Generation of Large Numbers of Dendritic Cells from Mouse Bone Marrow Cultures Supplemented with Granulocyte/Macrophage Colony-stimulating Factor

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Summary

Antigen-presenting, major histocompatibility complex (MHC) class II–rich dendritic cells are known to arise from bone marrow. However, marrow lacks mature dendritic cells, and substantial numbers of proliferating less-mature cells have yet to be identified. The methodology for inducing dendritic cell growth that was recently described for mouse blood now has been modified to MHC class II–negative precursors in marrow. A key step is to remove the majority of nonadherent, newly formed granulocytes by gentle washes during the first 2–4 d of culture. This leaves behind proliferating clusters that are loosely attached to a more firmly adherent "stroma." At days 4–6 the clusters can be dislodged, isolated by 1-*g* sedimentation, and upon reculture, large numbers of dendritic cells are released. The latter are readily identified on the basis of their distinct cell shape, ultrastructure, and repertoire of antigens, as detected with a panel of monoclonal antibodies. The dendritic cells express high levels of MHC class II products and act as powerful accessory cells for initiating the mixed leukocyte reaction. Neither the clusters nor mature dendritic cells are generated if macrophage colony-stimulating factor rather than granulocyte/macrophage colony-stimulating factor (GM-CSF) is applied. Therefore, GM-CSF generates all three lineages of myeloid cells (granulocytes, macrophages, and dendritic cells). Since >5 × 10^6 dendritic cells develop in 1 wk from precursors within the large hind limb bones of a single animal, marrow progenitors can act as a major source of dendritic cells. This feature should prove useful for future molecular and clinical studies of this otherwise trace cell type.

For some time it has been evident that proliferating precursors give rise to antigen-presenting dendritic cells within lymphoid organs (1–3). Yet it has been difficult until recently (4) to identify systems in which substantial production occurs in culture. The recent development involved the use of mouse blood cell cultures and the addition of GM-CSF. Proliferating cellular aggregates developed, and from these, many typical dendritic cells were released. The use of a liquid culture system provided access to the distinctive traits of the progeny dendritic cells, i.e., shape, motility, phenotype as detected with mAbs, potent stimulation of the MLR, and homing to T cell areas in situ.

The above studies with blood could not immediately be extended to bone marrow, from which dendritic cells ultimately arise (1, 2, 5, 6). This left open the possibility that the growth and maturation of dendritic cells, like the development of T lymphocytes, might proceed in extramedullary tissue rather than in the bone marrow itself. Here we modify the liquid culture method to document the extensive and rapid production of dendritic cells from MHC class II–negative precursors, again under the aegis of GM-CSF. In these cultures, GM-CSF is in effect stimulating three distinct systems of myeloid cells, i.e., granulocytes, macrophages, and dendritic cells, which can be readily distinguished on the basis of cytologic, cell surface, and functional properties.

Materials and Methods

Mice. Female BALB/C, male DBA/2, and female C57BL/6 mice, 7 wk old, were purchased from Japan SLC (Hamamatsu, Shizuoka, Japan). (BALB/C × DBA/2) F_1 of both sexes, 7–10 wk old, were from Japan SLC and the Trudeau Institute (Saranac Lake, NY).

Reagents. The culture medium was RPMI 1640 (Nissui, Tokyo, Japan; Gibco Laboratories, Grand Island, NY) supplemented with

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5% FCS, 50 μM 2-ME, and 20 μg/ml gentamicin. Murine rGM-CSF (10^8 U/mg protein) was kindly provided by Kirin Brewery Co. (Maebashi, Gumma, Japan). A panel of rat and hamster mAbs to mouse leukocyte antigens is described elsewhere (7, 8). FITC- and peroxidase-conjugated mouse anti-rat IgG were purchased from Boehringer Mannheim Biochemicals (Indianapolis, IN), and FITC- and peroxidase-conjugated goat anti-hamster Ig (γ and L chain) were from Jackson Immunoresearch Laboratory (Westgrove, PA) and Caltag Laboratories (San Francisco, CA), respectively.

Bone Marrow Cultures. After removing all muscle tissues with gauze from the femurs and tibias, the bones were placed in a 60-mm dish with 70% alcohol for 1 min, washed twice with PBS, and transferred into a fresh dish with RPMI 1640. Both ends of the bones were cut with scissors in the dish, and then the marrow was flushed out using 2 ml of RPMI 1640 with a syringe and 25-gauge needle. The tissue was suspended, passed through nylon mesh to remove small pieces of bone and debris, and red cells were lysed with ammonium chloride. After washing, lymphocytes and Ia-positive cells were killed with a cocktail of mAbs and rabbit complement for 60 min at 37°C. The mAbs were GK 1.5 anti-CD4, HO 2.2 anti-CD8, B21-2 anti-Ia, and RA3-3A1/6.1 anti-B220/CD45R (TIB 207, 150, 229, and 146, respectively; American Type Culture Collection, Rockville, MD). 7.5–10 × 10^5 cells were placed in 24-well plates (Nunc, Naperville, IL) in 1 ml of medium supplemented with 500–1,000 U/ml rGM-CSF. The cultures were usually fed every 2 d by gently swirling the plates, aspirating 75% of the medium, and adding back fresh medium with GM-CSF. An object of these washes was to remove nonadherent granulocytes without dislodging clusters of developing dendritic cells that were loosely attached to firmly adherent macrophages. To enrich for growing dendritic cells, we utilized a procedure similar to that described for mouse blood cell cultures (4). Briefly, the aggregates of attached cells were dislodged with Pasteur pipettes and applied to 6-ml columns of 50% FCS-RPMI 1640. Residual granulocytes in the cultures, often in aggregates as well, were easily dissociated at this step. Upon 1-*g* sedimentation of the dislodged cells, clusters moved to the bottom of the tube and single granulocytes were left at the top. The aggregates were subcultured at 2–3 × 10^5/ml in fresh medium with GM-CSF, typically for 1 d in 16-mm wells. After overnight culture, large numbers of typical dendritic cells were released. Adherent macrophages also expanded in these cultures, but most remained firmly adherent to the culture surface (see Results).

Cell Surface and Intracellular Antigens. Cell surface staining utilized cytofluorography (FACScan®; Becton Dickinson & Co., Mountain View, CA). Staining with primary rat or hamster mAbs was followed by FITC-conjugated mouse anti-rat or goat anti-hamster Igs as described (4). A panel of mAbs to cell surface (7, 8) and to intracellular antigens (9, 10) was tested on cytospin preparations. We studied both adherent and nonadherent populations, the former being dislodged in the presence of 10 mM EDTA (the adherent cells were rinsed twice with PBS and once with EDTA-PBS, and then incubated with EDTA-PBS for 20 min at 37°C). The cytospins were fixed in acetone and stained with mAbs followed by peroxidase-conjugated anti-rat or anti-hamster Ig. The peroxidase was visualized with diaminobenzidine, and the nuclei were counterstained with Giemsa.

Cytologic Assays. Giemsa stains were performed on cytospin preparations, as was the case for the nonspecific esterase (α-napthyl acetate as substrate) stain using standard methods (11), except that the cytospin preps were fixed with 2% glutaraldehyde in Hanks medium instead of buffered acetone formalin. Phase contrast observations, usually of living cells, were made with inverted microscopes (Nikon Diaphot, Melville, NY) at final magnifications of 100 and 400. Transmission electron microscopy (12) and [^{3}H]thymidine autoradiography (4) were performed on developing dendritic cells as previously described.

Mixed Leukocyte Reactions. Cells from the bone marrow cultures were exposed to 15 Gy of x-ray irradiation and applied in graded doses to 3 × 10^5 syngeneic or allogeneic T cells in 96-well flat-bottomed culture plates for 4 d. The T cells were prepared by passing spleen and lymph node suspensions through nylon wool and then depleting residual APCs with anti-Ia plus J11d mAbs plus complement. [^{3}H]Thymidine uptake was measured at 80–94 h after a pulse of 4 μCi/ml (222 GBq/mmol; American Radiolabeled Chemicals, Inc., St. Louis, MO).

Results

Development of Cells with the Cytologic Features of Dendritic Cells in Mouse Bone Marrow Cultures Supplemented with GM-CSF. In prior studies in which aggregates of growing dendritic cells were described in mouse blood (4), we were unable to identify such aggregates in comparable cultures of bone marrow. The difficulty was related to the fact that in marrow, in contrast to blood, there were large numbers of granulocytes that developed in response to GM-CSF. There were also many surviving B cells from the original marrow inoculum. Therefore, before culture, we treated the marrow suspensions with a cocktail of mAbs to B cells, T cells, and MHC class II antigens plus complement. At days 2 and 4 of culture, we gently swirled the plates to remove loosely adherent cells, which proved to be granulocytes typical in morphology and expression of the RB6 antigen (see below). With these steps, we recognized by day 4 cellular aggregates attached to a layer of adherent cells (Fig. 1 *A*). Some of the profiles in the aggregates had the veil- or sheet-like processes of dendritic cells. The aggregates could be dislodged by gentle pipetting and separated by 1-*g* sedimentation (Fig. 1 *B*). Within 3 h of replating, many spiny adherent cells emigrated from the clusters and had the appearance of fresh splenic adherent cells (13) (Fig. 1 *C*). After another day of culture, these adherent cells came off the surface and many typical dendritic cells were seen floating in the culture medium (Fig. 1 *D*). Optimal yields of dendritic cells were obtained when the aggregates were harvested on day 6 and then cultured overnight.

Attached to the surface of the culture wells were cells with the cytologic features of macrophages, and these also expanded in numbers during the first week of culture. These cells could be dislodged by pipetting after incubation at 37°C in the presence of 10 mM EDTA.

If the cultures were maintained in M-CSF, large numbers of macrophages grew out and were firmly attached to the plastic surface. However, no dendritic cells or dendritic cell aggregates were apparent. If a mixture of M-CSF and GM-CSF were applied, then colonies of adherent macrophages as well as aggregates of growing granulocytes and dendritic cells were noted (data not shown).

Development of Potent MLR Stimulator Cells in Bone Marrow Cultures. It is known that suspensions of mouse bone marrow are not active as MLR stimulators (14) and do not contain detectable dendritic cells (15). Given the cytologic observa-

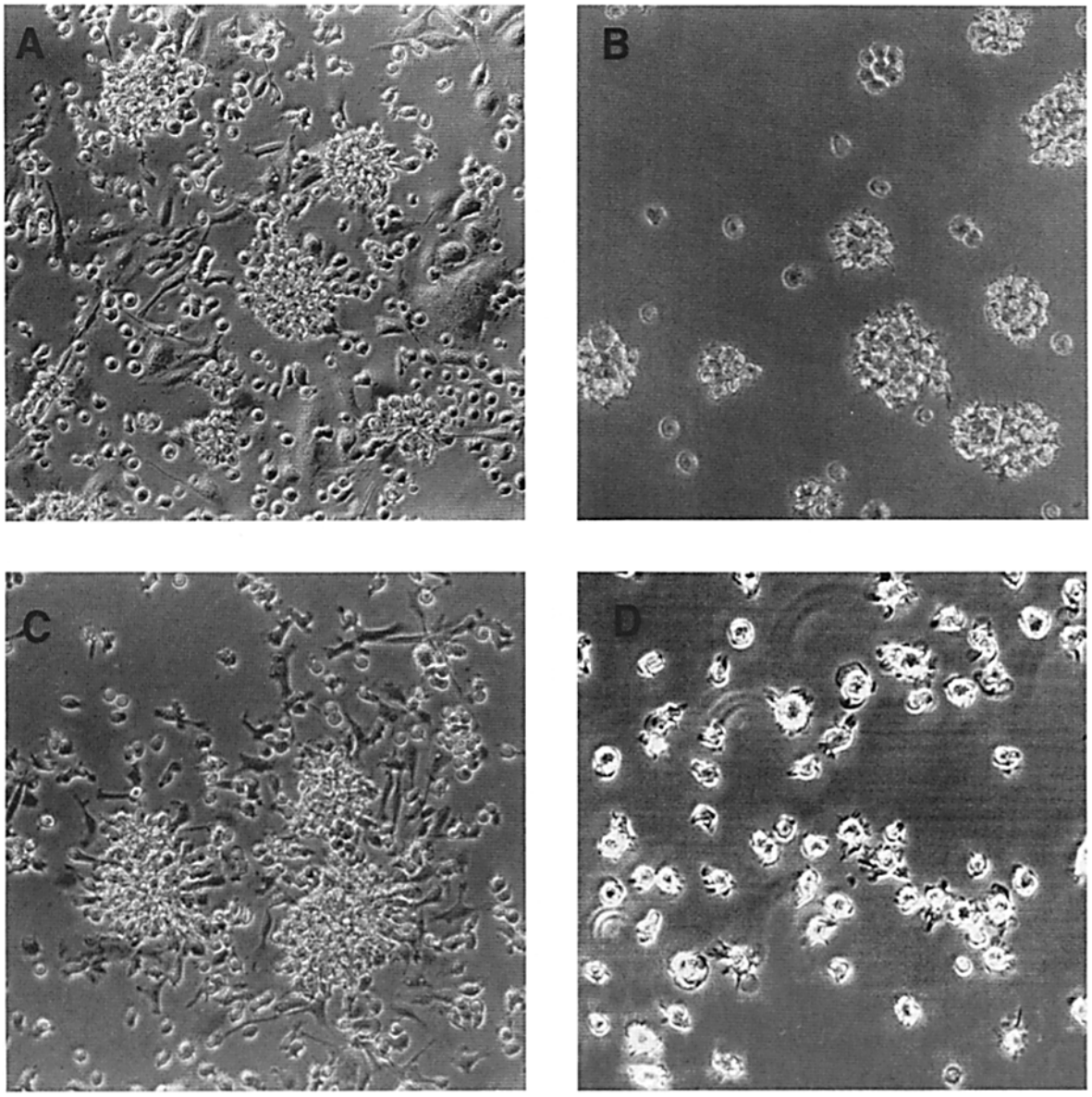

Figure 1. Phase contrast micrographs illustrating the development and isolation of dendritic cells from liquid cultures of mouse bone marrow nonlymphocytes supplemented with GM-CSF. (*A*) Low-power view of adherent bone marrow cells after 4 d of culture. The excess of nonadherent granulocytes was rinsed away at days 2 and 4 so that many attached aggregates are evident. ×100. (*B*) Purified aggregates obtained by dislodging 4-d marrow cultures (nonadherent granulocytes rinsed away at day 2) and separating aggregates from nonaggregates by 1-*g* sedimentation. ×100. (*C*) Isolated aggregates as in *B* were cultured for 3 h whereupon many cells emerge with the appearance of adherent dendritic cells. ×100. (*D*) Isolated aggregates as in C were cultured overnight, and the released cells transferred into a new well for examination at high power. Most have sheet-like processes or veils. ×400.

tions above, we cultured Ia-negative, bone marrow nonlymphocytes for 6 d and checked MLR-stimulating activity at daily intervals. As long as the cultures were supplemented with GM-CSF, strong MLR-stimulating activity developed (Fig. 2). The increase was progressive, and by day 6, as few as 100 of the marrow cells induced MLRs with stimulation indices of 20 or more.

To correlate the development of MLR-stimulating activity with the appearance of dendritic cells in these heterogenous cultures, we first separated the cultures into nonadherent and loosely adherent fractions (Fig. 3 *A*). The nonadherent cells, which were mainly granulocytes in the first 4 d, were obtained by gently swirling the plates and harvesting the cells. The loosely adherent cells, which contained the aggregates of presumptive dendritic cells at day 4 and at later times, were dislodged by pipetting over the surface of firmly adherent stromal cells. At days 2 and 4, the most potent stimulating activity was in the adherent fraction. By day 6, the nonadherent fraction was very active. If one tested firmly adherent macrophages, there was no MLR-stimulating activity (Fig. 3 *B*, *open squares*).

As mentioned above, in the presence of GM-CSF, the cul-

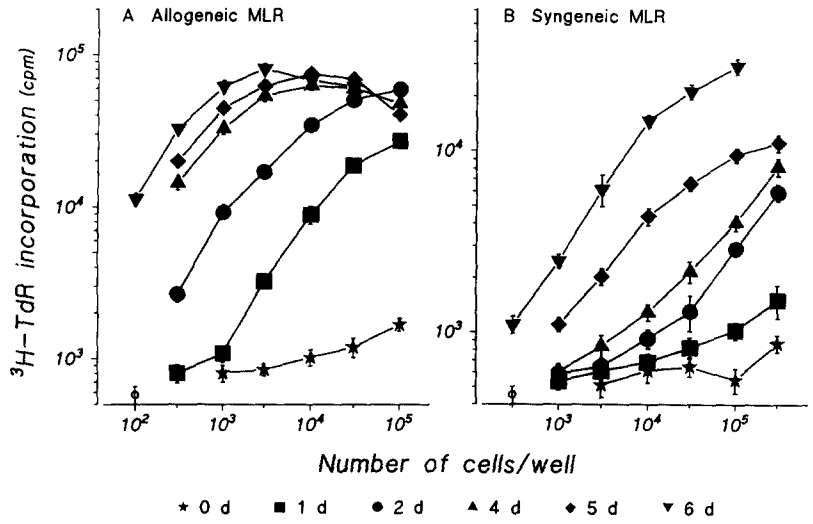

Figure 2. Progressive development of MLR-stimulating activity in bone marrow cultured in the presence of GM-CSF. Ia-negative, B and T cell–depleted marrow cells were cultured in GM-CSF with 75% of the medium being replaced every 2 d. At each time point, the cells were dislodged by gently pipetting. After irradiation, graded doses of marrow cells were applied to 3 × 10^5 allogeneic (C57BL/6, *left*) or syngeneic ([BALB/C × DBA/2]F_1) T cells and cultured for 4 d in the MLR. [^{3}H]TdR uptake was measured at 80–94 h (values are means of triplicates with SE bars).

tures developed aggregates of growing cells that release typical dendritic cells between days 4 and 8 of culture (Fig. 1). These aggregates could be isolated by gentle pipetting over the monolayer followed by 1-*g* sedimentation. When the aggregates were returned to culture, populations enriched in dendritic cells were released, and these released cells proved to have the very strong MLR-stimulating activity that is characteristic of dendritic cells (Fig. 3 *B*).

Cell Surface Markers: Cytofluorography. By cytofluorography, two populations of cells were readily distinguished in the nonadherent or easily dislodged cells. One population had a low forward light scatter, high levels of the RB6 antigen, and low levels of MHC class II. The other population was larger and had the reciprocal phenotype. The aggregated cells were enriched relative to unfractionated cultures in MHC class II–positive cells (Fig. 4, compare *left* and *middle*), and the level of MHC class II on individual cells increased when the aggregates were cultured overnight to release highly enriched populations of dendritic cells (Fig. 4, compare *middle* and *right*). More MHC class II–rich, RB6 antigen–negative cells were seen in day 6 vs. day 4 cultures (Fig. 4). None of the cells reacted with the mAbs to the B220 antigen of B cells or the SER-4 antigen of macrophages (not shown).

More detailed FACS® studies (Fig. 5) were performed on cells that had been released from the aggregates. The granulocytes were gated out on the basis of lower forward light scattering. The larger, dendritic cells had uniformly high levels of MHC class I and II, as well as CD44 and CD11b (Mac-1; M1/70). Intermediate level staining was noted for the heat-stable antigen (HSA; M1/69), CD45 (M1/9.3), and CD18 (2E6). Lower level staining was evident for the low-affinity IL-2 receptor (CD25, 7D4), interdigitating cell antigen (NLDC-145), Fcγ receptor (2.4G2), dendritic cell antigen (33D1), macrophage antigen (F4/80), and CD11c p150/90 β2-integrin (N418). Several antigens were not detectable, including phagocyte (SER-4 marginal zone macrophage, RB6 granulocyte) and lymphocyte (RA3-6.1 B lymphocyte; Thy-1, CD3, 4, 8 T lymphocyte) markers. This phenotype is similar in many respects to that seen in splenic and epidermal dendritic cells (7, 16, 17). The one exception is the high level

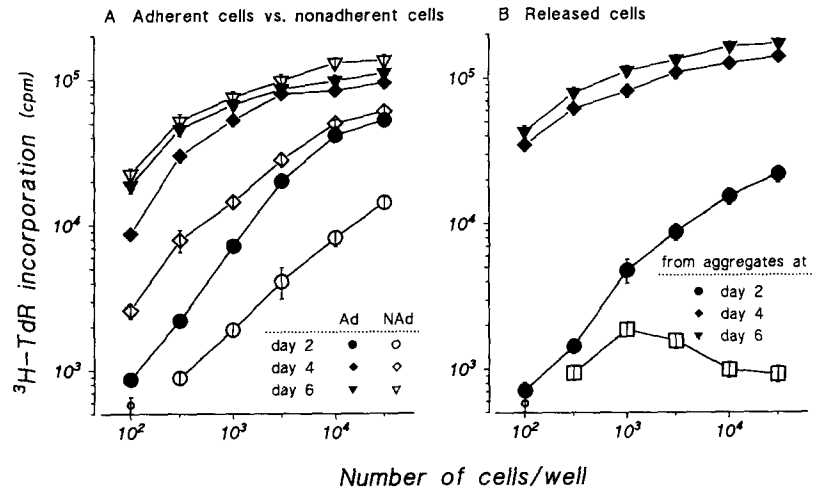

Figure 3. Physical properties of the MLR-stimulating cells that develop in GM-CSF-supplemented bone marrow cultures (see text). (*A*) Cultures similar to those in Fig. 1 were separated into nonadherent (*open symbols*) and loosely adherent fractions (*filled symbols*), the latter being cells that could be dislodged by gently pipetting over the monolayer. For the day 4 separations, nonadherent cells (mainly granulocytes) were rinsed away at day 2, and for the day 6 separation, granulocytes were rinsed away at days 2 and 4. The cells were irradiated and applied in graded doses to allogeneic T cells as in Fig. 2. (*B*) At the indicated time points, free cells and cell aggregates were dislodged from the stromal monolayer and separated by 1-*g* sedimentation. The aggregates were cultured for 1 d to provide released cells as in Fig. 1 *D*. These cells were irradiated and tested as MLR stimulators, as were firmly adherent cells that were dislodged in the presence of 10 mM EDTA (*open squares*).

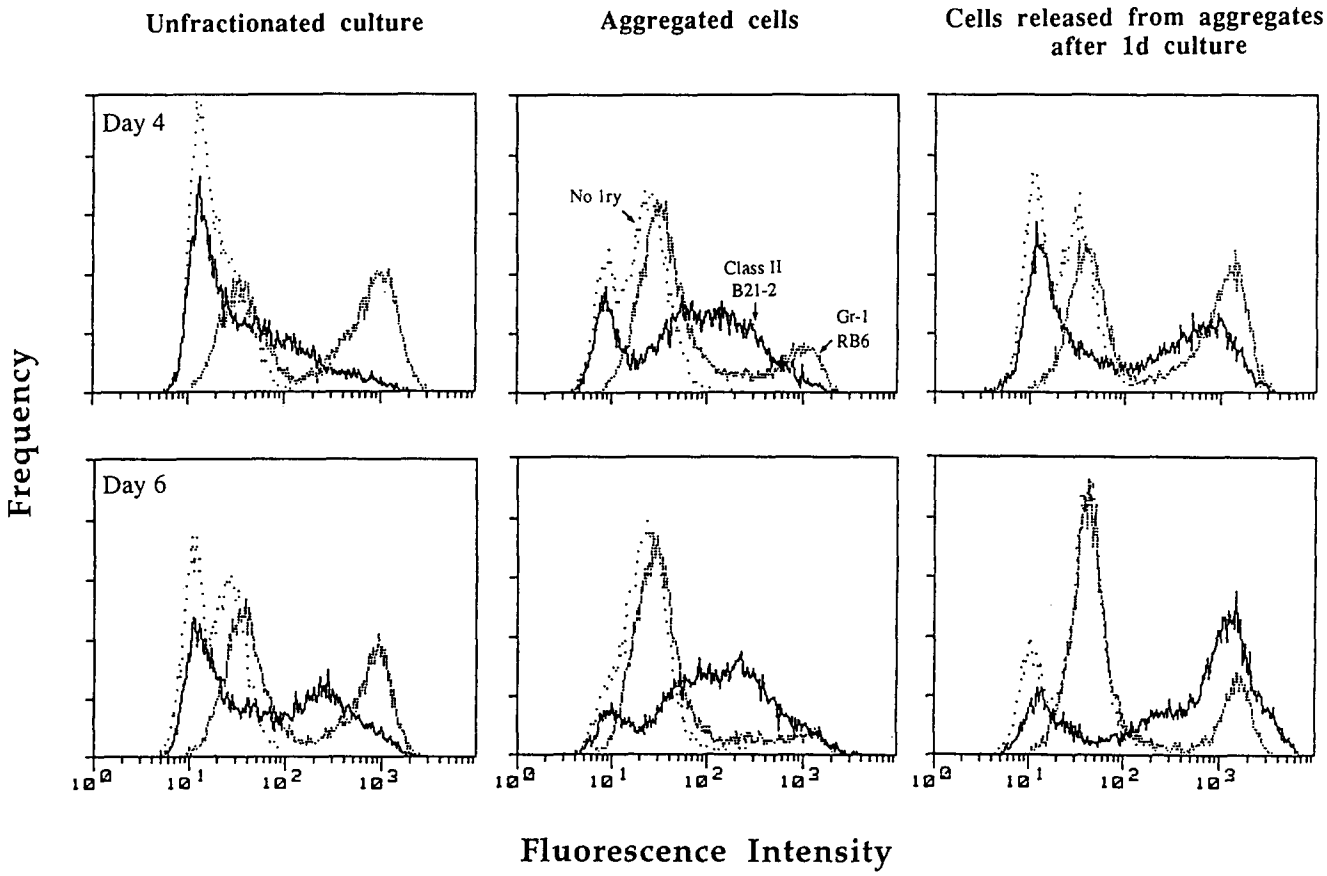

Figure 4. Development of Ia-positive cells from aggregates within bone marrow cultures supplemented with GM-CSF. GM-CSF-stimulated, bone marrow cultures (*left*, unfractionated) were compared with loosely attached cell aggregates (*middle*) and cells released from the aggregates after overnight culture (*right*). The cells were taken at days 4 or 6, so that the released cells were analyzed at days 5 and 7. The cells were stained with no primary mAb (no 1ry), or with mAb to granulocytes (RB-6) or MHC class II products (B21-2) followed by FITC-mouse anti-rat Ig.

in the marrow-derived cells of CD11b, an integrin that helps mediate emigration of myeloid cells from the vasculature.

Cytospin Preparations. Cytospins were prepared to further compare the released dendritic cells with the firmly adherent stromal population. By Giemsa stain, the cells that had been released from the aggregates had the typical stellate shape of dendritic cells, while the adherent cells were for the most part vacuolated macrophages (Fig. 6, *left*). Many of the dendritic cells had a perinuclear spot of nonspecific esterase stain, while the more adherent populations had abundant cytoplasmic esterase (not shown).

The released cells stained strongly for MHC class II products, except for the contaminants with typical granulocyte nuclei (Fig. 6, *top*, *arrows*). The strongly adherent cells contained a subpopulation of class II–positive cells (Fig. 6, *middle*, *arrows*).

Recently, antigens have been described that are primarily localized in intracellular vacuoles of dendritic cells and B cells but not mononuclear phagocytes. The antibodies are termed M342 (10) and 2A1 (Witmer-Pack et al., manuscript in preparation). Many of the dendritic cells had strong 2A1 stain (Fig. 6, *right*), and a smaller number expressed M342 (not shown). The adherent cells had a few profiles with weak 2A1 (Fig. 6, *bottom right*, *arrow*).

The development of Ia-positive cells and cells expressing granular intracellular antigens was quantitated on cytospins (Fig. 7). MHC class II antigens were expressed first, followed by the 2A1 and M342 granular antigens (Fig. 7). By day 8, the majority of the cells were dendritic and had high levels of MHC class II products and 2A1 antigen, as illustrated in Fig. 6. If granulocytes were not removed from the cultures, the yield of nonadherent cells was much larger, but the highest percentage of MHC class II–positive cells that we detected was 30% (not shown), and it was difficult to identify and isolate the aggregates that were the site of dendritic cell growth (see below).

When the cytospins were stained for other myeloid antigens, the released cells stained weakly and sometimes not at all above background with monoclonals to the Fc receptor (2.4G2) and macrophage-restricted antigen (F4/80). Most of the firmly adherent cells, in contrast, stained strongly for both antigens (not shown). This suggests that while low levels of 2.4G2 and F4/80 are found on the surface of the released dendritic cells, synthesis and expression are probably being

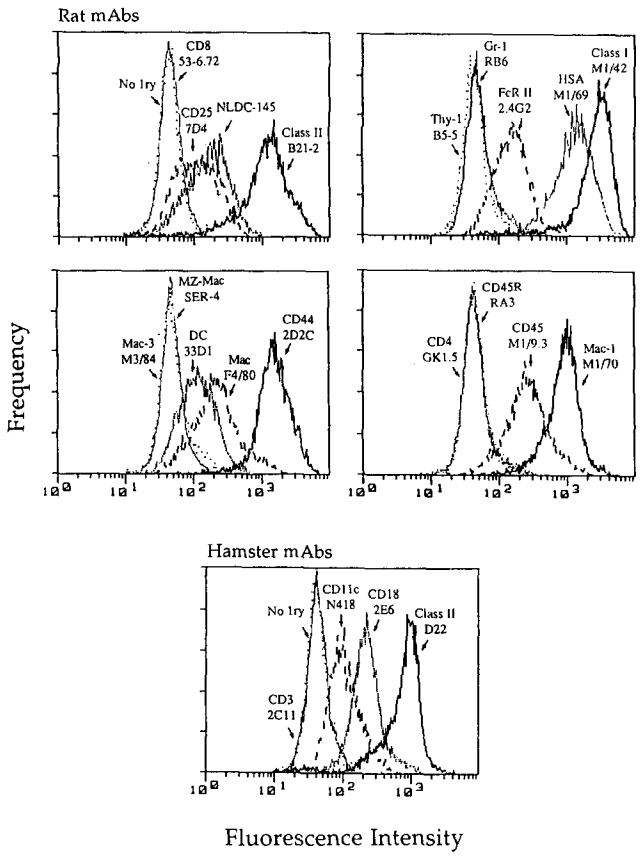

Figure 5. Detailed phenotype of the Ia-positive cells released from the growing dendritic cell aggregates. Contaminating, Ia-negative granulocytes were gated out on the basis of lower forward light scatter, so that one could examine the expression of many surface antigens on the larger cells using rat and hamster anti-mouse mAbs (7, 17) as indicated.

downregulated, as occurs when epidermal dendritic cells are placed in culture (16).

On day 4, some 3–5 × 10^4 Ia-positive cells were floating in the cultures, while on both days 6 and 8, another 5–10 × 10^4 Ia-positive cells were harvested. The quantitative data indicated that each well produced some 2 × 10^5 or more Ia-positive cells in 1 wk. Since we obtain ~20–30 wells of the starting Ia-negative marrow cells from two tibia and two femurs, the total yield of Ia-positive cells is 5 × 10^6 or more, exceeding the total estimated number of Langerhans cells in the skin of a mouse (16).

[³H]Thymidine Pulse Chase Experiments. To further document the proliferation and differentiation of dendritic cells in these cultures, clusters of cells were isolated on day 4, exposed to a 12-h pulse of [³H]thymidine, and examined by autoradiography immediately or after 1, 2, and 3 d of chase in [³H]thymidine-free medium. The majority of cells in the aggregate were labeled initially, and almost all cells released from the aggregates were labeled (Fig. 8). During the chase, increasing percentages of the released progeny expressed the 2A1 granule antigen of mature dendritic cells (Fig. 8).

Electron Microscopy. The released cells had many large veils or lamellipodia extending from several directions of the cell body (Fig. 9 *A*). The cytoplasm had many mitochondria, few electron-dense granules and lysosomes, but several electron-lucent vesicles, some with the cytologic features of multivesicular bodies (Fig. 9 *B*). The numerous cell processes extending from the dendritic cells were evident in the semi-thin sections of our preparations (Fig. 9 *C*).

Discussion

When suspensions of mouse bone marrow are cultured in the presence of GM-CSF, three types of myeloid cells expand in numbers. (*a*) Neutrophils predominate but do not adhere to the culture surface. Neutrophils have a characteristic nuclear morphology, express the RB-6 antigen, and lack MHC class II products. (*b*) Macrophages are firmly adherent to the culture vessel, express substantial levels of the F4/80 antigen, and for the most part express little or no MHC class II (but see below). (*c*) Dendritic cells arise from cellular aggregates that are attached to the marrow stroma. The aggregates become covered with sheet-like cell processes and eventually release typical single dendritic cells. The latter are distinguished by many traits: cell shape and motility, ultrastructure, high levels of MHC class II, and MLR-stimulating activity, a distinctive phenotype as detected with a group of monoclonals to cell surface and intracellular antigens. These features are stable so that, for example, the dendritic cell progeny do not become macrophages even when maintained in M-CSF.

Several procedural modifications are required to detect the growing dendritic cell aggregates in liquid, GM-CSF-supplemented, marrow cultures. The first and major point is to wash away the dominant granulocytes, the bulk of which are nonadherent. A second point is to deplete B cells from the starting population. While B cells and pre-B cells do not grow in response to GM-CSF, they represent ~50% of the initial marrow suspension and preclude the use of staining with anti-Ia mAbs to quickly enumerate dendritic cells. B cells also can express the M342 and 2A1 granular antigens that are useful markers for distinguishing dendritic cells from macrophages and granulocytes. A third point is to culture the Ia-negative marrow nonlymphocytes in high numbers (10^6/well). Liquid marrow cultures typically are set up at 10% of this dose, but it is then difficult to identify and isolate the aggregates of growing dendritic cells. A fourth and key step is to select the distinctive aggregates of proliferating, less mature dendritic cells away from the stroma and then return the aggregates to culture. If this is done after 4–6 d of culture, large numbers of dendritic cells are released. It is this released population that expresses the cardinal features of mature dendritic cells (reviewed above).

There have been two early reports of the development of dendritic cells in marrow culture (18, 19). These did not stress the use of GM-CSF and did not report the development of large numbers of dendritic cells. A more recent report described the development of dendritic cells in GM-CSF-supplemented marrow cultures (20). In the current study, efforts have been made to document the origin of the dendritic cells from MHC class II–negative precursors, to use

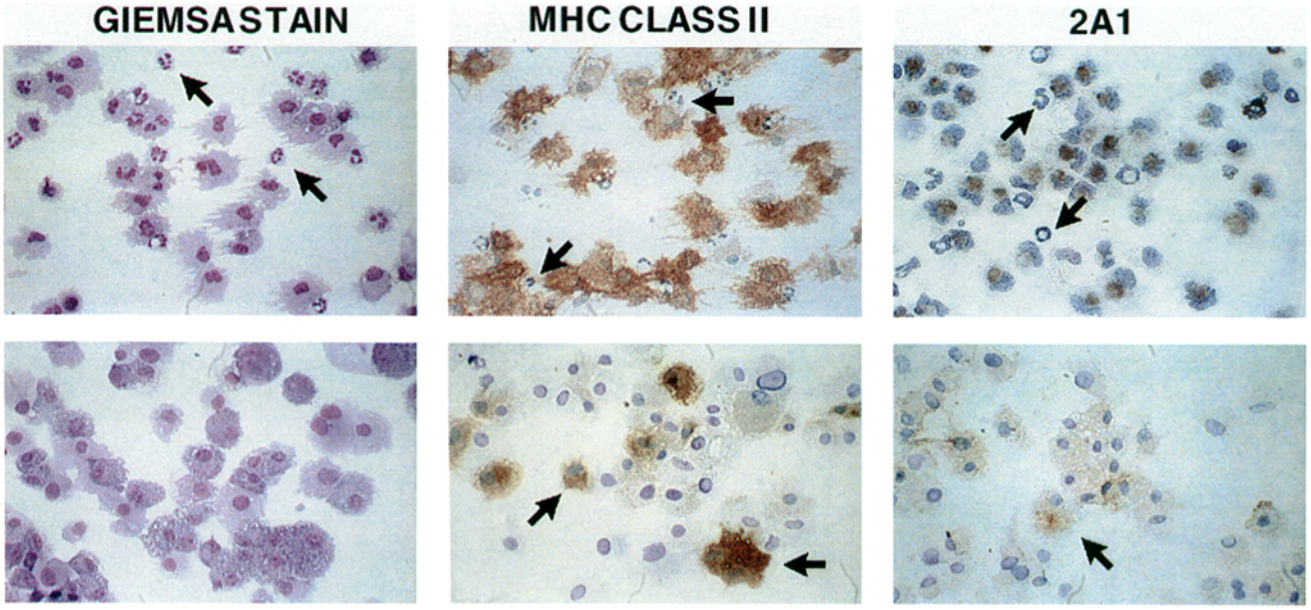

Figure 6. Comparison of the released (dendritic cell enriched; *top*) and adherent (macrophage enriched; *bottom*) fractions of 7-d bone marrow cultures. Ia-negative, bone marrow nonlymphocytes were cultured in GM-CSF. At days 2 and 4, nonadherent cells were gently washed away, and at day 6, the loosely attached cell aggregates were isolated by 1-*g* sedimentation. After 1 d in culture, the cells that released from the aggregates (*top*) were cytospun onto glass slides and stained with different mAbs plus peroxidase anti-Ig as well as Giemsa and nonspecific esterase (latter not shown). The firmly adherent cells in the original cultures were dislodged with EDTA and also cytospun (*bottom*). Many dendritic profiles are in the released fraction (a hand lens is useful to detect cell shape and contaminating granulocytes [*arrows*] in the Giemsa stain), while the adherent cells are for the most part typical vacuolated macrophages. Strong MHC class II expression occurs on all released cells but for a few typical granulocytes (*arrows*). Only a subset of the firmly adherent cells expresses class II (*arrows*). Most released cells express the 2A1 granule antigen, while the adherent cells are 2A1 weak or negative. ×160.

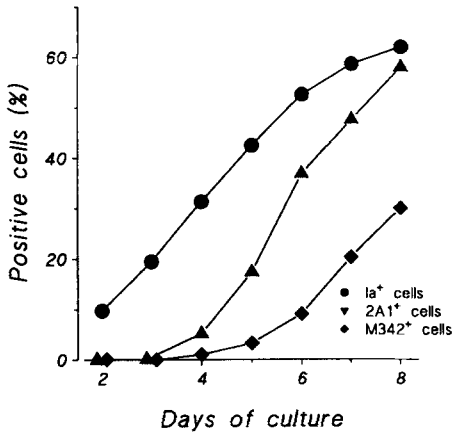

Figure 7. Quantitation of developing cells that bear the dendritic cell–restricted granule antigens 2A1 and M342. Dendritic cells contain intracellular granules that react with the M342 (10) and 2A1 (Witmer et al., manuscript in preparation) mAbs. Ia-negative nonlymphocytes from mouse marrow were cultured in GM-CSF, and the nonadherent granulocytes rinsed away at days 2 and 4. The data on days 2 and 4 represent cells that could be dislodged by pipetting, while the data on days 3 and 5–8 were cells released from the monolayer. At each of the indicated time points, at least 500 cells were counted in cytospins prepared and stained as in Fig. 6, *top*. When cultures are started at 10^6 cells/well and fed with 75% volume fresh medium every 2 d, the yields of total and Ia^+ cells were at day 2, 1.05×10^6 and 2.1×10^4, at day 4, 1.81×10^6 and 2.12×10^5, and at day 6, 1.54×10^6 and 3.21×10^5.

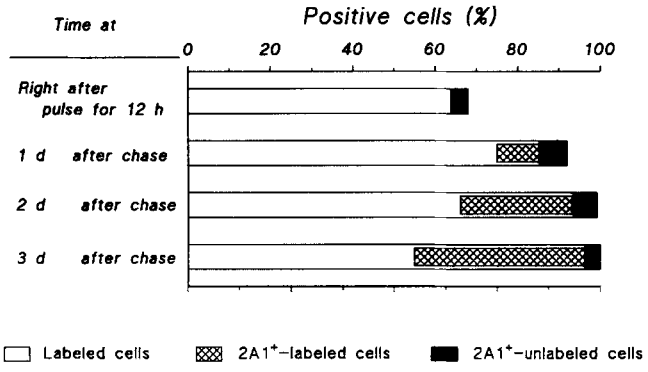

Figure 8. Progenitor-progeny relationships in growing dendritic cells. Growing aggregates were separated at day 4 from bone marrow cultures and pulsed with [^{3}H]TdR at 0.1 μCi/ml, 3×10^5 cells/well, for 12 h. All wells were replaced with fresh medium and returned to culture for 1, 2, or 3 d of chase. The yields of released cells during the chase were 2.0, 2.9, and 3.0×10^5, respectively, per well. The content of Ia^+ cells was 28% after the pulse, and 47%, 55%, and 62% on the day 1–, 2–, and 3–released cells, respectively. The data are shown as percentage of cells that were radiolabeled, with the filled bars being cells that express the 2A1 granule cell antigen of mature dendritic cells.

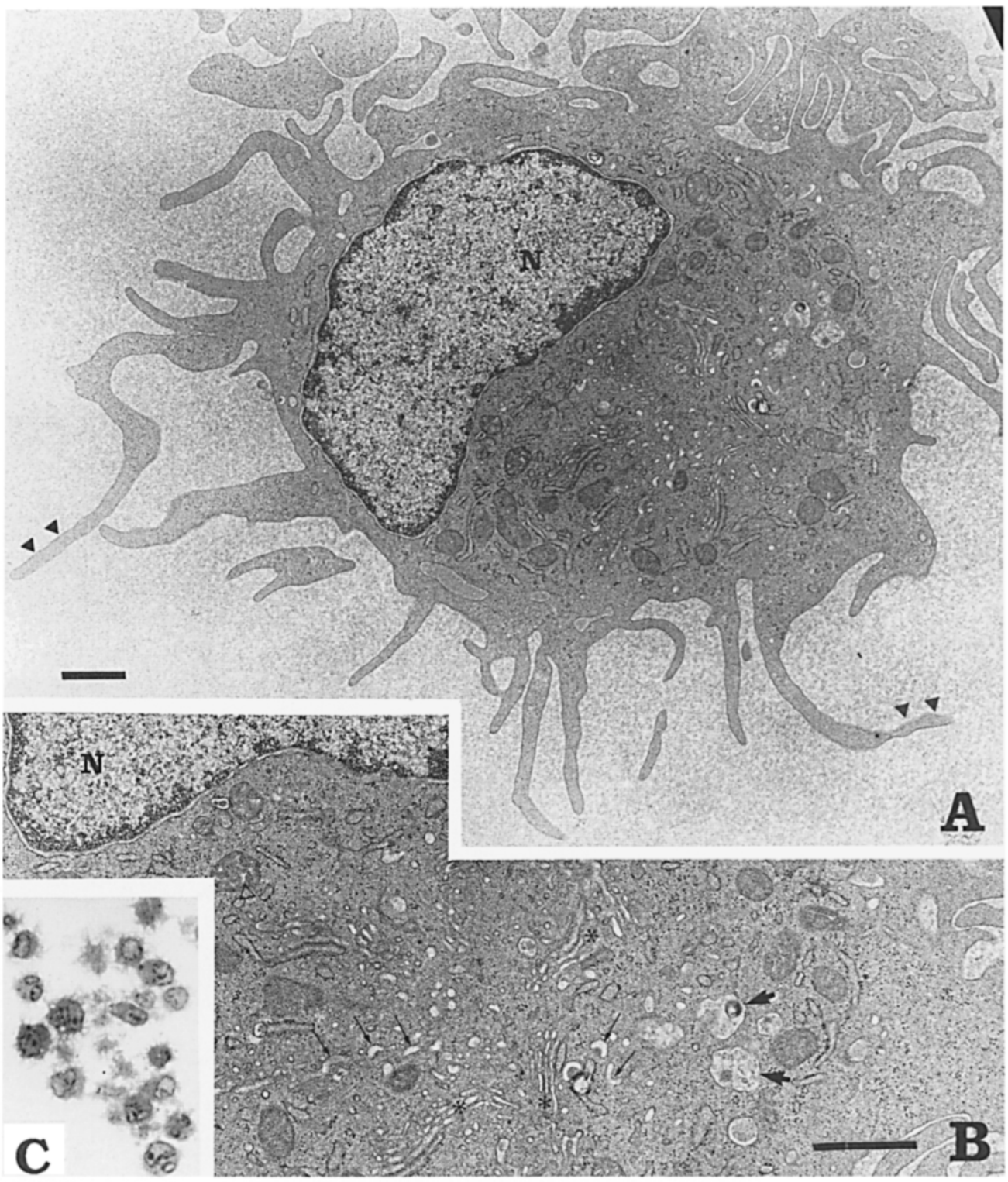

Figure 9. Electron microscopy of dendritic cells released from the proliferating cell aggregates. A bone marrow–derived dendritic cell at day 5 of culture shows many cytoplasmic veils (*A*, *filled triangles*). ×11,000; bar = 1 μm. A close up of the perinuclear region (*B*) shows profiles of smooth reticulum (*thin arrows*) and vacuoles (*thick arrows*). ×19,000; bar = 1 μm. There are few lysosomal or phagocytic structures. Golgi apparatus (*); nucleus (*N*). Magnifications are 11,000× and 19,000× [bars = 1 μm]. (*C*) The numerous cell processes in a semi-thin section. ×500.

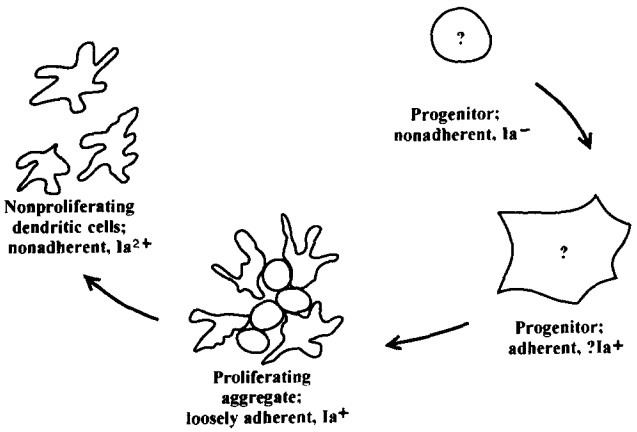

Figure 10. Diagram of the proposed pathway of dendritic cell development in marrow cultures supplemented with GM-CSF. A proliferating aggregate forms from a precursor that either attaches to the cell stroma or is itself adherent. During dendritic cell differentiation, which is evident at the periphery of the aggregate and in cells released therefrom, there is a progressive increase in cell processes, MHC class II, NLDC-145 surface antigen, and M342 and 2A1 intracellular antigen (see text), and a progressive decrease in adherence to plastic.

the proliferating aggregate as an enriched source of dendritic cells, and to phenotype the cells using several monoclonals to cell surface and intracellular antigens. The capacity of bone marrow to generate dendritic cells is striking: $>5 \times 10^6$ from the four major hind limb bones in a week.

In spite of the large numbers of dendritic cells that develop in these bone marrow cultures, it is likely that these events do not reach the same level of maturation in situ in the marrow. This is because mature dendritic cells are not detectable in mouse marrow or in mouse blood. We suspect that proliferation occurs in the marrow in situ, but that less mature progeny are released into the blood stream to populate tissues such as the epidermis and spleen. Further maturation into typical dendritic cells and perhaps additional proliferation then occurs, most likely under the aegis of GM-CSF (21–24).

The information at present suggests that the development of dendritic cells proceeds by the following pathway (Fig. 10). The precursors in both blood (4) and marrow (this paper) lack MHC class II antigens as well as B and T cell markers (CD45R, Thy-1, CD4/8), and the precursors are nonadherent. The precursors attach to the stroma and give rise to aggregates of class II–positive cells. Perhaps the growing aggregates arise from a subset of strongly class II–positive cells that are found in the firmly adherent monolayer even at later time points (Fig. 6, *middle*). However, these firmly adherent, class II–rich cells lack the MLR stimulatory activity of dendritic cells (Fig. 3 *B*, *open symbols*) and may express substantial levels of Fcγ receptors and the F4/80 antigen. The final stage of development is that the loosely attached aggregate releases mature, nonproliferating dendritic cells. The latter have even higher levels of MHC class II (Figs. 4–6) and can attach transiently to plastic, much like many of the dendritic cells released from spleen (25). As development occurs in the aggregate, there seems to be a reduction in the levels of cytoplasmic staining for Fcγ receptors and F4/80 antigen, and an increase in granule (M342, 2A1) and surface antigens (33D1, NLDC145) that are characteristic of dendritic cells. Last, accessory function for primary T-dependent immune responses increases as cells are released from the growing aggregates.

The development of monocytes, which is mediated by M-CSF, is known to involve a different set of events, which we confirmed (data not shown). Macrophage colonies in liquid culture systems are more dispersed and flattened (26). The nonproliferating monocyte progeny express little or no MHC class II, and in further contrast to dendritic cells, have strong phagocytic activity and persistent plastic adherence.

It is now more feasible to produce very large numbers of the specialized subset of dendritic cell APCs from small numbers of donors. Given the profound effects of GM-CSF on dendritic cell viability, function, and now growth (4, 21–24), this cytokine might logically be considered as a granulocyte, macrophage, dendritic cell–stimulating factor.

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