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REGULATED EXPRESSION OF THE Mac-1, LFA-1, p150,95 GLYCOPROTEIN FAMILY DURING LEUKOCYTE DIFFERENTIATION[1]

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The regulation of Mac-1, LFA-1, and p150,95 expression during leukocyte differentiation was examined. LFA-1 was present on almost all cell types studied. Both Mac-1 and p150,95 were present on the more mature cells of the myelomonocytic series, but only p150,95 was detected on some B cell lines and cloned cytotoxic T lymphocytes. Phorbol myristate acetate (PMA) stimulation of B chronic lymphocytic leukemia cells dramatically increased p150,95 expression. The resultant Mac-1, LFA-1, p150,95 phenotype resembled hairy cell leukemia, a B cell plasmacytoid leukemia. The promonocytic cell line U937 and the promyeloblastic cell line HL-60 expressed only LFA-1. Monocytic differentiation of U937 cells was stimulated by PMA, and induced the concomitant expression of Mac-1 and p150,95, with more p150,95 induced than Mac-1. Granulocyte/macrophage colony-stimulating factor (GM-CSF) stimulation of U937 cells gave similar results. PMA-stimulated monocytic differentiation of the HL-60 cell line also induced expression of both Mac-1 and p150,95. The number of p150,95 molecules on PMA-stimulated U937 and HL-60 cells were 5×10^5 and 3×10^5, respectively. Retinoic acid stimulated myeloid differentiation of HL-60 cells and induced expression of both Mac-1 and p150,95. These cells acquired a Mac-1, LFA-1, p150,95 profile that resembled that of granulocytes, with more Mac-1 than p150,95 induced. GM-CSF stimulation of HL-60 cells induced a similar Mac-1 and p150,95 phenotype. The contributions of Mac-1, LFA-1, and p150,95 to aggregation of PMA-differentiated U937 cells were assessed. Monoclonal antibodies to the β subunit and the LFA-1 α subunit, but not those to p150,95 or Mac-1 α subunit, inhibited this homotypic adherence.

Mac-1, LFA-1, and p150,95 are structurally related glycoproteins that function in cell-substrate and cell-cell adhesion reactions of myeloid and lymphoid cells (1). Study of the cell distribution of these glycoproteins is important to gain an understanding of how adhesiveness is regulated during leukocyte differentiation. Each of these three glycoproteins is an $\alpha\beta$ complex in which a unique α subunit is associated with a common β subunit (2). The Mac-1, LFA-1, and p150,95 glycoproteins are defined by monoclonal antibodies (MAb) that are specific for their unique αM, αL, and αX subunits, respectively. The α subunits have homologous amino acid sequences (3) (manuscript in preparation). Thus, this glycoprotein family is ideally suited for studying how closely related genes are regulated in leukocyte differentiation.

The α subunits appear to control the adhesive specificity of these glycoproteins. Mac-1 functions both as an iC3b receptor (CR3) (4) and a general adhesion molecule (5–9) in monocytes and granulocytes. There is evidence that the p150,95 molecule may also bind iC3b (10) and contribute to the adhesive properties of granulocytes (6). LFA-1 plays an important role in CTL-target adhesion (11), in the function of the natural killer (NK) cell (12), and in homotypic adhesion between lymphocytes (13–16). In addition to expression on the cell surface, Mac-1 and p150,95 are present in an intracellular pool in granulocytes (1, 8, 17). Rapid mobilization of this intracellular store to the cell surface in response to physiologic stimuli appears to regulate granulocyte adhesiveness (5, 6, 8).

Previous studies in the mouse and human systems have shown that Mac-1 is expressed on monocytes, macrophages, granulocytes, and NK cells (18–24). It is detected on nonspecific esterase-positive acute myelomonocytic leukemia cells (21). Mac-1 is absent from the mixed granulocyte and macrophage colony-forming unit (GM-CFU)[2] stem cell and is first detected during granulocyte differentiation at the myelocyte stage (25). Mac-1 is present on monocytoid precursor cells in the bone marrow (25, 26). Expression of p150,95 is found on monocytes, neutrophils, tissue macrophages, and acute myeloid and monocytic leukemia cells (17, 27, 28). It is absent from normal peripheral blood T and B cells, but is expressed in hairy cell leukemia, a B cell neoplasm (27).

LFA-1 is expressed in the mouse and human on T and B lymphocytes, NK cells, and myeloid cells (11, 12, 26, 29–31). Eighty percent of murine bone marrow cells express LFA-1, with early progenitors of erythroid burst-forming units, erythroid colony-forming units, GM-CFU, and monocytic colony-forming units positive for LFA-1, unlike Mac-1, which is expressed only on more mature forms (26).

In our present study we directly compared the expression of Mac-1, LFA-1, and p150,95. We focused on cells

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[2] Abbreviations used in this paper: B-CLL, B chronic lymphocytic leukemia; DMSO, dimethyl sulfoxide; fMLP, *N*-formyl-methionyl-leucyl-phenylalanine; GM-CFU, granulocyte/macrophage colony-forming unit; GM-CSF, granulocyte/macrophage colony-stimulating factor; MAb, monoclonal antibody; PMA, phorbol myristate acetate.

that express only one or two of the three glycoproteins, and on the relative expression of the three glycoproteins during differentiation in vitro of cell lines along lymphocytic, monocytic, or granulocytic pathways. We used the inducible human promonocytic U937 (32, 33) and promyelomonocytic HL-60 (34) cell lines to study the cell surface and intracellular expression of this family of adhesion proteins during myeloid and monocytic differentiation. Our studies address how the expression of each of the three α subunits and the common β subunit is regulated in leukocyte differentiation. We also studied the role of Mac-1, LFA-1, and p150,95 in the homotypic aggregation of U937 cells.

MATERIALS AND METHODS

Cells, cell lines, and activation conditions. U937 cells (32) were maintained in RPMI 1640 (Gibco Laboratories, Grand Island, NY) supplemented with 5×10^{-5} M β-mercaptoethanol, 2 mM glutamine, 5 μg/ml gentamicin (complete medium), and 10% FCS. HL-60 cells (34) were maintained in complete medium with 20% FCS. Stock solutions of the following agents were used in these studies: 14 μg/ml phorbol myristate acetate (PMA; Sigma Chemical Co., St. Louis, MO) in dimethyl sulfoxide (DMSO), 1 mg/ml retinoic acid (Kodak, Rochester, NY) in DMSO, 2×10^7 U/ml recombinant IFN-β and IFN-γ (generously provided by Dr. Daniela Novick, Weizmann Institute, Rehovot, Israel) stored at 4°C, recombinant GM-CSF in COS cell supernatants, and mock-transfected COS cell supernatants (35) (gifts of Dr. Steven Clark, Genetics Institute). Cells were stimulated in 4.5-cm diameter Teflon beakers (Fisher, Medford, MA) at 4 to 5×10^5 cells/ml in a total volume of 10 ml. PMA was used at a concentration of 2 ng/ml for U937 cells and 10 ng/ml for HL-60 cells. Retinoic acid was used at 300 ng/ml, IFN-β and IFN-γ at 1000 U/ml, and GM-CSF as indicated. Cryogenically preserved samples of B chronic lymphocytic leukemia (B-CLL) and hairy leukemia cells were kindly provided by Dr. Lee Nadler (Dana-Farber Cancer Institute) and Dr. Robert Humphreys (University of Massachusetts Medical Center, Worcester, MA), respectively. Cytotoxic T lymphocyte (CTL) clones were generously provided by Dr. Steven Mentzer (Dana-Farber Cancer Institute). B cell lines 3638, 1899, and 2p68 were from Dr. David Posnett (Rockefeller University), and KG-1 was from Dr. James D. Griffin (Dana-Farber Cancer Institute). Granulocytes and mononuclear cells were isolated from dextran-sedimented blood by centrifugation through Ficoll-Hypaque (d = 1.08) at 4°C. The pelleted granulocytes were hypotonically lysed to remove erythrocytes.

Monoclonal antibodies (MAb). SHCL3 anti-p150,95 MAb (27), TS1/18 anti-β, and anti-LFA-1α TS1/22 MAb were previously described (11). LM2/1, an IgG1 mouse anti-human Mac-1 α-chain hybridoma, was prepared from a BALB/c mouse immunized i.p. with TS1/18-Sepharose immunoprecipitates from granulocyte lysates and was boosted i.v. with 10^7 live human granulocytes. These spleen cells were fused with P3X63Ag8.653 myeloma cells, and the hybridomas were screened for binding to PMA-stimulated U937 cells and immunoprecipitation of Mac-1. TS1/22, TS1/18, RR1/1, X-63, LM2/1 IgG1, and W632 IgG2 were purified from ascites on a protein A-Sepharose column (Pharmacia, Piscataway, NJ) by using the method of Ey et al. (36). SHCL3 (available as LeuM5 from Becton Dickinson, Mountain View, CA) was purified from culture supernatant by eluting at pH 3.5 from a protein A-Sepharose column. TS1/22, TS1/18, and SHCL3 F(ab')$_2$, Fab', or Fab fragments were prepared according to Parham (37), as described (6). LM2/1 Fab' fragments were prepared by digesting IgG with preactivated papain for 16 hr (37). The digest was then reduced with 20 mM cysteine in 0.1 M Tris, pH 8.6, for 2 hr at 37°C and was alkylated with 30 mM iodoacetamide. After dialysis vs 5 mM Tris, pH 7.5, the Fab' was obtained in the flow-through of a DEAE-Sephacel column.

Immunoblotting. Lysate from 5×10^8 Sendai virus-induced leukocytes were ethanol precipitated overnight with 3 vol of absolute ethanol. The pellet was resuspended in 600 μl of nonreducing SDS sample buffer, loaded into a 3.5-cm-wide lane, and subjected to SDS-7% PAGE. Samples were electroblotted (Bio-Rad, Rockville Centre, NY) to nitrocellulose (Millipore, Bedford, MA) for 24 hr at 250 mA. The nitrocellulose was cut into strips and incubated with blocking buffer (1% BSA, 1% Hb, 10 mM Tris, pH 8, 150 mM NaCl, 0.025% sodium azide) for 2 hr at 37°C. Each strip was incubated with 3 ml of a 1/10 dilution of MAb culture supernatant for 1 hr at room temperature and washed six times (0.3% BSA, 10 mM Tris pH 8, 150 mM NaCl, 0.025% sodium azide) over 30 min. All strips were then incubated with 5×10^6 cpm of ^{125}I-goat anti-mouse IgG(H+L) (Zymed, South San Francisco, CA) for 1 hr and washed as above. Filters were aligned and autoradiographed for 4 days.

Immunoprecipitations. JY cells or granulocytes (2×10^7/ml) were iodinated with 2 mCi ^{125}I/ml by using Iodogen (Pierce Chemical Co., Rockford, IL) and were lysed at 5×10^7 cells/ml in 1% Triton X-100, 10 mM Tris, pH 8, 150 mM NaCl, 0.025% sodium azide, 1 mM phenylmethylsulfonylfluoride, 3×10^5 cpm of lysate, and 30 μl MAb-Sepharose; or 100 μl MAb culture supernatant + 50 μl 10% suspension *Staphylococcus aureus* were used for each immunoprecipitation. Immunoprecipitates were washed and subjected to SDS-7% PAGE under reducing conditions, as described (2).

PMA-activated U937 cells were iodinated with Iodogen at 5×10^6 to 1×10^7/ml and lysed in 1 ml of lysis buffer. Approximately 2.5×10^5 cpm of each dialyzed lysate were used per immunoprecipitation.

Site number analysis. U937 or HL-60 cells (10^5 cells in a final volume of 100 μl) were incubated in 96-well V-bottom plates (Falcon, Oxnard, CA) with increasing amounts of purified ^{125}I-SHCL3 IgG (553,390 cpm/μg) in triplicate. Duplicate wells containing a 100-fold excess of cold SHCL3 were used to determine nonspecific binding. The plates were shaken at 4°C for 2 hr, washed quickly, harvested, and quantitated on a Beckman Gamma 550 counter (Palo Alto, CA).

Flow cytometry. MAb culture supernatants were incubated with approximately 10^6 cells for 30 min at 4°C. Cells were washed three times with 5% FCS, HBSS, 10 mM HEPES, incubated with fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG(H+L) (Zymed, South San Francisco, CA) for 30 min, thrice washed, and fixed in a final concentration of 1% paraformaldehyde/PBS. Stained cells were analyzed on an EPICS V (Coulter Electronics, Hialeah, FL), with dead cells removed by scatter gating or propidium iodide staining. The log amplifier was set on channel 145 for the quarter-bright fluorescent microspheres (Coulter), and the linear amplifier was set on channel 68 (gain of 5). Microspheres of different intensities were analyzed with both log and linear amplifiers and were used to construct a standard curve. The peak log channel number of an experimental sample was converted to a linear value with the standard curve. The linear channel number of the X-63 nonspecific control was then subtracted, with the resulting difference deemed "specific fluorescence." The background linear fluorescence values for X-63 binding to the different lymphoid and uninduced and induced myeloid cell lines used here ranged from 2.8 to 7.3.

RESULTS

MAb. The subunit specificity of the MAb used in these studies is shown in Table I (38–40). The specificity of the newly obtained LM2/1 MAb for the α-chain of Mac-1 (αM) was demonstrated by an immunoblot of granulocyte lysate (Fig. 1, lane 2).

Distributions on cell lines. Leukocyte cell lines, leukemia cells, and normal peripheral blood leukocytes were analyzed by flow cytofluorometry for expression of Mac-1, LFA-1, and p150,95 (Table II). The level of expression was reproducible in different experiments; values shown are averages of one to 17 experiments. All B cell lines expressed LFA-1 except SLA, an Epstein Barr virus (EBV)-transformed line derived from a patient with a severe deficiency of cell surface Mac-1, LFA-1, and p150,95. Two of the B cell lines, JY and 3638, expressed p150,95, but none had Mac-1. The hairy leukemia spleen cells expressed high levels of p150,95, with low levels of LFA-1. Two B-CLL expressed LFA-1 but little or no p150,95 or Mac-1. Upon incubation of B-CLL cells with PMA, hairy projections appeared on 90% of the cells' surfaces, and p150,95 expression increased dramatically. In contrast, Mac-1 expression on B-CLL cells was not induced with PMA, and LFA-1 expression increased

TABLE I
Specificity of MAb

Subunit	MAb	Reference
LFA-1 αL	TS1/22	2, 38
Mac-1 αM	OKM1, OKM10, LM2/1	19, 40, this paper
p150,95 αX	SHCL3	16, 26, 39
Common β	TS1/18	2, 38

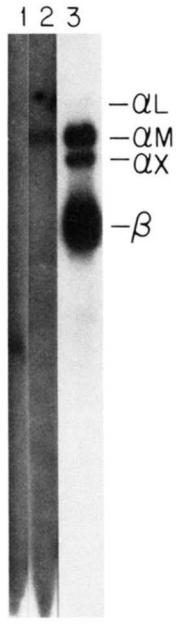

Figure 1. LM2/1 recognizes the Mac-1 αM subunit. Leukocyte lysate was subjected to nonreducing 7% SDS-PAGE and electrophoretically blotted onto a nitrocellulose filter. The filter was then cut into strips and incubated with MAb X63 (lane 1) or LM2/1 anti-Mac-1 (lane 2). An anti-β-chain immunoprecipitate from ^{125}I-granulocyte lysate (lane 3) was run and electroblotted as a M_r standard.

only slightly. T cell lines expressed LFA-1, but no Mac-1 or p150,95. CTL clones expressed high amounts of LFA-1, but differed from established T cell lines, as CTL also expressed small amounts of p150,95. The undifferentiated myelomonocytic cell lines, KG-1, U937, and HL-60, expressed LFA-1, but only KG-1 expressed (to a very low extent) Mac-1 and p150,95. When HL-60 and U937 cells were incubated with PMA, the levels of p150,95 and Mac-1 increased markedly. For comparison, the levels of Mac-1, LFA-1, and p150,95 expressed on granulocytes and monocytes are shown in Table II. In response to stimulation with the chemoattractant *N*-formyl-methionyl-leucyl-phenylalanine (fMLP), the amount of Mac-1 and p150,95 expressed on the surface of granulocytes and monocytes was increased, as previously reported (1, 8, 9, 17).

The expression of p150,95 on the JY B lymphoblastoid line was further examined and compared with expression on granulocytes by immunoprecipitation from iodinated cell lysates. Immunoprecipitation confirmed that JY cells expressed the p150,95 molecule as shown with both the anti-β MAb (Fig. 2*A*, lane 2, and Fig. 2*B*, lane 1) and the anti-p150,95 MAb (Fig. 2*B*, lane 2). No Mac-1 was immunoprecipitated from JY by anti-β MAb or by anti-αM MAb (Fig. 2*B*, lanes 1 and 4), confirming flow cytofluorometry results. The LFA-1 molecule from both JY cells and granulocytes contained the αL and β subunits (Fig. 2*A*, lanes 3 and 4; Fig. 2*B*, lane 3), as previously described. The αL and β subunits from JY cells were 8000 and 6000 g/mol lower in M_r, respectively, than those from granulocytes, which may reflect differences in glycosylation.

Expression of Mac-1, LFA-1, and p150,95 during myelomonocytic differentiation: PMA. U937 cells have been shown to differentiate along the macrophage/monocytic pathway in the presence of phorbol esters (41), lymphokine preparations, vitamin D_3, and IFN-γ (42–44).

TABLE II

Antigen expression measured by fluorescence flow cytometry on normal and transformed cells

Cell Line	Cell Surface Antigen Expression[a]		
	LFA-1	p150,95	Mac-1
B cell			
JY	27•	2.1•	0.0•
CO-1[b]	7.0•	0.0•	0.0•
SLA[b]	0.0•	0.0•	0.0•
3638	4.0	2.0	0.2
1899	27	0.0	0.0
2p68	2.0	0.0	0.0
HCL[c] 67 spleen cells	4.0	31	0.3
B-CLL 8	0.4•	0.2•	0.1•
B-CLL 10	2.1	0.3	0.5
B-CLL 10 + PMA[d]	4.0	17	0.4
T cell			
SKW-3	86•	0.0•	0.0•
Jurkat	2.3•	0.0•	0.0•
CTL clone a	53	5.0	0.1
CTL clone b	40	1.0	ND[g]
CTL clone c	51	6.0	0.5
Myelo/monocytic			
KG-1	22•	1.0•	1.0•
HL-60	8.6•	0.0•	0.0•
HL-60 + PMA[d]	67•	30•	72•
U937	16•	0.2•	0.2•
U937 + PMA[d]	39•	64•	23•
Granulocytes	6.0•	1.4•	5.4•
Gran. + fMLP[e]	5.6•	5.1•	37.1•
Monocytes[f]	18•	1.6•	7.1•
Mono. + fMLF[e,f]	20•	7.2•	26•
Pre-erythroid			
K562	6.0•	1.0•	0.7•

[a] Specific fluorescence units were determined as described in *Materials and Methods*. Asterisked (•) values represent averages of two to 17 determinations. In all cases at least 10,000 cells were counted.

[b] CO-1 and SLA are EBV-transformed lines: CO-1 from a normal healthy donor, and SLA from a patient with a severe deficiency of the Mac-1/LFA-1/p150,95 family of proteins.

[c] HCL, hairy cell leukemia.

[d] B-CLL and HL-60 cells were cultured with 10 ng PMA/ml, and U937 cells with 2 ng PMA/ml, for 3 days.

[e] Granulocytes or monocytes from healthy donors were incubated with 10^{-8} M fMLP for 30 min at 37°C.

[f] Monocytes in mononuclear cell preparations were identified by light scatter gating and their identity confirmed by positive staining with Mo2 MAb (21).

[g] Not determined.

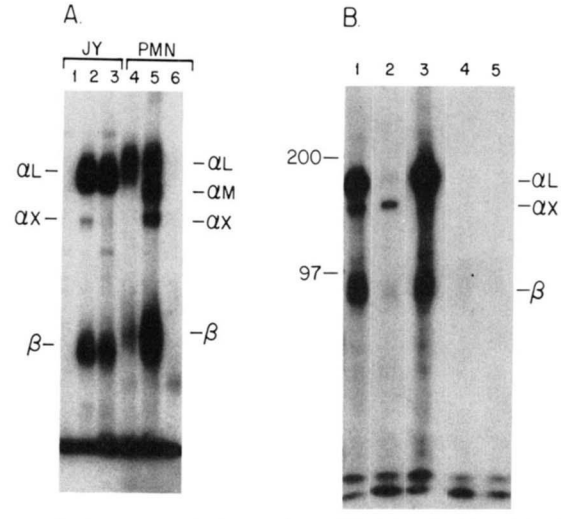

Figure 2. Immunoprecipitation from JY B lymphoblastoid cells and granulocytes. *A*, Immunoprecipitates from ^{125}I-JY lysate (lanes 1, 2, and 3) or ^{125}I-granulocyte lysate (lanes 4, 5, and 6) were formed with anti-β-subunit MAb-Sepharose (lanes 2 and 5), anti-LFA-1 αL subunit MAb-Sepharose (lanes 3 and 4), or with quenched CNBr-activated Sepharose (lanes 1 and 6). *B*, ^{125}I-JY lysates were immunoprecipitated with anti-β-chain MAb (lane 1), anti-p150,95 MAb (lane 2), anti-LFA-1 αL subunit MAb (lane 3), LM2/1 anti-Mac-1 αM subunit MAb (lane 4), or control MAb Sepharose (lane 5). Reduced immunoprecipitates were subjected to SDS-7% PAGE and autoradiography.

U937 cells were cultured in the presence of PMA for varying lengths of time. After 3 days, cell viability began to decrease. Binding of α-chain-specific MAb was measured by flow cytofluorometry. Unstimulated U937 cells were negative for p150,95 and Mac-1 (Fig. 3*A*). After PMA stimulation, p150,95 and Mac-1 were induced with similar kinetics, with p150,95 reaching higher levels than Mac-1. LFA-1 increased twofold. Representative immunofluorescence flow cytometry histograms from day 0 and day 3 showed that PMA induced essentially all U937 cells to express p150,95 and Mac-1 (Fig. 4*A* and *B*).

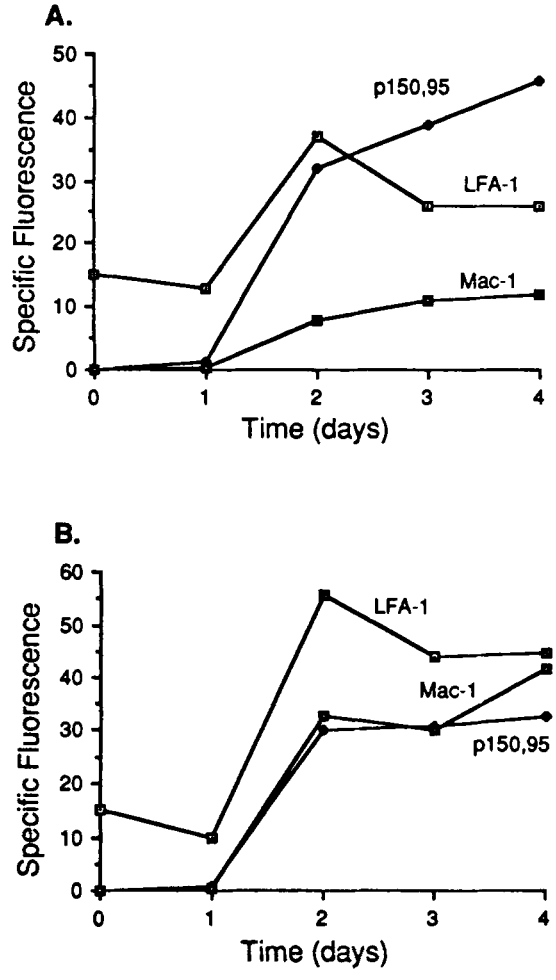

Figure 3. Both U937 and HL-60 increase expression of Mac-1 family proteins after PMA induction. (*A*) U937 or (*B*) HL-60 cells (5×10^5/ml) were incubated with 2 ng PMA/ml or 10 ng PMA/ml, respectively, with fresh medium and PMA added every 2 days. Cells were then incubated with MAb specific for LFA-1 (□), Mac-1 (■), or p150,95 (◆), or with control MAb, washed, incubated with FITC-goat-anti-mouse IgG(H+L), and subjected to flow cytofluorometric analysis. Specific linear fluorescence was calculated as described in *Materials and Methods*.

In parallel to U937 cell induction, HL-60 cells were stimulated to differentiate along the monocytic pathway with PMA. The promyeloblastic line HL-60 is developmentally bipotential: although PMA and vitamin D_3 are inducers for monocytic development (45, 46), DMSO and retinoic acid induce granulocytic differentiation (47, 48). PMA-stimulated HL-60 cells began to clump tightly, adhere to plastic, and stop dividing within 24 hr of incubation with PMA. As on U937 cells, Mac-1 and p150,95 expression increased dramatically between day 1 and day 2 (Fig. 3*B*). However, Mac-1 was induced to higher levels than p150,95 on HL-60 cells in five of six experiments (1.9 ± 0.9-fold, n = 6), whereas p150,95 was induced to higher levels than Mac-1 on U937 cells in six of six experiments (4.1 ± 2.4-fold, n = 6). Incubation of U937 or HL-60 with PMA for 6 to 8 days indicated that p150,95 expression continued to increase on U937 cells; conversely, only Mac-1 and LFA-1 levels continued to rise on HL-60 cells (data not shown).

Expression of the three antigens during PMA-stimulated monocytic differentiation of U937 cells was also examined by immunoprecipitation from iodinated lysates of U937 cells (Fig. 5). On day 0, p150,95, visualized in anti-β subunit immunoprecipitates, was absent or was present in only small amounts (Fig. 5, lane 6), whereas it was strongly induced by PMA on days 1 through 7 (Fig. 5, lanes 7–10). Mac-1 was not detected on day 0 (Fig. 5, lane 1) and was present after PMA stimulation from day 1 to day 7 (Fig. 5, lanes 2–5). LFA-1 was detected on all days (Fig. 5, lanes 11–15). Mac-1 and p150,95 were induced coordinately in nine of nine immunofluorescence experiments and in two of two immunoprecipitation experiments. The time of onset of p150,95 and Mac-1 induction (day 1 or day 2) varied, perhaps because the immunoprecipitation experiments were done 3 yr earlier than the immunofluorescence experiments and the U937 subline or culture conditions may have differed.

The amount of p150,95 on PMA-differentiated U937 and HL-60 cells was determined by saturation binding of p150,95 MAb (Fig. 6). There were approximately 5×10^5 anti-p150,95 IgG binding sites per PMA-induced U937

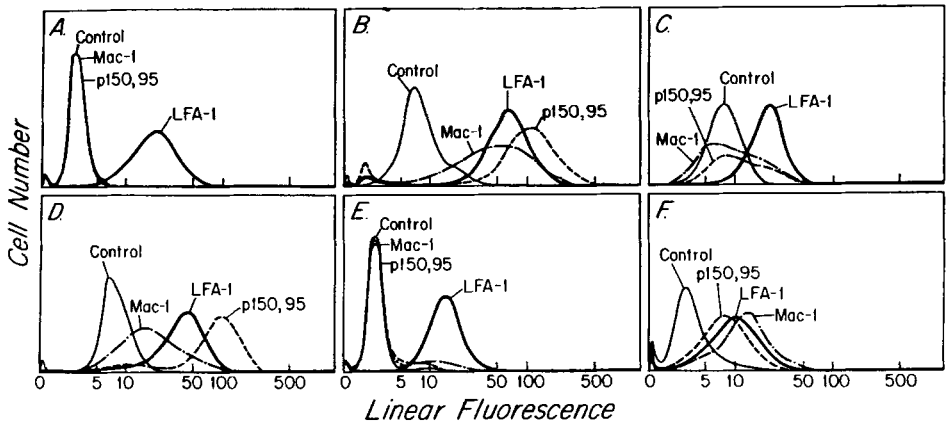

Figure 4. Flow cytofluorometry profiles of untreated and differentiated U937 and HL-60 cells. *A*, Normal untreated U937 cells are only positive for LFA-1. *B*, U937 cells cultured with 2 ng PMA/ml for 3 days are positive for all three members of the Mac-1/LFA-1 family. *C*, U937 cells incubated for 8 days with 1000 U IFN-γ/ml show a slight increase in the number of cells positive for both Mac-1 and p150,95. *D*, U937 cells stimulated for 4 days with recombinant GM-CSF become positive for Mac-1 and p150,95. *E*, Normal, untreated HL-60 cells are positive for LFA-1, but devoid of both Mac-1 and p150,95. *F*, HL-60 cells cultured with 300 ng/ml retinoic acid for 6 days become weakly positive for Mac-1 and p150,95. In all histograms, Mac-1 was identified with MAb LM2/1, p150,95 with MAb SHCL3, and LFA-1 with MAb TS1/22.

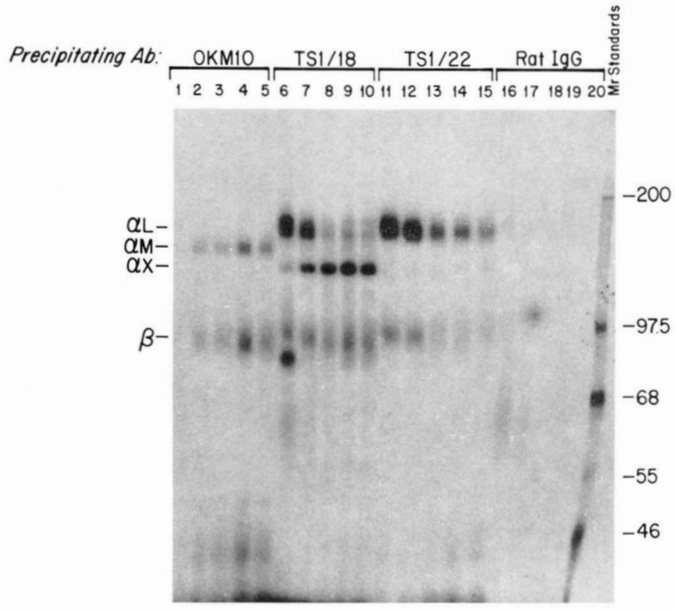

Figure 5. Immunoprecipitates from PMA-activated U937. Immunoprecipitations with OKM10 and anti-Mac-1 MAb (lanes 1–5), anti-β-chain MAb (lanes 6–10), anti-LFA-1 αL MAb (lanes 11–15), or control rat Ab (lanes 16–20) were carried out with 250,000 cpm of iodinated lysates from U937 cells activated with PMA for 0 days (lanes 1, 6, 11, 16), 1 day (lanes 2, 7, 12, 17), 3 days (lanes 3, 8, 13, 18), 5 days (lanes 4, 9, 14, 19), or 7 days (lanes 5, 10, 15, 20). Immunoprecipitates were reduced and subjected to SDS-7% PAGE and autoradiography.

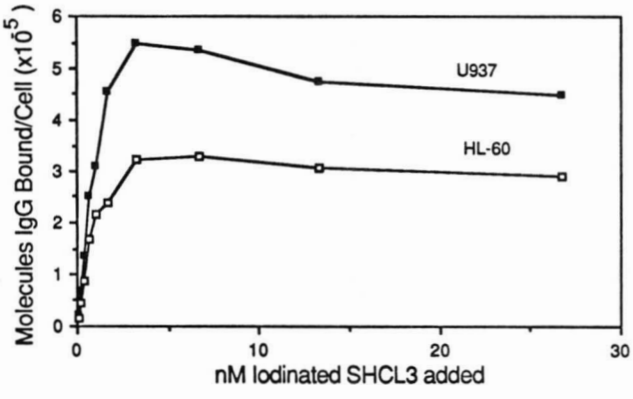

Figure 6. Saturation binding of ^{125}I-anti-p150,95 MAb to PMA-differentiated U937 and HL-60 cells. U937 (■) or HL-60 (□) cells (1×10^5) stimulated for 3 days with PMA were incubated with increasing concentrations of ^{125}I-SHCL3 IgG, as described in *Materials and Methods*. A 100-fold excess of unlabeled p150,95 MAb was added to duplicate wells to determine nonspecific binding (≤8.2% of the total counts bound) and subtracted from total counts bound to obtain specific binding. The data shown are the average of six determinations.

cell, and 3×10^5 binding sites per PMA-induced HL-60 cell. The K_d was approximately 6×10^{-10} M^{-1} as estimated by Scatchard analysis.

GM-CSF. Human granulocyte/macrophage colony stimulating factor (GM-CSF) has been found to stimulate formation of mixed granulocyte/macrophage colonies from myeloid precursor cells (49). We tested the ability of GM-CSF to stimulate U937 and HL-60 cell maturation.

The kinetics of GM-CSF induction of Mac-1 and p150,95 expression were examined on both U937 and HL-60 cells with a 1/500 dilution of GM-CSF (24 times greater than saturating GM-CSF colony-forming activity; Steven Clark, personal communication) (Fig. 7*A* and *B*, Fig. 4*D*). GM-CSF reduced proliferation by 75% and caused some aggregation of U937 cells, but had little

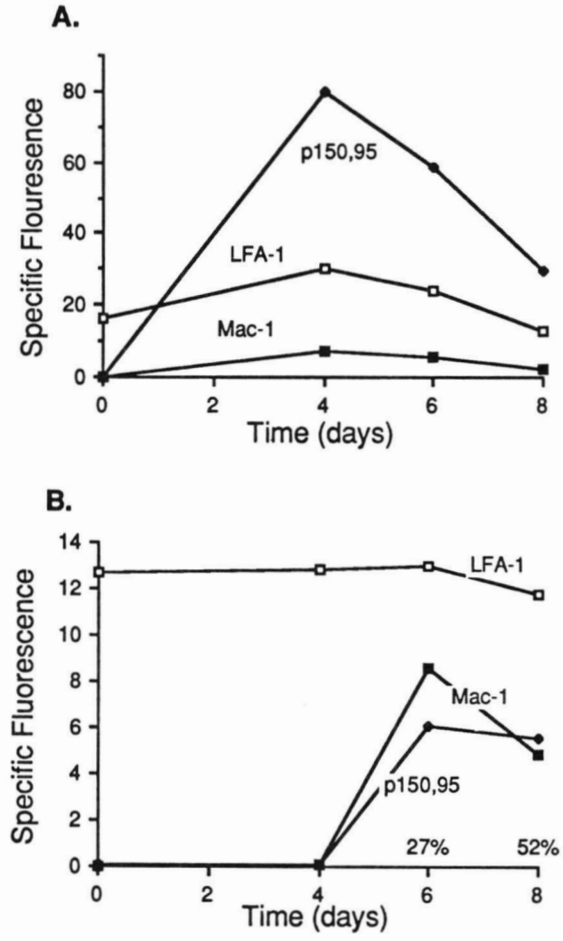

Figure 7. Effect of recombinant GM-CSF on U937 and HL-60 expression of Mac-1, LFA-1 and p150,95. U937 (*A*) or HL-60 cells (*B*) (5×10^5/ml) were incubated with a 1/500 dilution of a COS cell supernatant containing recombinant human GM-CSF for the indicated number of days. Cells were incubated with anti-p150,95 MAb (◆), LM2/1 anti-Mac-1 MAb (■), or anti-LFA-1 MAb (□), and specific fluorescence was determined. The percentage of HL-60 cells positive for p150,95 and Mac-1 was identical and is shown at the bottom of the graph. One hundred percent of the HL-60 cells were positive for LFA-1 on all days tested. Mock-transfected COS cell supernatants had no effect on antigen expression (not shown).

visible effect on HL-60 cells. On U937 cells GM-CSF induced much more p150,95 than Mac-1 expression, and the two antigens were induced with similar kinetics. The major portion of that increase occurred between days 1 and 2 (data not shown). HL-60 cells responded quite differently. Mac-1 and p150,95 expression began to increase only after day 4. The effect of GM-CSF was not uniform on the entire population of HL-60 cells. On day 6 only 27% of the cells were expressing Mac-1 and p150,95, but by day 8 approximately half were positive for these two cell surface differentiation antigens.

The dose response of U937 cells at day 4 was tested with various dilutions of recombinant GM-CSF. GM-CSF had different effects on Mac-1 and p150,95 expression. As the concentration of GM-CSF increased, the percentage of Mac-1$^+$ cells increased (Fig. 8, *bottom*), but the amount of antigen per Mac-1$^+$ cell did not change (Fig. 8, *top*). In contrast, p150,95 was moderately induced on a small percentage of cells by low concentrations of GM-CSF, but expressed to a higher level on larger percentages of cells with increased concentrations of GM-CSF. Concentrations of GM-CSF higher than those shown did not

further increase p150,95 expression. GM-CSF did not affect LFA-1 expression on U937 cells.

Retinoic acid. Differentiation along the granulocytic pathway was studied by incubation of HL-60 cells with retinoic acid (Fig. 4*F* and Fig. 9). Proliferation of HL-60 cells was greatly reduced by retinoic acid; they doubled once during the first 4 days of incubation, then were quiescent. Morphologically, they failed to become adherent and were smaller than their uninduced counterparts. When examined by flow cytofluorometry, Mac-1 expression increased the most, p150,95 increased slightly, and LFA-1 declined by almost two-thirds. The phenotype on day 8 resembled that of freshly isolated, unstimulated granulocytes, which have less LFA-1 and p150,95 than Mac-1 on their surfaces. For comparison, the amount of these antigens expressed on unstimulated granulocytes is shown in Figure 9.

Interferons. IFN-γ has been shown to stimulate antigen expression on monocytic cell lines (50). IFN-γ (1000 U/ml) induced little or no increase in aggregation or adherence of U937 and HL-60 cells. IFN-γ caused from 11 to 15% of U937 cells to become positive for both Mac-1 and p150,95 (Fig. 4*C*). The same concentration of IFN-β had no effect on U937 cell expression of Mac-1, p150,95 and LFA-1. Incubation of HL-60 with either IFN-β or IFN-γ for 7 days caused only a small increase in Mac-1 and p150,95 expression. LFA-1 expression on HL-60 was unaffected by both agents (data not shown).

Lack of latent Mac-1 and p150,95 pools in differentiated myelomonocytic cell lines. One of the responses of mature granulocytes and monocytes to fMLP stimulation is a rapid increase in the amount of some cell surface molecules, including Mac-1 and p150,95 (Table II) (1, 8, 9, 17). This appears to be due to the translocation of Mac-1 and p150,95 stored in secondary granules to the cell surface (8). Neither Mac-1 nor LFA-1 nor p150,95 were upregulated when PMA-differentiated U937 or HL-60 cells were stimulated with fMLP (not shown). In another experiment, U937 or HL-60 cells were cultured with GM-CSF for 8 days. Some cells were then incubated with or without fMLP, other cells were fixed with paraformaldehyde-glutaraldehyde, and another group was fixed and detergent permeabilized with 0.1% TX-100 to access any internal preformed pools. Subsequent flow cytofluorometry showed that Mac-1, p150,95, and LFA-1 levels were unaffected by fMLP stimulation, and detergent-accessible pools were absent (not shown). HL-60 cells induced to differentiate with retinoic acid for 8 days were also tested, with similar negative results. Granulocytes treated in parallel contained detergent-accessible pools of Mac-1 and p150,95 that were fMLP responsive (not shown). Taken together, these data indicate that Mac-1, LFA-1, and p150,95 are not upregulatable in differentiated HL-60 or U937 cells, and no detectable internal pools of these molecules existed in the differentiated cells.

The role of Mac-1, LFA-1, and p150,95 in PMA-differentiated U937 cell aggregation. The ability of subunit-specific MAb to reproduce the defects in cellular adhesion seen with Mac-1/LFA-1-deficient patients' cells has been reported (6, 7, 51). Anti-Mac-1 MAb have been shown to inhibit PMA or fMLP-stimulated granulocyte aggregation (6), and anti-LFA-1 MAb have been shown to inhibit PMA-stimulated lymphoblastoid cell line aggregation (13,

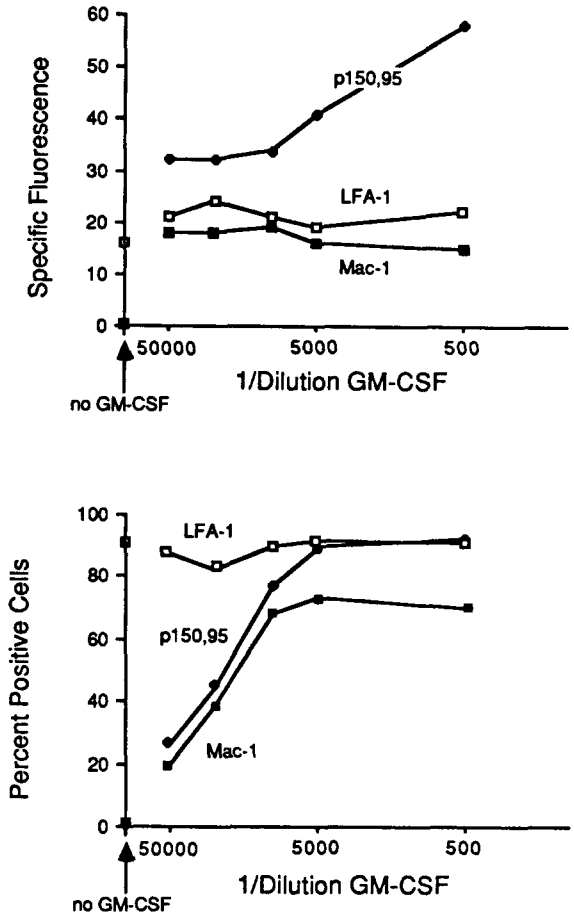

Figure 8. Dose response of U937 cells to culture with various dilutions of GM-CSF. U937 cells (5×10^5/ml) were incubated for 4 days with the indicated dilutions of GM-CSF cos cell supernatant, or none. The cells were then incubated with either LM2/1 anti-Mac-1 MAb (■), anti-p150,95 (◆), anti-LFA-1 MAb (□) or control MAb. After washing and staining with FITC-conjugated goat anti-mouse IgG(H+L), the cells were subjected to flow cytofluorometry. *Top*, Specific fluorescence. *Bottom*, percent positive cells.

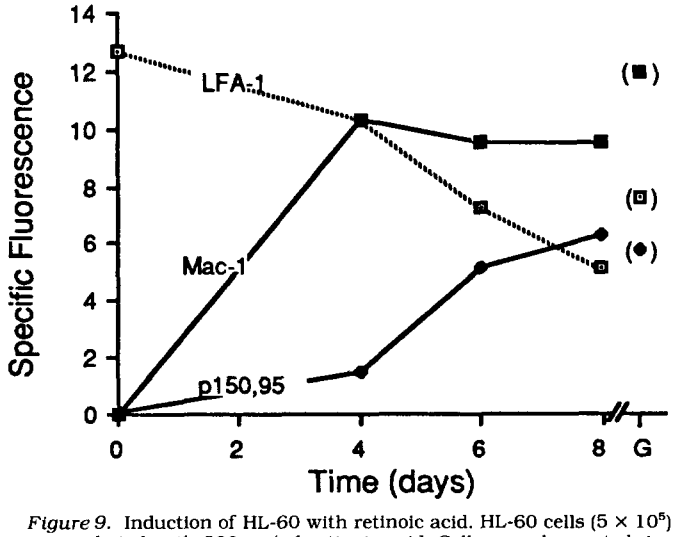

Figure 9. Induction of HL-60 with retinoic acid. HL-60 cells (5×10^5) were incubated with 300 ng/ml retinoic acid. Cells were harvested, incubated with LM2/1 anti-Mac-1 MAb (■), anti-p150,95 MAb (◆), or anti-LFA-1 MAb (--□--), then with FITC-goat anti-mouse IgG(H+L), and analyzed by flow cytofluorometry. Purified unstimulated granulocytes were also subjected to flow cytofluorometric analysis. Their fluorescence values are represented in parentheses.

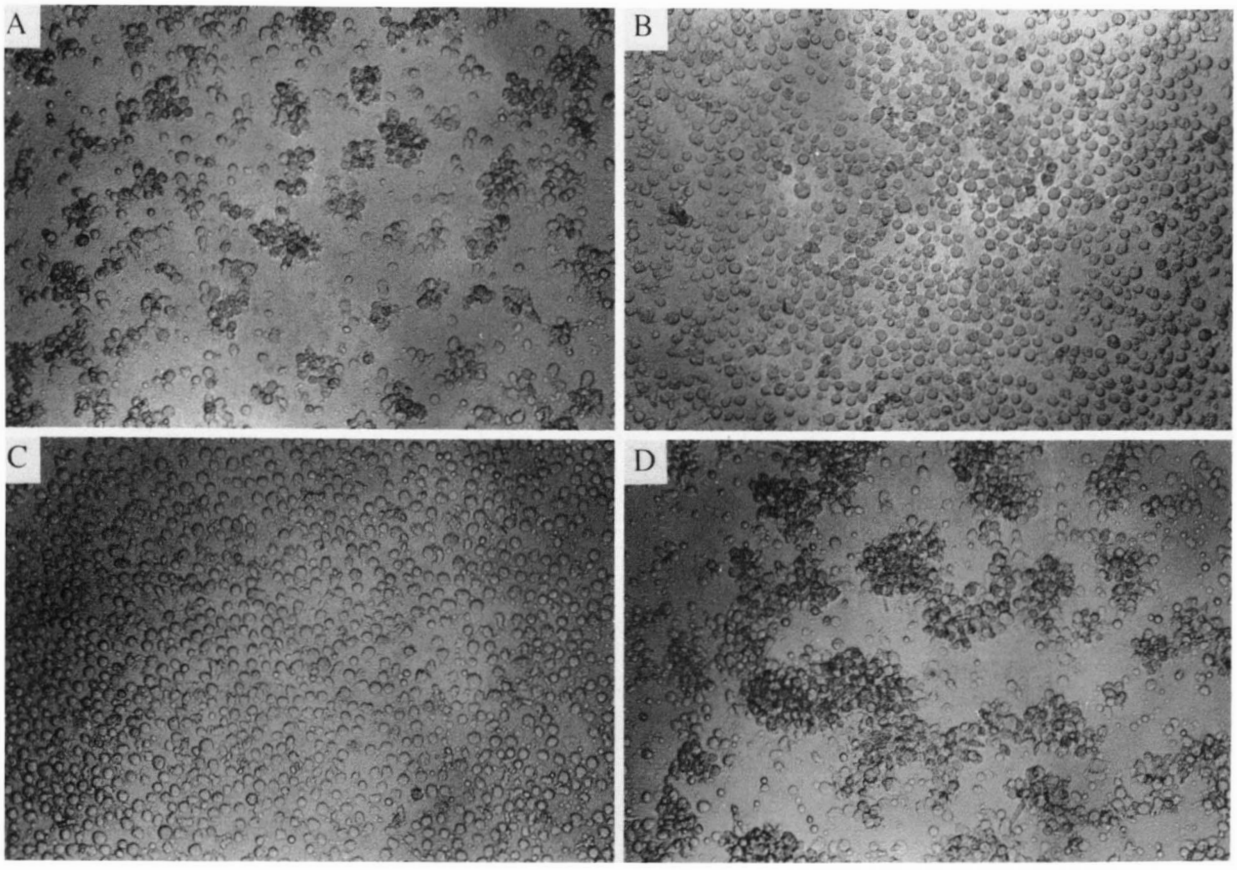

Figure 10. Effect of EDTA or MAb incubation on PMA-differentiated U937. U937 cells were activated for 3 days with 2 ng/ml PMA and dispersed to single cells by vortexing. Cells (4×10^5 in 25 μl HBSS/HEPES) were added to 50 μl HBSS/HEPES (*A*), HBSS/HEPES/5 mM EDTA (*B*), TS1/18 anti-β MAb culture supernatant (*C*), or LM2/1 anti-αM MAb culture supernatant (*D*), incubated for 1 hr at 37°C in a Labtech 8-well slide, and photographed with an inverted microscope.

TABLE III

Inhibition of U937 cell homotypic aggregation with MAb and comparison to the amount of MAb bound

Assay	Addition to Incubation Mixture							
	TS1/18 IgG/F(ab′)$_2$	TS1/22 IgG/Fab′	SHCL3 IgG/Fab	LM2/1 IgG/Fab′	OKM1 IgG	W632 IgG	X63 IgG	EDTA
Aggreg.[a]	−/−	+/+	++++/++++	++++/++++	++++	++++	+++	−
Spec. Fl.[b]	110/ND[c]	55/ND	93/ND	42/ND	38	250	0	ND

[a] Aggregation studies were performed in flat-bottom 96-well plates. One hundred microliters of 20 μg/ml IgG, Fab, Fab′, or F(ab′)$_2$ in HBSS/10 mM HEPES, or HBSS/10 mM HEPES/EDTA were added to 2×10^5 PMA differentiated U937 cells. After 1 hr at 37°C, the plate was scored as follows: −, single cells; +, mostly single cells, a few doublets and small groups; ++, mixture of single cells and small clusters; +++, mostly clusters (and equivalent to the buffer only control); ++++, large clusters.

[b] Specific fluorescence was determined as described *Materials and Methods*.

[c] ND = not determined.

14, 16). Because PMA-stimulated U937 cells express all three members of this adhesion protein family, we studied their individual contributions to the aggregation of this cell line. U937 cells stimulated with PMA for 3 days were dispersed, washed, and then incubated with or without MAb or EDTA. In the absence of any additions, aggregates formed within 1 hr (Fig. 10*A*). Divalent cations are necessary for this aggregation, as EDTA abolishes the phenomenon (Fig. 10*B*, Table III). Anti-β subunit MAb completely inhibited the aggregation (Fig. 10*C*, Table III). MAb specific for LFA-1 also inhibited clustering (Table III). In contrast, MAb specific for p150,95 and two MAb specific for Mac-1 did not inhibit aggregation (Fig. 10*D*, Table III). These results were confirmed with Fab, Fab′, or F(ab′)$_2$ fragments. There was no correlation between relative antigen density on the cell surface, as measured by immunofluorescence, and inhibitory effects (Table III). MAb to HLA and p150,95, which were both noninhibitory, bound to a higher number of sites per cell than LFA-1 MAb, which were inhibitory. The Third International Leukocyte Workshop myeloid (146 MAb), activation (35 MAb), non-lineage (65 MAb), and thymic epithelium (19 MAb) panels were also screened (1/100, 1/200, or 1/1000 dilutions of each sample) for inhibition of PMA-induced U937 aggregation. No Mac-1 (one tested) or p150,95 (eight tested) MAb inhibited aggregation. Four of four anti-LFA-1 MAb and two of four β-subunit-specific MAb inhibited PMA-induced U937 aggregation (52). The specificity of these MAb was established independently in immunoprecipitation experiments (53). These results suggest that

LFA-1, Mac-1, and p150,95 are not equivalent in their ability to mediate U937 cell aggregation, and that LFA-1 plays a selective role in this process.

DISCUSSION

In contrast to previous studies on the individual Mac-1, LFA-1, and p150,95 antigens (7, 8, 11, 14, 17, 20–25, 27, 29, 30, 42, 43, 51), we have carried out extensive comparative studies on all three antigens. Our findings also extend knowledge about the distribution and inducibility of the individual antigens, particularly p150,95.

We first studied the distribution of these antigens on normal leukocytes and representative lymphoid and myeloid cell lines and leukemias. These results confirm that LFA-1 has the broadest distribution of these three antigens and is present on cells of the lymphocytic, granulocytic, and monocytic series. It is also expressed in small amounts on the K562 line, a preerythroid cell line. Mac-1 is present on granulocytes, monocytes, and on differentiated myeloid cell lines, but not on myeloid precursor cells, in agreement with previous studies (25, 26).

Previous studies have shown p150,95 to be absent from blood B and T lymphocytes and present in low amounts on populations enriched for large granular lymphocytes (27, 39), but we found an interesting pattern of expression on cell lines. In small amounts, p150,95 is present on several B cell lines, as shown by immunofluorescence and immunoprecipitation, and is absent from other B cell lines and two B-CLL. It is strongly expressed on hairy cell leukemia, a B cell neoplasm. PMA stimulation of B-CLL also leads to strong expression of p150,95, in agreement with a recent report (54). Although p150,95 is not expressed on peripheral T lymphocytes, it is clearly expressed on CTL clones. These findings show that p150,95 can be expressed on certain types of activated or transformed B and T lymphocytes. Solubilized p150,95 has been shown to bind iC3b (10). Our findings thus predict that induction of p150,95 expression on activated B and T lymphocytes may result in acquisition of complement receptors by these cells.

We next studied the modulation of Mac-1, LFA-1, and p150,95 expression during differentiation in vitro of myeloid cell lines. Our aim was to compare directly the regulation of these three surface antigens. We also extended previous studies on the induction of Mac-1 on myeloid cell lines (42, 43, 55–57) and of p150,95 on PMA-treated U937 cells (2, 27). Mac-1 has been detected in normal bone marrow on myelocytes, metamyelocytes, band cells, peritoneal macrophages, monocytes, granulocytes, and null cells (25), and murine LFA-1 is found on stem cells of almost all hematopoietic lineages (26). We found that when U937 or HL-60 cells are differentiated along either monocytic or granulocytic pathways they change from LFA-1$^+$, Mac-1$^-$, p150,95$^-$ to LFA-1$^+$, Mac-1$^+$, p150,95$^+$. Mac-1 and p150,95 are always induced with similar kinetics. However, the ratio of p150,95 to Mac-1 varies.

Induction of U937 differentiation along the monocytic pathway with PMA results in expression of about fourfold more p150,95 than Mac-1. This high expression of p150,95 compared with Mac-1 resembles that of tissue macrophages (27, 28). PMA-stimulated maturation of HL-60 cells results in the inverse phenotype: Mac-1 is twofold more strongly expressed than p150,95. PMA has been reported to induce monocytic differentiation in both cell types, as shown by nonspecific esterase, lysozyme, and surface antigens (32, 33, 58). The resultant phenotypes of PMA-stimulated U937 and HL-60 cells may reflect differences in the initial developmental states of these cell lines. U937 cells can only be differentiated along the monocytic pathway, whereas the HL-60 cell line can be differentiated along both the monocytic and the granulocytic pathways.

Retinoic acid has been shown to initiate HL-60 cell maturation along the granulocytic differentiation pathway (48). We found that retinoic acid induces both Mac-1 and p150,95 expression, but only after a lag period in which cell division slows. In this case LFA-1 expression diminishes, resulting in a Mac-1, LFA-1, and p150,95 expression profile reminiscent of peripheral blood granulocytes.

GM-CSF stimulates maturation of monocytes and granulocytes from myelomonocytic precursor cells (35, 49, 59). As a more physiologic means of stimulating U937 and HL-60 cell maturation, we tested the ability of GM-CSF to induce Mac-1 and p150,95 expression. On U937 cells, GM-CSF induced a higher level of expression of p150,95 than of Mac-1, resembling the phenotype after PMA induction. On HL-60 cells, GM-CSF induces lower levels of Mac-1 and p150,95 expression than PMA and does not increase LFA-1. In this regard, GM-CSF resembles retinoic acid in its effect on HL-60 cells. IFN-γ induces moderate levels of Mac-1 and p150,95 on a subpopulation of U937 cells and has no effect on LFA-1 expression. IFN-γ and IFN-γ-containing lymphokine preparations have been shown to induce Mac-1 on U937 cells (42, 43). IFN-β had no effect on Mac-1 or p150,95 expression on U937 cells.

Peripheral blood granulocytes and monocytes contain intracellular pools of Mac-1 and p150,95 that can be mobilized to the cell surface with chemoattractants such as fMLP or by activation with PMA (1, 6, 8, 17). Our studies on fMLP stimulation and detergent permeabilization of differentiated U937 and HL-60 cells indicate that these cells are not equivalent to their peripheral blood counterparts, since they lack such intracellular pools (not shown).

We can draw the following conclusions about the regulation of LFA-1, Mac-1, and p150,95 gene expression during leukocyte differentiation. The LFA-1 antigen appears to be expressed on all leukocyte lineages, and LFA-1 expression precedes that of Mac-1 and p150,95 in myeloid differentiation. LFA-1 is expressed on all circulating leukocytes and remains after induction of differentiation in all the myeloid cell lines examined here. Work in the mouse has shown that LFA-1$^+$ circulating monocytes, after emigration into tissues, give rise to cells of the mononuclear phagocyte series, many of which are LFA-1$^-$ (60, 61). Activation of macrophages with IFN-γ or LPS leads to reexpressison of LFA-1 (62).

Differentiation of myeloid precursor cells along the monocytic or granulocytic pathways results in coordinate expression of Mac-1 and p150,95. In contrast, p150,95, but not Mac-1, is expressed by some activated B and T lymphocytes. We speculate that myeloid specific regulatory sequences are located near the promoters of the p150,95 and Mac-1 α subunit genes, and there is an

additional sequence near the p150,95 α subunit gene for controlling expression during lymphoid cell activation. At least one member of this glycoprotein family is expressed on all leukocyte cell types examined to date; thus the β subunit gene must be universally expressed in leukocyte differentiation.

In the mouse, Mac-1 and LFA-1 α subunit mRNA have been assessed by translation in vitro, and the presence of Mac-1 α subunit mRNA has additionally been probed with a cloned DNA (63, 64). In the limited number of cell lines thus far examined, Mac-1 mRNA and LFA-1 α subunit mRNA is found only in cells that express the relevant $\alpha\beta$ complex on the cell surface. Differentiation of the murine myeloid cell line M1 stimulated by IFN-γ led to the induction of Mac-1 α subunit mRNA, as shown by both DNA probe hybridization and translation in vitro. Cloning of the human Mac-1, p150,95, and LFA-1 α subunit genes is currently in progress. The studies presented here provide a framework for investigations on the transcriptional control of these genes with DNA probes.

Long-term, but not short-term, PMA induction of U937 cells induces a change in the adherence properties of the cell. They adhere to one another (homotypic aggregation) and also adhere to glass and plastic substrates. In granulocytes, PMA or chemoattractants stimulate a fivefold increase in Mac-1 surface expression and also stimulate aggregation (5, 9). Aggregation is Mac-1 dependent, since it is inhibited by anti-β subunit MAb and anti-Mac-1 α subunit MAb (6). Homotypic aggregation by several lymphoid cell types is LFA-1 dependent (14). Because U937 cells express all three members of the Mac-1, LFA-1, p150,95 glycoprotein family, we assessed the contribution of each of these molecules to homotypic aggregation. The relative abundance of these antigens on the cell surface was p150,95 > LFA-1 > Mac-1. Anti-LFA-1 MAb, but not numerous anti-Mac-1 and anti-p150,95, were found to inhibit U937 cell aggregation. The LM2/1 anti-Mac-1 MAb used here has been shown to inhibit inflammatory mediator-stimulted granulocyte aggregation by 88%, and the anti-p150,95 and Mac-1 MAb have been shown to inhibit granulocyte adherence to substrates (6). Our findings suggest that Mac-1, LFA-1, and p150,95 are not equivalent in their ability to mediate adhesiveness of U937 cells. Whether this reflects differences in the adhesive specificity of these glycoproteins, differences in their clustering on the cell surface, or the expression of a ligand for LFA-1 on U937 cells and a different ligand for Mac-1 on granulocytes has not yet been determined. It is of interest that differentiation of U937 cells with PMA induces expression of the intercellular adhesion-1 molecule (ICAM-1), which may be a ligand for LFA-1 (65, 66). Whatever the explanation, these findings show that there are important functional differences between LFA-1, Mac-1, and p150,95 in their ability to mediate homotypic adhesion by different cell types.

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