scored if they consisted of more than 50 cells.

Harvesting of colony cells for morphologic and functional studies. Colonies identified on the inverted microscope were plucked under direct vision with a bent glass pasteur pipette and taken into 200 μL of phosphate-buffered saline (PBS) or IMDM with or without antibody. Cytospin preparations were then air dried and either processed within 24 hours or frozen at −20°C for later use.

Cytchemistry and immunologic phenotyping. The air dried cytospin preparations were fixed either in methanol (for Giemsa staining), formalin-acetone for (α-naphthyl acetate esterase stain), or methanol-acetone before immunoenzymatic staining with the alkaline phosphatase-antialkaline phosphatase method (APAAP) as described.13 The following monoclonal antibodies (MoAbs) were used: NA/134 (anti-CD1a; Dako 1:5); Leu 4 (anti-CD3; Beckton-Dickinson 1:40); T4 (anti-CD4; Dako neat); anti-HLA-DR (Beckton-Dickinson 1:2); Leu 10 (anti-HLA-DQ Becton Dickinson 1:10); Y1/82a17 (monocyte/macrophage cytoplasmic marker, courtesy of DY Mason, neat); and 33D1 (anti-mouse DC 1:2, kindly supplied by R.M. Steinmann).18 Nonspecific binding of antibody was excluded by simultaneous incubations with irrelevant antibody as well as by the clear differential staining properties of Fc-receptor bearing macrophages and DC within the same preparation (see Results).

Electron microscopy. Colonies were transferred directly into 400 μL of IMDM with monoclonal antibody (MoAb) at the appropriate concentration (33D1: 1 in 2, NA/134: 1 in 5). Following a 1-hour incubation at 4°C, 20 μL of 0.1% sheep red cells were added to facilitate processing of the pelleted cells. After washing three times in PBS containing 1% BSA, the cells were immunolabeled with protein A/gold (10 nm) for 1 hour at 4°C. Two washes in PBS/BSA then preceded fixation in 3% glutaraldehyde in 0.1 mol/L phosphate buffer pH 7.4 for 1 hour at room temperature. After a wash in the
same buffer, the cells were postfixed in 1% osmium tetroxide in phosphate buffer for 45 minutes at room temperature. This was followed by a further wash in phosphate buffer. To minimize cell loss, the cells were next enrobed in 1.5% low melting point agar at 37°C. The resulting block was dehydrated in graded acetone and infiltrated with Spurr resin which was polymerized overnight at 70°C.

The osmicated cells could be seen within the agar block and the semithin sections were stained with toluidine blue to ascertain when an appropriate area had been reached. Ultrathin sections were mounted on copper grids and stained with aqueous uranyl acetate at 50°C and with lead citrate at room temperature.

**Mixed leukocyte cultures.** Peripheral blood mononuclear cells (PBMC) were cultured overnight on Petri dishes (Nunc) and the nonadherent cells were layered onto metrizamide gradients (14.5 g metrizamide [Nygaard] plus 100 mL of medium consisting of Dutch modification of RPMI 1640 [Flow Laboratories] and 10% FCS). The cells were centrifuged at 600 g for 10 minutes at room temperature and the interface cells were enriched DC and the pellet cells, which were diluted from the hypertonic metrizamide slowly with warm medium, were used as responder lymphocytes. Lymphocytes (10⁴) were cultured with and without enriched syngeneic or allogeneic peripheral blood DC (40 to 200) in 20 μL of hanging drop cultures in Terasaki plates. Some cultures received cells from
Fig 2. Light microscopic appearances, cytochemistry, and immunoenzymatic (APAAP) labeling of cytospin preparations of plucked colonies. (A) Giemsa stained DC; (B) α-naphthyl esterase, macrophage+ DC−; (C) HLA-DR, macrophage+ (left) and DC− (right); (D) Leu10 (DQ), DC+ macrophage−; (E) Y1/82A, macrophages− DC+.
Fig 2. (Cont'd). (F) NA/134 (CD1a), DC- macrophage; (G) Leu4 (CD3), T cells; macrophage DC; (H) T4 (Dako CD4), DC-; (I) Autoradiography of 24-hour $^3$HdR labeled colony: nuclear labeling of both NA/134 +ve and -ve cells.
colonies plucked from the methylcellulose cultures. Uptake of 
'2H-thymidine (1 μL added per culture to give 1 μg/mL of thymidine at 2 Cl/mmOL/2 hours before harvesting) was assessed on day 6 of culture in cells blotted onto filter discs and processed for β scintillation counting.

RESULTS

Microscopic appearances of DC colonies. In addition to colonies of granulocytes and macrophages that were readily recognized as myeloid in character (CFU-GM), three other colony types were identified in generally low numbers in the cultures. Type 1 (Fig 1A, B): Cells with long cytoplasmic processes in diffuse clusters of from 4 to 50 cells. In many, but not all of these colonies, the cells were clearly adherent to the base of the culture dish. Type 2 (Fig 1C): Clusters of cells as in type 1 (but generally not adherent to the dish) together with variable numbers of large round granular cells with a regular contour that resembled macrophages. Type 3 (Fig 1D): Tight clusters of small round cells either occurring singly or in closely associated groups. In some cases these groups of clusters seemed to form discrete colonies of very large size and were visible macroscopically. These clusters were usually observed only at cell densities greater than 1 x 10^5/mL.

Cell morphology by light microscopy. Giemsa staining (Fig 2A) of cells from type 1 and from type 2 colonies showed the presence of large cells of irregular shape and with an irregular or lobulated nucleus. Some cells had very long thin dendritic processes but others appeared more rounded than in the cultures but with either numerous pseudopods or short hair-like processes. The cytoplasm was homogeneous with few or absent vacuoles. In type 2 colonies such cells of dendritic appearance were found together with large round granular cells with a few or absent vacuoles. In type 2 colonies pseudopods or dendrites of varying shapes and sizes were present in the cytoplasm. The cells had many blunt pseudopodia or dendrites of varying shapes and sizes. The cytoplasm within these contained only a few ribosomes and some microfilaments. Birbeck granules were not observed.

Electron microscopic studies. The appearances of DC from each colony type were relatively uniform. The cells were large and many had multilobed nuclei. The cytoplasm was generally pale with little endoplasmic reticulum, but many free ribosomes and polyribosomes. Dense lipid droplets were present in the cytoplasm. The cells had many blunt pseudopodia or dendrites of varying shapes and sizes. The cytoplasm within these contained only a few ribosomes and some microfilaments. Birbeck granules were not observed.

The morphology of these cells was readily distinguished in the preparations from macrophages, which had simple oval or ovoid nuclei, relatively dense cytoplasm, small finger-like surface microvilli, and many large vacuoles, probably lysosomal in nature. Immunogold studies (as described above) using the murine DC marker 33D1 (Fig 3A,B) and NA/134 (Fig 3C,D) demonstrated labeling with both antibodies. Though this was weak with the murine marker, the labeling of the membrane CD1a was intense and corresponded to the findings by immunoenzymatic analysis (APAAP). Macrophages did not label with these antibodies.

Linearity studies. The relationship between cell numbers in culture and the numbers of DC colonies observed was studied in both bone marrow (five experiments) and peripheral blood (four experiments). The results for type 1 (pure DC) and type 2 colonies (DC/macrophage) are analyzed separately and the mean values (SEM) over the range of 1.0 to 6.0 x 10^4 cells/mL are shown in Fig 4. In these later experiments cultures were inspected at 7 to 9 days and it was noted that some type 1 colonies easily recognized at this time became necrotic when reexamined 1 week later. This was not the case for type 2 mixed colonies which, in contrast, seemed to mature later and gave higher scores at 14 to 16 days in culture. For this reason linearity studies for type 1 colonies are recorded at the earlier culture time. The scatter of data is large, however, at these low cell numbers, the relationship is linear and extrapolates through the origin. At higher cell numbers this relationship was lost and types 1 and 2 DC colonies became harder to discern. This appeared to be due, at least in part, to the appearance of large type 3 lymphocyte-containing colonies.

Table 1. Antibody Labeling (APAAP) of Colony DC and Macrophages

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Dendritic Cell</th>
<th>Macrophage</th>
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<tbody>
<tr>
<td>NA/134* (CD1a)</td>
<td>++</td>
<td>–/±</td>
</tr>
<tr>
<td>HLA-DR</td>
<td>++</td>
<td>++</td>
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<tr>
<td>Leu 10 (HLA-DQ)</td>
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<td>–</td>
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<td>Y1/82A</td>
<td>–</td>
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<td>Dako T4 (CD4)</td>
<td>+</td>
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<tr>
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<td>–</td>
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<td>33D1†</td>
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*Also labeled by immunogold electron microscopy (IEM).
†Antibody to mouse DC by IEM only.
Numbers of DC containing colonies in blood and bone marrow. Total numbers of DC containing colonies (type 1 and type 2) were assessed in 14-day cultures of PBMC from nine normal controls, nine patients with either acute leukemia, or non-Hodgkin lymphoma during the regenerative phase after chemotherapy and in three of these patients before treatment. Bone marrow samples were obtained from eight patients (seven pretreatment and two posttreatment samples) and from six control individuals without malignant disease (four normal donors and two patients with unexplained macrocytosis). The levels in bone marrow and the highest levels recorded in the bone are shown in Fig 5. In peripheral blood the mean peak values (SEM) were 35.7 (9.4) and 3.7 (2.6) per 1 x 10^5 cultured cells, respectively, in patients during bone marrow regeneration (R) or in a steady state before chemotherapy (S). In normal blood the mean value was less than 1.0/1 x 10^5 cultured cells. In the normal bone marrows the mean value was 20.4 (5.8) in five individuals but was much higher (175/1 x 10^5 cells) in one of the normal donors. In contrast, the mean level in seven patient bone marrows just before a treatment course was only 4.0 (1.5). All these patients had received prior chemotherapy. In one patient whose marrow was assessed both before and after treatment the level increased from zero (pretreatment) to 12.0 during hematopoietic recovery.

Correlation between circulating myeloid (CFU-GM) and dendritic progenitors during hematopoietic recovery. The levels of myeloid progenitors in the circulation are known to increase from ten- to 100-fold above normal after chemotherapy-induced aplasia in some patients. It is possible that the high values of types 1 and 2 DC colonies we observed in patients might also be a feature of recovery hematopoiesis. Therefore, all scores of DC containing colonies were plotted against CFU-GM numbers in the same cultures in six patients studied (on 40 separate occasions). Positive correlation coefficients (r) of between +0.61 and +0.98 were observed in these six sets of observations. For the sake of clarity, data from three of the six individuals are shown in Fig 6. In order to assess the significance of this apparent relationship between myeloid and DC colony numbers, Fisher's z-transformation was used to determine the variance and error of all the values for (r). The result was highly significant (P < .001).

Autoradiography. Cultures of PBMC were labeled at 6, 7, or at 8 days of culture by the addition of 1 μCi of tritiated thymidine (3H-TdR specific activity 2 Ci/mmol/L), and returned to the incubator for a further 24 hours. Individual colonies were then plucked and added to 200 μL of PBS, and cytospin preparations were made in the usual manner. Following staining by APAAP with anti-CD1a MoAb (NA/134) the cytoplasmic nuclei were coated with photographic emulsion and exposed for 4 weeks before development.

Cells that were synthesizing DNA (ie, in S-phase of cell cycle) during the 24-hour labeling period were identifiable by heavy grain appearance over the nucleus (Fig 21). As can be seen, both NA/134 positive as well as negative cells showed autoradiographic evidence of DNA synthesis.

Activity of DC and macrophages in mixed leukocyte responses. DC were dividing in the colonies but after 6 days of culture in hanging drops there was no evidence of uptake of 3H-thymidine in cultures of these cells (Fig 7A). When 40 to 200 freshly isolated peripheral blood DC (7D) or DC or macrophages from the colonies were added to syngeneic lymphocytes, little stimulation was seen. However, in each of four experiments, high levels of stimulation of lymphocytes were observed when allogeneic DC from the colonies were added (Fig 7C). The lymphocyte responses to the allogeneic DC from colonies were greater than that seen with an equivalent number of freshly isolated DC from the peripheral blood of the same donor (Fig 7B; P < .05). Allogeneic macrophage colony cells caused little stimulation (Fig 7E). The DC from the colonies thus showed high potency for stimulating primary mixed leukocyte responses (MLR), a property shared with DC from other sources.

DISCUSSION

Since their original description in the murine lymphoid system1 DC have been demonstrated in other species including humans. They are widely distributed and different populations of DC have been described in different anatomical locations. Thus interdigitating cells (IDC) in the lymph node paracortex, LC in the skin, and veiled cells in the blood and lymph resemble each other in their gross morphology and their common expression of HLA-DR on the cell surface. Cells of the monocyte-macrophage type are also HLA-DR+ and although DC, unlike monocytes, are not generally phagocytic, there are a number of other features that they hold in common. The overlap in phenotypic characteristics of DC and monocytes suggests that they may have a common origin in the bone marrow and it has even been suggested that monocytes may be capable of differentiation into LC in the skin. There is good evidence both from transplantation in mice and in humans that LC are derived from the bone marrow, however, until recently, direct evidence for this has been lacking. It is also unclear whether the aforementioned subsets of DC are separate lineages or whether their differences relate only to their anatomical location.

The present study demonstrates for the first time that recognizable DC colonies proliferate in cultures of cells from both human peripheral blood and bone marrow. The highly distinctive appearance of these cells readily identifies the colonies in the culture dish (Fig 1A,B) and the striking dendritic morphology of these cells both by light and electron microscopy, conforms to previous descriptions of DC from various anatomical locations and distinguishes them from macrophages.

Our observations indicate that the colony cells are either identical with or closely related to the Langerhans subset of lymphoid DC. The strongest evidence for this derives from the clear immunoenzymatic labeling with antibody to the CD1a (T6) antigens (confirmed by immunoglobulin electron microscopy) as well as to class II (HLA-DR) antigen. CD1a is expressed on prothymocytes but is found together with HLA-DR only on LC. Our observation that HLA-DQ (Leu 10) is expressed strongly on these cells but not on colony...
macrophages strongly supports this conclusion since it is consistent with previously reported data on circulating DC. It has been suggested that membrane expression of HLA-DQ is important in the capacity of these cells to present antigen to virgin T cells.\(^\text{15,26}\)

As might be expected, colony DC were CD3\(^+\) in contrast to the CD3\(^-\) T cells that clustered in close apposition to the cell membrane in type 3 colonies. However, we have shown that both DC and macrophages in the cultures express the T-helper cell antigen, CD4 (Fig 2H) and this agrees with the observations of others.\(^\text{29}\)

A number of MoAbs that label monocytes are also known to label DC but in many cases (eg, Leu M3, 63D3, Mo2, and 3C10) this labeling is weak or absent on DC and indeed this feature has been used to distinguish these populations from each other.\(^\text{19-22}\) We have found that the antibody Y1/82A that labels a cytoplasmic antigen strongly expressed in macrophages\(^\text{17}\) failed to label colony cells with typical dendritic morphology. The low level or absence of nonspecific esterase (NSE) activity that was found in these cells is consistent with previous evidence that weakly positive LC may lose such activity in culture and that lymph node IDC are generally NSE negative.\(^\text{6}\)

We have been unable to demonstrate Birbeck granules in colony cells and this appears to be inconsistent with their identification as LC. However, these organelles have not
been observed in all skin LC and they are absent in the 'indeterminate cells' of the dermis. Since there is evidence for loss of the granules in cultured LC, the failure to identify them in this study may be due to the effects of culture or may relate to the level of cellular maturation.

The immunostimulatory properties of dendritic colony cells in mixed lymphocyte culture (Fig 7C) are consistent with the well described ability of all DC to present transplantation antigens. Cells from dendritic colonies showed a much enhanced ability to stimulate allogeneic T cells in MLR when compared with colony macrophages (Fig 7E). The ability to elicit such a response at a very low target to effector ratio (1:500) is typical for DC and it may be seen that these cells were even more effective than peripheral blood DC from the same donor freshly prepared over a metrizamide gradient (Fig 7B). It is probable that the dense clusters of CD3+ cells closely associated with CD1a+ DC in the type 3 colonies (Figs 1D and 2G) resulted from aggregation and proliferation of T cells as in an autologous MLR. It is noteworthy that these colonies were only observed when cells were cultured at higher numbers (ie, >1 x 10⁵/mL).

The autoradiographic studies reported here (Fig 2I) indicate that the cells we have observed in these colonies have arisen as a result of cell division rather than simply by aggregation. Less than 1% of skin LC are observed to be in mitosis but the possibility that type 1 DC colonies are derived from a very small number of mature circulating or bone marrow CD1a+ cells is not excluded by the present data. It is very unlikely that lymphoid DC in either blood or bone marrow are precursors for these colonies since these cells are thought to be incapable of cell division and DC-enriched preparations from metrizamide gradients showed no enrichment for DC colonies (data not shown).

Cells that coexpress CD1a(T6) and myeloid markers have been identified previously in adult human bone marrow and Bowers and Berkowitz have demonstrated the appearance of DC from proliferating la- precursors in 5-day cultures of rat bone marrow. It has recently been reported that small numbers (1% to 8%) of CD1a+ (T6) cells can be identified in granulocyte-macrophage colonies cultured from human bone marrow. The discrete nature of the mixed macrophage DC colonies that we have observed suggests that these cells have a common origin in a single myeloid progenitor. This is supported by the linear relationship between cell numbers cultured and colony scores (Fig 4). However, such data may be misleading since isoenzyme studies of clonality have previously shown that at colony densities of more than 30 per dish multicellular origin is common even though linearity is maintained. Type 1 colony numbers were always well below this value so the linear relation for pure DC progenitors may be acceptable as evidence of clonality. However, macrophage colony numbers were generally high in these cultures so that a clonal origin for mixed colonies must remain tentative until formally proven using genetic markers that can be shown to be common to both cell populations.
Vitortors (CFU-GM, BFU-E, and pluripotent CFU-mix) have peripheral lymphoid organs of mice. J Exp Med

Antigen-presenting cells, including Langerhans cells, veiled cells and ALL antibody responses in mouse and man. Proc Natl Acad Sci USA

CFU-GM and DC colony numbers during this recovery induced bone marrow aplasia.2~8 The correlation between been described during the recovery from chemotherapy-induced lymphoma. Very considerable overshoots in myeloid progenitors (CFU-GM, BFU-E, and pluripotent CFU-mix) have been described during the recovery from chemotherapy-induced bone marrow aplasia.12~18 The correlation between CFU-GM and DC colony numbers during this recovery phase (Fig 6) makes it likely that this finding was fortuitous and related to a transient expansion of the circulating pool of yet another class of hematopoietic progenitor rather than being a feature of the underlying malignant disease.

The identification of these highly distinctive DC/LC in in vitro culture of human blood and bone marrow will now make possible the further characterization of their progenitor (DL-CFU) and its response to recombinant growth factors. The ability to obtain even small numbers of pure 'virgin' DC rather than the very impure populations prepared on density gradients, will provide possibilities to study antigen presentation by cells previously unexposed to these antigens in vivo.

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