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ABSTRACT The developmental origin of dendritic cells, a specialized system of major histocompatibility complex (MHC) class II-rich antigen-presenting cells for T-cell immunity and tolerance, is not well characterized. Granulocyte–macrophage colony-stimulating factor (GM-CSF) is known to stimulate dendritic cells, including growth and development from MHC class II-negative precursors in suspension cultures of mouse bone marrow. Here we studied colony formation in semi-solid methylcellulose cultures, a classical bioassay system in which GM-CSF induces the formation of mixed granulocyte–macrophage colonies. When colonies were induced from MHC class II-negative precursors, a small subset (1–2%) of typical dendritic cells developed alongside macrophages and granulocytes. The dendritic cells were distinguished by their cytologic features, high levels of MHC class II products, and distinct intracellular granule antigens. By using differential adherence to plastic, enriched populations of the various myeloid cell types were isolated from colonies. Only the dendritic cells stimulated a primary T-cell immune response, the mixed leukocyte reaction, and the potency was comparable to typical dendritic cells isolated from spleen. Macrophages from mixed or pure colonies were inactive as stimulator cells. Therefore, three distinct pathways of myeloid development—granulocytes, macrophages, and dendritic cells—can develop from a common MHC class II-negative progenitor under the aegis of GM-CSF.

Dendritic cells constitute a system of antigen-presenting cells in the T-cell-dependent areas of lymphoid tissues and at the portals of antigen entry into the body such as skin, airway epithelium, and afferent lymph. These cells express high levels of antigen-presenting major histocompatibility complex (MHC) class II products, cell surface adhesion and costimulatory molecules (1), and certain cytoplasmic antigens localized primarily within intracellular granules (2, 3). Dendritic cells act as specialized accessories to induce immunity (4) and tolerance (5–7). In contrast to other hematopoietic cells, the developmental lineage of dendritic cells is not worked out.

A relationship of dendritic cells to phagocytes is suggested by the shared responsiveness of these cells to granulocyte–macrophage colony-stimulating factor (GM-CSF). GM-CSF maintains dendritic-cell viability (8–11) and mediates their development from less-mature but nonproliferating precursors in skin (8, 9). More recently, GM-CSF has been shown to induce the extensive growth of dendritic-cell precursors in suspension cultures (12–14). In these cultures, the mature nonproliferating progeny express high levels of MHC class II and other marker antigens that are characteristic of dendritic cells. Interestingly, dendritic cells do not respond to the more

lineage-restricted macrophage and granulocyte colony-stimulating factors (M-CSF and G-CSF, respectively) (8, 10, 12).

Since GM-CSF can induce the formation of mixed populations of granulocytes and macrophages in semi-solid colony systems (15), we asked whether dendritic cells could also arise from a colony-forming precursor that is common to phagocytes. Cells with some of the features of dendritic cells have been detected in human cell colonies that were induced with lectin-conditioned medium (16) and more recently with a mixture of tumor necrosis factor and GM-CSF (17). Here we isolate cells from colonies that are induced by recombinant GM-CSF from MHC class II-negative precursors and show that the colonies contain significant numbers of dendritic cells by several criteria. The dendritic cells are readily distinguished from phagocytes particularly in their high levels of MHC class II expression, content of intracellular antigens, and antigen-presenting-cell function. Dendritic cells, therefore, represent a distinct immunostimulatory differentiation pathway that is induced by GM-CSF but shares a common MHC class II-negative precursor with phagocytes.

MATERIALS AND METHODS

Marrow Cells and Cultures. Bone marrow suspensions were prepared from 6- to 8-week-old (BALB/c × DBA/2)F$_1$ mice as described, including treatment with monoclonal antibodies and complement to deplete cells bearing MHC class II, CD4, CD8, and B220 markers (13). Approximately 6×10^4 cells from BALB/c × DBA/2 adult male mice were suspended in 1 ml of culture medium [RPMI 1640 medium/10% (vol/vol) fetal calf serum/50 μM 2-mercaptoethanol, gentamicin (20 μg/ml)] in the presence of 1% methylcellulose (Nakarai Tesque, Kyoto) and recombinant mouse GM-CSF (2000–4000 units/ml, 9.2×10^7 units/mg; Kirin Brewery, Takasaki, Gunma) and applied to 35-mm Petri dishes (Falcon, 3001).

Phenotype of Marrow Cells. After 5 or 6 days of culture, ≈50 mixed colonies were plucked with a Pasteur pipette from the methylcellulose, resuspended, washed, and cytocentrifuged onto glass slides (Shandon, Sewickley, PA). The cytocentrifuged cells were stained with Giemsa (Wako Pure Chemical, Osaka) or were fixed with absolute acetone and stained with monoclonal antibodies (see Results) followed by peroxidase-conjugated anti-immunoglobulin as described (13). To observe the distinctive morphology of dendritic cells in live preparations, the colonies were examined at a final magnification of ×40 to ×100. To separate the various cell types that are found in colonies, 200–300 colonies were pooled and separated into three fractions on the basis of differential adherence to plastic.

Abbreviations: MHC, major histocompatibility complex; GM-CSF, granulocyte–macrophage colony-stimulating factor; MLR, mixed leukocyte reaction.
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The cells were applied in culture medium to 35-mm tissue culture dishes and after 2 hr were separated into nonadherent (granulocytes and some dendritic cells), weakly adherent (dislodged by gentle pipetting), and strongly adherent [dislodged after 20 min of culture at 37°C in phosphate-buffered saline (PBS) with 10 mM EDTA].

Stimulation of the Mixed Leukocyte Reaction (MLR). Enriched populations of dendritic cells and macrophages were prepared from pools of bone marrow colonies as above, treated with mitomycin C (Kyowa Hakko, Tokyo), and applied in graded doses to 3×10^5 purified T cells from allogeneic C3H/He mice. DNA synthesis in the responding T cells was measured with a pulse of 1 μCi of [^{3}H]thymidine (1 Ci = 37 GBq) administered at 72–90 h of culture. Data with syngeneic T cells as responders were <10% of the corresponding response with allogeneic T cells.

RESULTS

A slightly modified colony-forming assay was used to evaluate the effect of GM-CSF on mouse bone marrow progenitors. Since a hallmark of the mature dendritic cell is the abundant expression of MHC class II products, we first treated the marrow suspensions with antibodies and complement to deplete cells that express MHC class II and the lymphocyte antigens B220, CD4, and CD8. Treatment with antibody and complement removed detectable B and T lymphocytes from suspension cultures (12, 13). Small numbers of the treated marrow cells (or unmanipulated bone marrow that gave colonies similar to those described below)

Table 1. Yields of colony-forming units from bone marrow depleted of T, B, and MHC class II-rich cells

Type of colony	Colony-forming units, no. per 10^5 bone marrow cells
Granulocyte	79 ± 12
Mixed-type	108 ± 15
Macrophage	57 ± 14
Eosinophil	10 ± 2
Burst	1.6 ± 0.9

Values are the means ± SEM of quadruplicate assays in four experiments.

were then suspended in medium that was made semi-solid by adding methylcellulose and was supplemented with fetal calf serum and various cytokines. We found it helpful to omit the horse serum, a standard supplement in this culture system. At 5–8 days, colonies appeared, many of which were of the "mixed" type containing more compact round cells and many dispersed cells. The yield of different types of colonies is shown in Table 1. By several criteria, we were able to show that some of the dispersed cells were typical dendritic cells as long as GM-CSF (but not interleukin 3, M-CSF, or G-CSF) had been used to generate the colonies.

Identification of Cells with the Phenotype of Dendritic Cells. Under phase-contrast microscopy, the presumptive dendritic cells in individual colonies extended distinct processes in many directions from the cell body. These large lamellipodia or "veils" are characteristic of dendritic cells from many tissues and species (18–20). Colonies were picked and pooled
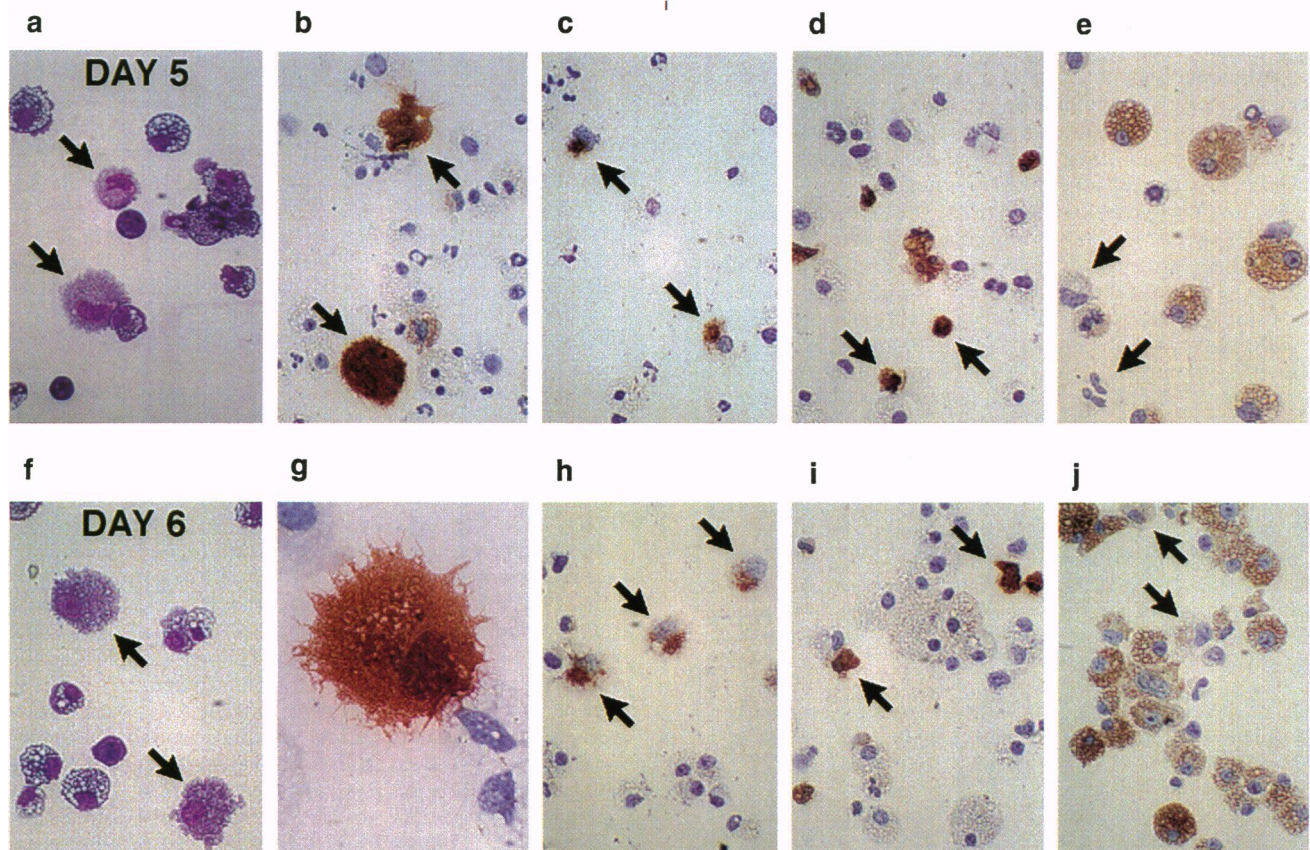

FIG. 1. Identification of dendritic cells in mixed colonies of macrophages and granulocytes at day 5 and 6 of culture in GM-CSF. Presumptive dendritic cells in these cytocentrifuged preparations of pooled colonies are indicated by arrows, except in *d* and *i* where granulocytes are identified. (*a–f* and *h–j*, ×100; *g*, ×250.) (*a* and *f*) Giemsa stain. Macrophages are round and heavily vacuolated; dendritic cells (arrows) are larger, less-vacuolated, and have many spinous processes. (*b* and *g*) MHC class II antigens (monoclonal B21-2, ATCC TIB229) are strongly expressed on a subset of larger spiny cells. (*c* and *h*) 2A1 intracellular antigen, found in the perinuclear region of dendritic cells but not phagocytes (unpublished data), stains a subset of the colony cells. (*d* and *i*) RB6-8C2 granulocyte antigen. (*e* and *j*) FA11 intracellular granule antigen of macrophages (21). A few mononuclear cells, presumably the dendritic cells, are FA11-negative.
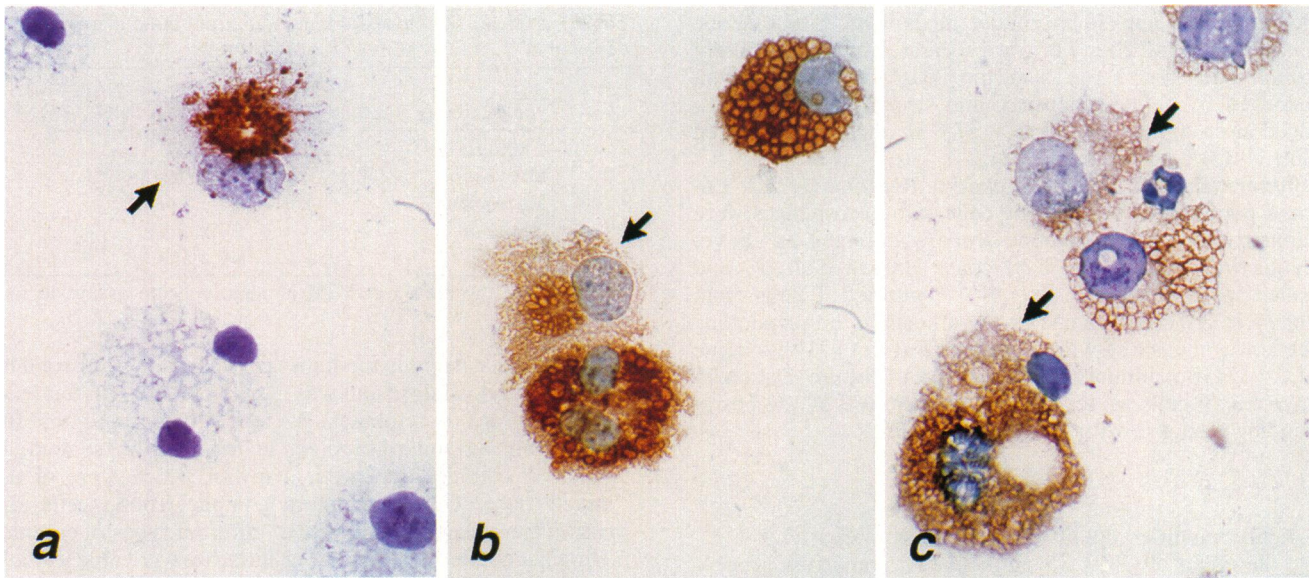
FIG. 2. Higher-power views of intracellular antigens within the endocytic vacuolar system. Cytocentrifuged cells from 6-day colonies were stained by 2A1 anti-dendritic cell (*a*), FA11 anti-macrophage (*b*), and MAC-3 anti-lysosome (*c*) antibodies. The less-vacuolated dendritic cells (arrows) stain strongly for 2A1 but more weakly for FA11 and MAC-3 antigens. The reciprocal applies to heavily vacuolated macrophages.

for examination with the standard Giemsa hematologic stain (Fig. 1 *a* and *f*). Three cell types were evident. There were typical macrophages (i.e., large round mononuclear cells with many cytoplasmic vacuoles). There also were granulocytes (cells with a pale cytoplasm but characteristic irregularly shaped condensed nuclei often assuming a "doughnut" shape). Then, there were mononuclear profiles (Fig. 1 *a* and *f*, arrows; usually larger than most of the phagocytes, with an irregular "frilly" shape and few cytoplasmic vacuoles).

After staining with antibodies to MHC class II antigens, the large cells were stained very strongly and selectively, and the dendritic processes were clearly evident (Fig. 1 *b* and *g*, arrows). When we applied the M342 (3) or 2A1 (unpublished data) monoclonal antibodies that recognize cytoplasmic vacuoles within dendritic cells and some B cells (but not phagocytes), a subset of cells again stained selectively (Fig. 1 *c* and *h*, arrows; Fig. 2*a*). Granulocytes were further identified with the RB6 monoclonal antibody (Fig. 1 *d* and *i*, arrows). The FA11 monoclonal antibody (22), which identifies a "macrosialin" that is abundant in cytoplasmic granules of macrophages (21), stained most of the mononuclear cells in the colonies. However, some FA11-weak presumptive dendritic cells were also noted (Fig. 1 *e* and *j*, arrows; Fig. 2*b*). A monoclonal antibody (MAC-3) to a lysosomal membrane glycoprotein stained the phagocytes strongly relative to the weak stain on presumptive dendritic cells (Fig. 2*c*).

The yield of the subset of MHC class II-rich and 2A1 cytoplasmic antigen-positive cells was determined in colonies at different times. At the peak, day 5–6, the presumptive dendritic cells represented 0.5–1.5% of the progeny (Table 2).

Table 2. Frequency of dendritic cells arising in mixed colonies generated from MHC class II-negative bone marrow precursors

Day of colony assay	MHC class II-rich cells, %	2A1-positive cells, %
4	0.11 ± 0.09	ND
5	0.55 ± 0.23	0.07 ± 0.04
6	1.47 ± 0.34	0.48 ± 0.23
7	1.39 ± 0.28	0.50 ± 0.21
8	0.73 ± 0.12	0.21 ± 0.08

Values are the means ± SEM of three experiments in which >400 cells were counted in cytocentrifuged cells harvested from mixed colonies.

Prior work has shown that the 2A1 and M342 cytoplasmic antigens appear relatively late in dendritic-cell development, after the cell has stopped proliferating, whereas MHC class II is expressed during and after growth (12, 13). We conclude that GM-CSF induces MHC class II-negative precursors to form mixed colonies that contain typical phagocytes (macrophages and granulocytes) plus some dendritic cells, as assessed by cytologic features and surface and intracellular antigens.

Separation of Colony-Derived Macrophages and Dendritic Cells. To verify that all the distinctive markers of dendritic cells (Figs. 1 and 2) were being expressed by the same population, we pooled the cells from many colonies and prepared macrophage- and dendritic-cell-enriched populations by using adherence criteria. It is known that dendritic cells, unlike macrophages, do not stick firmly to plastic surfaces (19, 20, 23).

When colony-derived cells were applied to plastic, the bulk of the granulocytes and some of the dendritic cells were nonadherent and could be removed by swirling the plates. When we gently pipetted buffer over the remaining adherent cells, most of the dendritic cells were dislodged as verified by double labeling for MHC class II and the 2A1 intracellular dendritic cell antigen (Fig. 3 *a–c*; e.g., cells at arrows), but not the FA11 macrophage granule antigen (data not shown). The vacuolated phagocytes remained firmly attached and could only be dislodged after warming the cultures in PBS supplemented with 10 mM EDTA. Almost all the cells in the firmly attached population (Fig. 3*d*) had low or absent MHC class II (Fig. 3*e*), and expressed the FA11 antigen that is abundant in macrophage granules (Fig. 3*f*), but lacked 2A1 (data not shown). In addition, macrophages expressed much higher levels of the lysosomal membrane glycoprotein detected by the MAC-3 monoclonal antibody than did dendritic cells (data not shown). With additional GM-CSF, we could not convert the phagocytes to dendritic cells or vice versa.

Function of Colony-Derived Dendritic Cells and Macrophages. The enriched populations (nonadherent, weakly adherent, and firmly adherent) were then evaluated for immunostimulatory activity by using the MLR as a test system. In the MLR, leukocytes from one strain of mice, particularly dendritic cells (25, 26), initiate a strong proliferative response in T cells from other strains that differ in MHC. Stimulation of the MLR requires presentation of the foreign MHC prod-
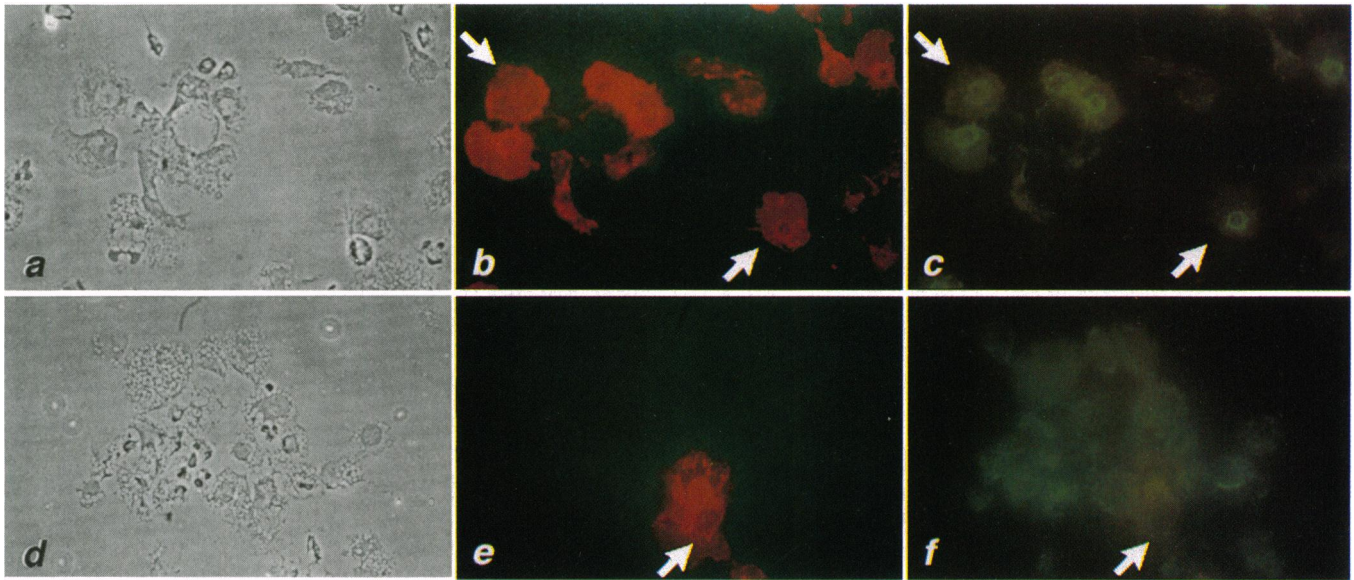
FIG. 3.   Two-color immunofluorescence to test for coexpression of MHC class II and other antigens in enriched bone-marrow-colony-derived dendritic cells and macrophages. Cells were double-labeled for MHC class II and either the 2A1 antigen of dendritic-cell intracellular granules (unpublished data) or the FA11 antigen of macrophage intracellular granules (21). (*a* and *d*) Phase-contrast images of the cytocentrifuged cells fixed in acetone. (*b* and *e*) MHC class II monoclonal antibody N22 (24) (ATCC HB225). (*c* and *f*) Double-labeled cells with rat monoclonal antibodies to intracellular antigens (2A1 and FA11, respectively). (*a–c*) Loosely attached dendritic cell-enriched population. (*d–f*) Firmly attached macrophage-enriched population. (*b* and *c*) The dendritic-cell fraction stains strongly for MHC class II and 2A1 intracellular antigens. Two such dendritic cells are indicated by arrows. (*e* and *f*) The macrophage fraction expresses little or no MHC class II but high levels of the FA11 intracellular antigen (a single class II-rich profile is indicated by arrows). The staining sequence was N22 hamster anti-mouse class II, biotin-conjugated rabbit anti-hamster immunoglobulin (Jackson ImmunoResearch), Texas red-streptavidin (Biomedia, Foster City, CA), 2A1 or FA11 rat monoclonal antibody, and fluorescein-conjugated mouse anti-rat immunoglobulin (Boehringer Mannheim). (×250.)

uct plus other accessory functions [e.g., the ligation of several adhesion or costimulatory molecules including CD2, CD11a, and CD28 (1)].

The nonadherent cells from the colonies (containing granulocytes and dendritic cells) and the loosely adherent cells (containing primarily dendritic cells; Fig. 3 *a–c*) were very much enriched relative to the bulk or unfractionated population in MLR stimulating activity (Fig. 4, compare data for weakly adherent and nonadherent GM-colony populations with that for the bulk GM-colony population). The strongly adherent GM-colony macrophages were inactive. The weakly adherent dendritic-cell component was indistinguish-

able from splenic dendritic cells in MLR stimulating activity (Fig. 4, compare weakly adherent GM-colony population and spleen dendritic cells). Some of the methylcellulose colonies were not of the "mixed" type but contained either typical macrophages and granulocytes as described (15). When cells from these colonies were tested, no MLR stimulating activity was apparent.

DISCUSSION

Bowers and Berkowitz (27) first reported that dendritic cells could be generated from MHC class II-negative precursors in
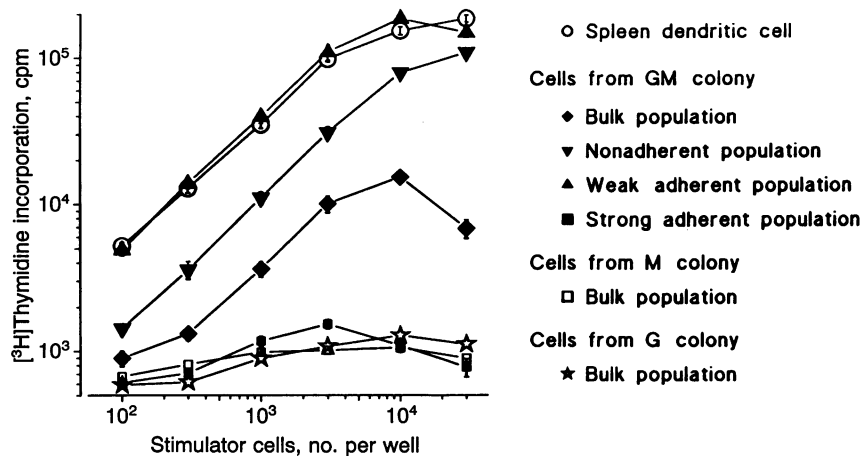

FIG. 4.   MLR stimulating activity of various cell types derived from GM-CSF-induced bone marrow colonies. GM-CSF was used to induce the formation of colonies from MHC class II-negative precursors as in Fig. 1. Cells from mixed colonies (containing both macrophages and granulocytes, GM colony) or from pure macrophage (M colony) and granulocyte (G colony) colonies were plucked from the methylcellulose semi-solid cultures, washed, mitomycin C-treated, and used to stimulate an allogeneic MLR in 3×10^5 purified T cells from C3H H-2^k mice. The cells in the mixed GM colonies were separated into fractions as in Fig. 3. The nonadherent fraction contains both granulocytes and dendritic cells, the weakly adherent fraction is primarily dendritic cells, and the strongly adherent fraction is primarily macrophages. The MLR was monitored by measuring DNA synthesis in the T cells ([^{3}H]thymidine uptake) at 72–90 h of culture.

serum-free suspension cultures of rat bone marrow. By adding GM-CSF, much larger numbers of granulocytes, macrophages, and dendritic cells can be generated (13). Reid *et al.* (16) observed that bulk populations of human bone marrow or blood cells would generate mixed colonies of macrophages and dendritic cells upon supplementation with a lectin-stimulated leukocyte-conditioned medium that likely contained GM-CSF. Recently, Reid *et al.* (17) have documented that GM-CSF and tumor necrosis factor work together to generate colonies containing macrophages and dendritic cells or dendritic cells only.

Here we show that dendritic cells share a common MHC class II-negative colony-forming precursor with phagocytes (granulocytes and macrophages), by using the classical GM-CSF semi-solid bioassay. We were unable to detect pure dendritic-cell colonies. In mixed colonies, the dendritic cells were enumerated and distinguished from typical phagocytes by the following features: a dendritic or veiled morphology, weak adherence to plastic, and distinct antigenic markers including high levels of MHC class II products and new intracellular antigens. Since this small subset of dendritic cells could be enriched by adherence methods, it was possible to study the T-cell stimulating activity of different cell types from mixed colonies and from pure macrophage and granulocyte colonies. The MLR stimulating activity of colony-derived dendritic cells was very strong and comparable to that seen in mature lymphoid dendritic cells, whereas the phagocytes lacked detectable activity (Fig. 4). Therefore, by the criteria that we have studied, the dendritic cells differ markedly from the macrophages and granulocytes that are also arising from MHC class II-negative progenitors within individual colonies.

The extent to which this distinct immunostimulatory, differentiation pathway occurs in bone marrow *in vivo* is unclear. Typical dendritic cells have not been identified in fresh bone marrow suspensions and are rare in blood, whereas mature granulocytes and macrophages are found in both sites. *In vivo*, dendritic cell development may not proceed to the same extent in marrow as in these semi-solid colony-forming systems. Prolonged exposure to GM-CSF, or other cytokines yet to be identified, may be required to induce the full development of MHC class II-rich immunostimulatory dendritic cells.

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