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Effect of Hematopoietic Growth Factors on Human Blood Monocytes/Macrophages in In Vitro Culture

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The production of mature monocytes/macrophages is regulated by a group of hematopoietic growth factors, or colony-stimulating factors (CSF). We investigated the in vitro effect of human hematopoietic growth factors on human blood monocyte/macrophage differentiation and proliferation in short- and long-term in vitro cultures. The addition of macrophage CSF, granulocyte-macrophage CSF, and granulocyte CSF and interleukin-6 and interleukin-3 growth factors to monocyte/macrophage cultures induced morphological changes in cultured cells, including enhancement of cell growth and the formation of multinucleated giant cells, spindle-like cells, and fibroblast-like cells. In addition, CD4 and HLA-DR antigen expression was down regulated by the addition of growth factors without a change in the expression of other surface antigens, including CD3, CD11B, CD14, CD15, NK H1, and B1. The proliferating cell nuclear antigen was not detected in growth factor-treated nonadherent monocytes/macrophages in long-term cultures. Bromodeoxyuridine was incorporated in the adherent monocytes/macrophages, and intense staining in the small rounded cells which occur above the adherent cells in these cultures was observed after a 72-h pulse, indicating that monocytes/macrophages are slowly dividing cells.

Mononuclear phagocytes comprise a widely distributed cell system that includes promonocytes in the bone marrow, circulating monocytes, and tissue macrophages. The promonocyte is an actively dividing cell in the bone marrow that undergoes several intramarrow mitoses before differentiating into the monocyte. Tissue macrophages are replaced from the blood monocyte and exist as both fixed and free macrophages (13, 19, 20). Free macrophages are found in the pleural, synovial, peritoneal, and alveolar spaces. The less-motile fixed tissue macrophages include liver Kupffer cells, splenic macrophages, bone osteoclasts, and brain microglia (5, 6, 13, 19, 20). Monocytes/macrophages play a central role in the induction, regulation, and amplification of specific immune responses; in homeostasis; and in the synthesis of potent regulatory proteins (cytokines) (5, 6, 13, 19, 20). The production of mature monocytes and macrophages is regulated by a group of hematopoietic growth factors, or colony-stimulating factors (CSF) (4, 13, 19). Macrophage CSF (M-CSF), granulocyte-macrophage CSF (GM-CSF), and interleukin-3 (IL-3), or multi-CSF, affect the differentiation and functional activities of monocytes/macrophages (18, 19, 21). M-CSF stimulates predominantly the proliferation of progenitors committed to macrophage lineages (19). The addition of M-CSF to serum-free monocyte cultures increases the levels of intracellular lysosomal enzyme acid phosphatase and superoxide production (1). GM-CSF stimulates the proliferation and differentiation of precursor cells for both macrophages and granulocytes (16). It also enhances macrophage oxidative metabolism, antibody-dependent cellular cytotoxicity, microbial phagocytosis, and tumoricidal activity (2). IL-3 acts on primitive progenitors of the hematopoietic cell lineages, including the monocytes/macrophages (10). Moreover, IL-3 enhances endotoxin-induced cytotoxicity of macrophages through tumor necrosis factor secretion (2). GM-CSF and M-CSF enhance the production of human immunodeficiency virus type 1 (HIV-1) in human blood monocyte/macrophage cultures (10, 12, 15), through an unidentified mechanism(s).

In this study, we have further characterized the in vitro effects of human hematopoietic growth factors on human blood monocyte/macrophage differentiation and proliferation. We investigated the effects of hematopoietic growth factors, including M-CSF, GM-CSF, and IL-3, on morphological changes, cell proliferation, and cell surface receptor expression of human blood monocytes in short- and long-term in vitro cultures.

MATERIALS AND METHODS

Isolation and culture of human blood monocytes. Human peripheral venous blood was collected from healthy donors in plastic tubes with preservative-free heparin. Mononuclear cells were separated by centrifugation with Ficoll-Hypaque (Pharmacia, Piscataway, N.J.) (13) and layered on gelatin-coated plastic flasks to purify the blood monocytes as previously described (7, 8). The blood monocytes collected were at least 98% pure (8). Cells were also analyzed for the presence of stem cells and early progenitor cells with CD34 antibody (Becton Dickinson, Mountain View, Calif.). Cells were washed once with Dulbecco's modified Eagle medium (DMEM) (GIBCO, Grand Island, N.Y.) at 1,200 rpm and 4°C for 10 min. Monocytes were counted in a hemacytometer and suspended in DME with 10% pooled heat-inactivated human AB serum supplemented with 50 U of penicillin per ml, 50 μg of

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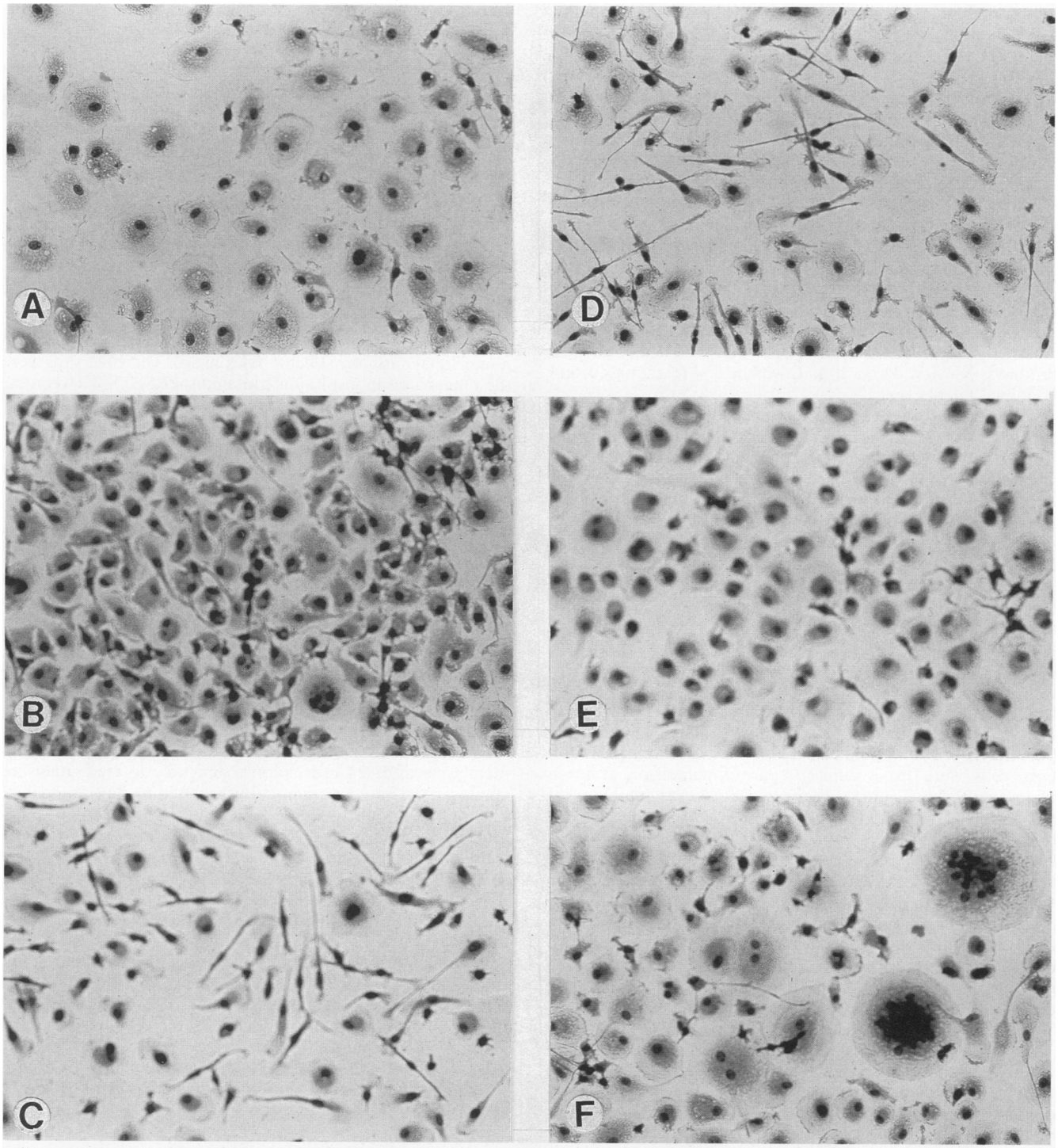

FIG. 1. Blood monocytes/macrophages were maintained in in vitro cultures on glass coverslips for 10 days in the presence and absence of growth factors. Growth factors were added every 48 h to cultures, and cultures were refed only on day 5. After 10 days in culture, cells were fixed and stained with Dif-Quik stain. In the absence of growth factors, macrophages were rounded or oval and were 50 to 80 μm in diameter, with central or eccentric nuclei and cell cytoplasm that stained light blue (A). The addition of GM-CSF to cultures increased cell spread and also cell density (B), and G-CSF (C) and M-CSF (D) induced the formation of fusiform, or spindle-like, cells. The addition of IL-3 to monocyte cultures increased the cell density (E), and IL-6 induced the formation of multinucleated giant cells (F). All magnifications are ca. ×340.

streptomycin per ml, 4 mM L-glutamine, and 100 μM nonessential amino acids (GIBCO Laboratories). Cells were adjusted to a concentration of 5×10^6/ml, and 100 μl of the mixture was plated on a prewashed glass coverslip (Fisher Scientific, King of Prussia, Pa.) in the presence and absence of different hematopoietic growth factors. Blood monocytes were maintained for long-term in vitro culture in a humidified atmosphere of 95% air and 5% CO_2 at 37°C. After 5 days, the

culture medium was removed from the monocyte/macrophage cultures and floating cells were discarded. Cultures were then refed with fresh medium supplemented with hematopoietic growth factors.

Morphological characterization of growth factor-treated blood monocytes/macrophages in short-term in vitro cultures. Blood monocytes/macrophages were maintained in in vitro cultures for 10 days in the presence or absence of growth factors. Different growth factors including human recombinant M-CSF (CSF-1) (1,000 U/ml) (kindly provided by Peter Ralph, Cetus Corporation, Emeryville, Calif.), human recombinant GM-CSF (100 U/ml), and human recombinant IL-3 (1:1,000 dilution) (kindly provided by Gordon Wong, Genetics Institute, Cambridge, Mass.) were added to the cultures every 48 h. Cultures were refed once on day 5 and were ended on day 10. Coverslips were removed from petri dishes and stained with modified Wright-Giemsa stain (Dif-Quik; American Scientific Products, McGaw Park, Ill.). Coverslips were mounted and examined microscopically.

Cell surface antigen characterization of growth factor-treated monocytes/macrophages in long-term in vitro cultures. Human blood monocytes/macrophages were isolated and maintained in in vitro cultures as described above. Cultures were refed with fresh medium and growth factors including M-CSF, GM-CSF, and IL-3 every 5 days and maintained in in vitro cultures for more than 4 weeks. The nonadherent cells in growth factor-treated and untreated cultures were removed every 5 days during refeeding of cultures. Cells were centrifuged and stained for the expression of various surface antigens. Cells were incubated for 30 min at 4°C with monoclonal antibodies against CD14 and CD15 (Becton Dickinson), CD11B (gift from Donald Anderson, Baylor College of Medicine, Houston, Tex.), CD4 and CD3 (Ortho Diagnostic Systems, Raritan, N.J.), NK H1 (gift from Giorgio Trinchieri, The Wistar Institute, Philadelphia, Pa.), and HLA-DR and B1 (Coulter Electronics, Hialeah, Fla.). To assess cell proliferation, cells were fixed with 1% paraformaldehyde at room temperature for 2 min, suspended in absolute methanol at −20°C for 10 min, and washed with phosphate-buffered saline (PBS) containing 0.1% Triton X-100. Cells were then incubated with monoclonal antibody to proliferating cell nuclear antigen (anti-PCNA) (Boehringer Mannheim Biochemicals, Indianapolis, Ind.). Isotype-matched mouse monoclonal antibodies immunoglobulin G2A (IgG2a), IgG2b, and IgG1 were obtained from Coulter Electronics and used as control antibodies. Cells were then incubated with fluorescein-conjugated goat $F(ab')_2$ anti-mouse IgG (Tago, Burlingame, Calif.) for 30 min at 4°C and analyzed by flow cytometry (Cytofluorograph System 50HH; Ortho Diagnostics Systems, Westwood, Mass.).

Assessment of cell proliferation in monocyte/macrophage cultures. Monocyte/macrophage proliferation was assessed in vitro with modified Wright-Giemsa stain and by a 5-bromo-2′-deoxyuridine (BrdU) cell incorporation assay. Monocytes/macrophages were grown on plastic coverslips in the presence of growth factors as described above. After 3 weeks in culture, coverslips were washed with PBS and stained with Dif-Quik (modified Wright-Giemsa) stain. Cells were examined by light microscopy for the presence of active proliferating cells in mitotic stages. For assessment of proliferating cells in in vitro cultures, BrdU, a thymidine analog, was used. Monocytes/macrophages cultured for 21 days in the presence or absence of growth factors were pulsed with 10 μM BrdU for 72 h. Coverslips were washed three times with PBS, fixed in absolute methanol at −20°C for 10 min, and washed in distilled water for 5 min. The coverslips were then suspended in 2 ml of 1.5 M HCl at 20°C for 20 min, washed twice with 5 ml of PBS, and resuspended for 1 h in PBS containing 0.5% Tween 20 (Sigma), 0.5% bovine serum albumin, and anti-BrdU mouse monoclonal antibody (Becton Dickinson). The coverslips were then incubated in a solution containing PBS, 0.5% Tween 20, and biotin-labeled goat anti-mouse gamma globulin. BrdU incorporation in cells was detected with a biotin-avidin detection system and an *O*-phenylenediamine substrate. Coverslips were then washed, dried, mounted, and examined with a Zeiss light microscope equipped with a camera.

TABLE 1. Effect of growth factors on formation of fusiform cells in short-term in vitro cultures of human purified blood monocytes/macrophages

Growth factor	% Fusiform cells in expt:				
	1	2	3	4	5
None	16	18	14	14	5
IL-3	16	10	10	5	29
CSF-1	33	35	18	24	5
GM-CSF	16	10	8	5	19
G-CSF	28	35	23	18	32
IL-6	11	18	11	8	25

RESULTS

Morphological characterization of growth factor-treated blood monocytes/macrophages in short-term cultures. Human purified blood monocytes/macrophages stained negative for stem cells or early progenitor cells (data not shown). Blood monocyte/macrophage cultures maintained in vitro for 10 days in the presence or absence of growth factors displayed several morphological changes. In the absence of growth factors, purified blood monocytes adhered to the glass coverslip in a few hours; these cells spread and formed a thin cytoplasmic membrane after 24 h in culture, and they formed small cell clumps after 2 to 3 days in culture. After 10 days in culture, macrophages were rounded or oval and were 50 to 80 μm in diameter, with central or eccentric nuclei. The cell cytoplasm stained light blue with Dif-Quik stain (Fig. 1A). The addition of GM-CSF to the cultures increased cell spreading and cell density (Fig. 1B). In contrast, the presence of M-CSF and granulocyte colony-stimulating factors (G-CSF) in blood monocyte cultures induced the formation of fusiform, or spindle-like, cells (Fig. 1C and D). Fusiform cells constituted 5 to 35% and 18 to 35% of the total cells in both CSF-1- and G-CSF-treated cultures, respectively (Table 1). The addition of IL-3 to monocyte cultures increased the cell density seen in cultures in comparison with that in control cultures (without growth factors added) (Fig. 1E). Moreover, cultures treated with IL-6 for 10 days contained multinucleate giant cells (Fig. 1F). In four different experiments with cells from different donors, the fusion rate was 9 to 23% (Table 2). The fusion rate was calculated for cells which contained three or more nuclei. In addition, many binucleate cells were observed.

Cell surface antigen regulation on monocytes/macrophages in growth factor-treated cultures. The simultaneous addition of GM-CSF, M-CSF, and IL-3 to monocyte/macrophage cultures altered the expression of cell surface antigens, including CD4 and HLA-DR. Cultures were maintained for 3 weeks in the presence of growth factors, and detached cells were collected and analyzed for CD3, CD4, CD11B, CD14, CD15, NK H1, B1, and HLA-DR weekly. In six different experiments, fewer cells in the growth factor-treated group than in the control group expressed CD4 and HLA-DR antigens (Table

TABLE 2. Effect of growth factors on formation of giant cells in short-term in vitro cultures of human purified blood monocytes/macrophages

Growth factor	Fusion rate (%) in expt:			
	1	2	3	4
None	4	0	4	5
IL-3	5	3	4	3
CSF-1	4	5	5	1
GM-CSF	8	4	2	3
G-CSF	0	0	1	5
IL-6	11	23	22	9

3). However, no change in CD3, CD11B, CD14, CD15, NK H1, and B1 antigen expression occurred (data not shown). This difference in antigen expression was observed throughout the 3-week culture period (Table 3). Moreover, no significant change in the percentage of cells expressing CD4 and/or HLA-DR was noticed in the control groups during the 3-week culture period.

The effect of growth factors on monocyte/macrophage proliferation in long-term cultures. The addition of growth factors GM-CSF, CSF-1, and IL-3 induced different morphological changes in monocyte/macrophage cultures, including the appearance of giant multinucleated cells and small rounded mononuclear cells (Fig. 2A). The giant cells are 100 to 600 μm in diameter and contain 3 to 40 central nuclei and many cytoplasmic vacuoles. The rounded cells are 40 to 100 μm in diameter, with a central nucleus and pale cytoplasm. The rounded cells occur in groups attached to the upper surface of the giant cells; these cells subsequently detach and are distributed throughout the culture (Fig. 2A). The rounded cells occurred more frequently in the long-term in vitro cultures than in the short-term cultures. However, when the cells were stained with modified Giemsa stain, cells in mitoses were observed (Fig. 2B).

Monocyte/macrophage proliferation in in vitro cultures in the presence and absence of growth factors was determined by two different methods, detection of PCNA and detection of BrdU incorporation. The presence of PCNA in detached cells in monocyte/macrophage cultures was determined in six different experiments. During the 3-week culture period, no significant change was observed in the percentage of cells that were positive for PCNA in both the control and test groups (Table 3). However, heavy staining due to BrdU incorporation in the adherent population of monocyte/macrophage cultures was observed only in the nuclei of the rounded cells present on the upper surface of the giant cells (Fig. 2C).

DISCUSSION

The production of mature monocytes and macrophages is regulated by a group of hematopoietic growth factors, or CSF (4). CSF including GM-CSF, M-CSF, and IL-3 (multi-CSF) also exert different regulatory effects on different monocyte/macrophage functions (9, 11, 13, 16, 20). In this study, the addition of the growth factors separately to short-term monocyte/macrophage cultures induced specific morphological changes in cultured cells. GM-CSF and IL-3 enhanced monocyte/macrophage growth (Fig. 1A, B, and E). This effect is possibly related to the fact that more cells survive in CSF-treated cultures, while a small percentage of cells do detach in the control cultures.

Moreover, the addition of GM-CSF and M-CSF to monocyte/macrophage short-term cultures induced the formation of spindle-like, fibroblast-like cells (Fig. 1C and D). This effect has been shown previously with the addition of M-CSF to mouse peritoneal macrophage cultures (3). This induction of spindle-like, fibroblast-like cells may be due to the effect of M-CSF on the specific cell population in monocyte/macrophage cultures. G-CSF has not previously been shown to induce spindle-like, fibroblast-like cells in monocytes/macrophages. The effect observed with G-CSF may be due to its direct effect or to stimulation of the monocytes/macrophages to secrete M-CSF.

IL-4 induces the formation of multinucleated giant cells in monocyte/macrophage cultures (14). We observed similar effects after the addition of IL-6 to monocyte/macrophage short-term cultures for 10 days (Fig. 1F). The mechanism of formation of multinucleated giant cells is partially mediated via protein kinase C activation. This mechanism has previously been described by us and by others as an important factor in the formation of multinucleated giant cells in monocyte/macrophage cultures (9). Other inducers of multinucleate giant cells include anti-class II major histocompatibility complex antibodies (17). It will be important to determine if IL-6 activates protein kinase C in monocytes/macrophages and induces the formation of multinucleated giant cells.

CSF-treated monocytes/macrophages were studied for the

TABLE 3. Effect of growth factors on regulation of cell surface antigens and cell proliferation in long-term in vitro cultures of monocytes/macrophages

Wk and culture	No. of cells (10^4) in expt:						% of cells in indicated expt positive for:																	
							CD4						HLA-DR						PCNA					
	1	2	3	4	5	6	1	2	3	4	5	6	1	2	3	4	5	6	1	2	3	4	5	6
1																								
Control	6.1	1.8	8.5	6.0	4.7	10.6	27	27	29	ND[a]	30	ND	92	84	61	75	53	ND	10	2	10	ND	1	4
Treated	13.3	11.5	7.2	3.0	10.3	10.3	7	14	16	ND	25	ND	81	60	26	57	39	ND	2	0	0	ND	10	4
2																								
Control	4.7	7.2	12.9	2.7	2.1	20.0	28	66	41	15	19	30	85	84	57	46	66	72	8	0	0	ND	33	3
Treated	3.2	12.2	11.5	7.9	1.9	28.0	3	48	8	9	10	27	75	50	30	35	38	54	18	0	2	ND	14	0
3																								
Control	ND	11.8	1.1	2.0	3.4	9.3	ND	ND	ND	23	25	25	ND	ND	ND	59	65	50	ND	ND	46	ND	13	18
Treated	ND	8.8	0.6	3.7	2.3	10.8	ND	ND	ND	13	21	8	ND	ND	ND	49	61	32	ND	ND	25	ND	7	26

[a] ND, not determined.

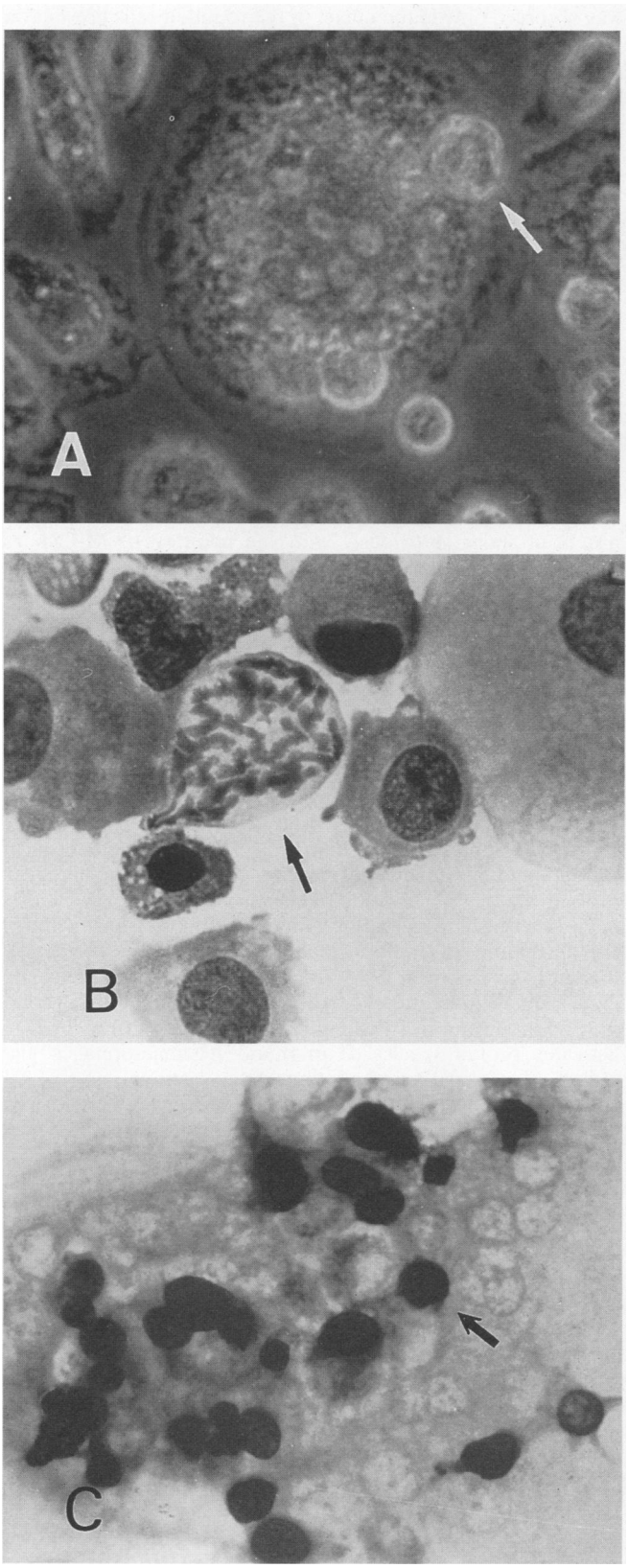

FIG. 2. Monocytes/macrophages were grown on glass coverslips in the presence of growth factors GM-CSF, M-CSF, and IL-3 for 3 weeks in culture. Monocyte/macrophage proliferation was assessed in in vitro cultures with modified Wright-Giemsa stain and by BrdU incorporation. Following staining, cells were examined by light microscopy. The addition of growth factors GM-CSF, M-CSF, and IL-3 induced the development of giant multinucleated cells (100 to 600 μm in diameter) and small rounded mononucleated cells (40 to 100 μm) (arrow) attached to the upper surface of the giant cells (A). With modified Wright-Giemsa stain, cells in the mitotic phase (arrow) are visible within rounded cells (B). BrdU incorporation resulted in heavy staining in the nuclei of the rounded cells (arrow) present on the upper surface of the giant cells (C). All magnifications are ×1,000.

expression of different cell surface antigens, including CD3, CD4, CD11B, CD14, CD15, NK H1, B1, and class II HLA-DR. In all experiments performed, only CD4 and HLA-DR antigen expression was down regulated by the addition of growth factors (Table 3) while no change was observed for the other surface antigens (data not shown). The addition of GM-CSF, M-CSF, and IL-3 to monocytes/macrophages enhances HIV-1 infection in these cells. The observed enhancement of HIV-1 infection is probably mediated via mechanisms unrelated to CD4 and HLA-DR cell surface antigens since these determinants were down regulated by the addition of growth factors.

CSF induce mouse macrophage proliferation in in vitro cultures (3); this effect has not been described for human macrophages. In order to assess the proliferative capacity of human monocytes/macrophages in the presence of growth factors in in vitro cultures, we tested for the presence of contaminating stem cells or early precursor cells in the purified blood monocyte preparations. In all experiments carried out, stem or early progenitor cells were not detected on the basis of analysis for the presence of CD34 (MY10), a specific marker for myeloid progenitor cells (data not shown). The PCNA was not detected in nonadherent growth factor-treated monocytes/macrophages, which may be due to the decrease of intracellular PCNA, as the cells may be in the G_2/M phase of the cell cycle. The PCNA usually begins to accumulate during the G_1 phase of the cell cycle and declines during the G_2/M phase. Moreover, BrdU incorporation in the adherent monocytes/macrophages resulted in heavy staining only in the small rounded cells present on the upper surface of the adherent cells. The incorporation of BrdU was detected after 72 h of incubation, suggesting that monocytes/macrophages are slowly dividing in this in vitro culture system. The expression of other cellular determinants on these small round proliferating cells, which may represent immature forms or early precursors of monocytes/macrophages, and the susceptibility of these cells to HIV-1 infection require further investigation.

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