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EXPRESSION AND INDUCTION *IN VITRO* OF MACROPHAGE DIFFERENTIATION ANTIGENS ON MURINE CELL LINES[1]

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Macrophage antigens were studied on 12 macrophage cell lines and variant clones, as well as on 11 other lines representative of a variety of hematopoietic lineages, to determine the relation of surface antigen expression to cell differentiation, maturation, and function. Biosynthetic labeling followed by immunoprecipitation with monoclonal antibodies and gel electrophoresis showed that Mac-1 polypeptides of 95,000 and 170,000 M_r and the Mac-2 polypeptide of 32,000 M_r were found in lysates of mature macrophage lines but not in other lines, including myeloid or immature leukemias. The Mac-3 antigen was found in large amounts in all macrophage lines and to lesser degrees in some myeloid and B lymphoid lines. The M_r of Mac-3 varied from 100,000 to 170,000, perhaps due to differential glycosylation. Analysis of Mac-1 and Mac-2 antigens by flow cytometry showed expression on all macrophage lines. Similarly, all three Mac antigens were detected in high amounts on macrophage lines by ^{125}I-labeled antibody binding. Mac-1 and Mac-2 were not routinely seen on other hematopoietic lines, but Mac-3 was expressed in variably low amounts on some lines of myeloid and B cell lineage. These results with macrophage lines and variants exclude a simple relationship between Mac differentiation antigens and certain macrophage characteristics (5′ nucleotidase, cytotoxic capacities, antibody-dependent phagocytosis, and production of oxygen radicals). Mac-1 and Mac-3 but not Mac-2 could be induced in the M1 myeloblast line by corticosteroid, lipopolysaccharide, and several conditioned media containing myeloid colony-stimulating activity. These agents are known to induce a number of other macrophage markers in M1, e.g., Fc and C receptors, phagocytosis, and lysozome production. Therefore, it appears Mac-2 antigen is not required for expression of these characteristics. The levels of Mac-1 and Mac-3 in M1 cells increased slowly over 6 days of stimulation, approaching those found on mature macrophage lines. Although anti-Mac-1 does not block the detection of Mac-3 antigen on induced M1 cells, the presence of anti-Mac-1 antibody during induction partially blocked the appearance of Mac-3 antigen. Thus, the surface structure associated with Mac-1 antigen appears to be involved in differentiation of macrophages.

Differentiation is a process involving the coordinated control of many genes to produce different cell types. Some of these changes affect the cell surface. Antigens that are expressed on some but not all cells have been termed differentiation antigens (1). Differentiation antigens may be restricted to a particular cell lineage, to a stage of differentiation, or in a fashion related to neither of these (2). We previously described hybridoma rat anti-mouse cell surface antibodies (MAb)[3] that recognize antigenic structures on macrophages. The Mac-1 antigen of molecular mass (M_r) 170,000 and 95,000 is present on granulocytes, macrophages, and natural killer cells (3–6). It recently was found to be identical to or associated with the function of the complement (C) receptor type three (reviewed in Reference 7).[4] Mac-2 antigen of 32,000 M_r (8) and the 110,000 M_r Mac-3 antigen (Reference 9)[5] have been detected on mononuclear phagocytes, but not on lymphocytes.[6] Differentiation from blood monocyte to thioglycollate-induced peritoneal macrophage is accompanied by a large increase in the expression of Mac-1 and Mac-2 in parallel with a large decrease in expression of a heat-stable antigen found on a number of hematopoietic cell types (10).

Murine macrophage-related cell lines have been described with different degrees of maturation or functional capacity (11–13). Another model for studying differentiation is provided by the myeloblast line M1, which can be induced to express many macrophage characteristics (14). The experiments presented here were designed to investigate the presence of macrophage antigens on a variety of cell lines with different functional phenotypes and on the uninduced and induced myeloblast M1 line.

MATERIALS AND METHODS

Hybridoma lines. M1/70, M3/38, and M3/84 are subcloned rat spleen cell × NSI hybridoma lines that secrete IgG2b anti-Mac-1, IgG2a anti-Mac-2, and IgG1 anti-Mac-3 MAb (3, 15), respectively. M1/70 and M3/84 are spontaneous HL variants secreting only specific heavy and light chains, whereas M3/38 HL variants were selected after agar cloning as described (8). MAb used in Table III have been described (2, 7, 16). Cells are usually maintained in DMEM (GIBCO, Grand Island, NY) supplemented with 10% horse serum or fetal calf-serum (FCS).

Purification of MAb and coupling to Sepharose. Purified MAb were prepared from culture supernatants by ammonium sulfate precipitation and DEAE and Sephadex G-200 chromatography (6). Pure MAb and normal rat IgG (Miles Laboratories, Elkhart, IN) were coupled to CNBr-activated Sepharose CL-4B (Pharmacia, Piscataway, NJ) at 1 to 2 mg protein/ml of wet

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[3] Abbreviations used: M_r, relative molecular mass; MAb, monoclonal antibody; CM, conditioned medium; PEC, peritoneal exudate cells; PBS, 0.01 M $NaPO_4$, 0.14 NaCl, pH 7.3; PMSF, phenylmethylsulfonyl fluoride; EDTA, ethylenediaminetetraacetic acid; FITC, fluorescein isothiocyanate; HEPES, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid.
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gel as described (4).

Cells. Thioglycollate-elicited peritoneal exudate cells (PEC) were obtained by peritoneal lavage of C57BL/6 mice (The Jackson Laboratory, Bar Harbor, ME), that had been injected with 1.5 ml Brewer's thioglycollate broth (Difco Laboratories, Detroit, MI) per mouse 4 days previously. Lymphoma cells YAC (17) and EL-4 (18), myeloid leukemia line C1498.3 (18), mastocytoma P815, and monocyte/macrophage-like lines (reviewed in Reference 12) P388D_1, WEHI-3A, RAW 264.7, PU5-1R, and J774 were obtained from the Salk Cell Distribution Center. Dr. P. Edelson kindly supplied E2 and E8 (both of which are subclones of P388D_1), WEHI-3, and the 5′-nucleotidase-positive and -negative clones of J774 (J774N$^+$ and J774N$^-$, respectively) (19). WEHI-3A and WEHI-3 are not known to be different but have been maintained in different laboratories for 7 yr. We described previously macrophage-like lines PU5-1.8, 426C, J774.16C3C, RAW 264.10, and myeloblast M1 (12, 13), thymoma line R1 (18), B lymphoma lines 18-8 and WEHI-231 (20), myeloma X63 (8), and erythroleukemia GM-86 (21). RAW264.10 is very similar to RAW 264.7. Myeloblast line RFM was obtained from J. Greenberger (22) and macrophage line NCTC1469 was acquired from H. van Lovern (23). A2 is a macrophage line derived from long-term marrow culture of A/J bone marrow cells (12). Cell lines were grown in DMEM supplemented with 10% horse serum or RPMI 1640 medium with 10% FCS. M1 cells were induced by culturing at 3 × 10^5/ml in RPMI 1640 medium plus 10% FCS with WEHI-3 CM (12), lymphokine (supernatant of *Mycobacterium* BCG-primed spleen cells stimulated with tuberculin PPD, Reference 13), dexamethasone (Sigma Chemical Co., St. Louis, MO), or lipopolysaccharide (LPS, *S. typhosa* W0901, Difco). Fc and C receptors were detected on 20 to 50% of induced cells compared to 0 to 4% on control M1 cells, assayed by EA and EAC rosettes, respectively (24).

Biosynthetic labeling and immunoprecipitation. PEC or adherent tumor cells were plated on T25 tissue culture flasks (Costar, Cambridge, MA) and were incubated overnight at 37°C. Nonadherent cells were washed off and adherent cells were labeled with 200 μCi L-^{35}S-methionine (New England Nuclear, Boston, MA) in 1 ml of methionine-free DMEM supplemented with 10% dialyzed FCS for 6 hr. Nonadherent tumor cells were washed, suspended to 10^7/ml, and labeled similarly. After labeling, the cells were washed 3× in phosphate buffered saline (PBS) and were solubilized in 0.01 M Tris-HCl, pH 8.0, 0.14 M NaCl, 1% Triton X-100, 1% bovine hemoglobin, 1 mM phenylmethylsulfonyl fluoride (PMSF), and 2 mM iodoacetamide at 4°C. After centrifugation at 100,000 × G for 1 hr, the supernatant was recovered. For immunoprecipitation, 6 to 9 μg of purified MAb coupled to Sepharose CL-4B were incubated with cell lysates in a final volume of 200 to 300 μl. After shaking on a microshaker for 1.5 hr at 4°C, the beads were washed three times with 0.01 M Tris HCl, pH 8, 0.14 M NaCl, 0.1% hemoglobin, once with 0.01 M Tris-HCl, pH 8, 0.14 M NaCl, and once with 0.05 M Tris-HCl, pH 6.8. After the addition of sodium dodecyl sulfate (SDS) sample buffer containing 5% 2-mercaptoethanol, immunoprecipitates were heated in a boiling water bath for 5 min. Samples were then analyzed by SDS-polyacrylamide gel electrophoresis (PAGE) as described (3). To visualize ^{35}S-labeled molecules, gels were impregnated with EN^{3}HANCE (New England Nuclear) before exposure to Kodak SB-5 film.

Iodination of purified MAb. Purified MAb (0.1 mg/ml) were iodinated with 0.5 to 1 mol carrier-free ^{125}I per mole protein using IODO-GEN (Pierce, Rockford, IL) according to Fraker and Speck (25), except the reaction was terminated by transfer to fresh tubes containing 20 μl of 0.4 mg/ml L-tyrosine.

Binding of ^{125}I-labeled MAb to cells. ^{125}I-labeled MAb was stored in a solution containing 1% bovine serum albumin carrier protein and 4 mg/ml human γ-globulin (Miles Laboratories, Elkhart, IN) to block binding by Fc receptors. Cells (10^6 in 0.1 ml PBS) were incubated 60 min at 4°C, with 10 μl ^{125}I-MAb (approximately 70 ng/150,000 cpm). Cells were then washed four times and were assayed for remaining radioactivity. This time period and amount of antibody were shown to give plateau levels of binding to macrophage cell lines and M1 myeloblasts. Incubations without cells showed 100 to 400 cpm, and these backgrounds were subtracted.

Immunofluorescent flow cytometry. Nonadherent tumor cells were recovered from tissue culture flasks, washed 2× with PBS, and resuspended to 2 × 10^7/ml. Tumors growing as adherent monolayers were detached by incubating with PBS + 1 mM EDTA[3] for 15 min at 37°C. They were then processed as for nonadherent cells. Cells (50 μl) were incubated with equal volumes of MAb-containing spent culture supernatant for 30 min, or with control M1/69 HK supernatant or NSI supernatant plus 50 μg/ml normal rat IgG. The cells were washed 3× and resuspended in 50 μl of affinity purified FITC[3]-rabbit $(Fab')_2$ anti-rat IgG absorbed with mouse IgG (diluted to 60 μg/ml in L15 medium; fluorescein/protein, 19 μg/mg) for 30 min. After another three washes, the cells were analyzed on a Becton-Dickinson FACS-II using glutaraldehyde-fixed sheep red cells as standards. All procedures were carried out in L15 medium + 0.5% bovine serum albumin + 10 mM HEPES[3] + 11 mM glucose at 4°C. During the second stage incubation and subsequent washes, 0.01 M sodium azide was included in the medium to prevent patching.

RESULTS

Immunoprecipitation of Mac-1, Mac-2, and Mac-3 from tumor cell lines. Tumor cell lines have been used extensively for the study of macrophage differentiation and function (11–13). To determine whether lines representing cells at different maturational states expressed different macrophage antigens, (^{35}S)-methionine-labeled tumor cell lysates were immunoprecipitated with M1/70, M3/38, and M3/84 MAb. As seen in Figure 1, Mac-1 polypeptides of 170,000 and 95,000 M_r could be detected in lysates of monocytic/macrophage-like lines, such as J774 and its variants (lanes 6–8), RAW264.7 (lane 9), WEHI-3A (lane 10), P388D1 and its subclones (lanes 11–13), WEHI-3 (lane 14), and 426 C (lane 16). T lymphoma lines YAC (lane 1) and EL-4 (lane 2), however, as well as P815 mastocytoma cells (lane 3) were negative. Two lines of myeloid origin, C1498.3 (lane 5) and M1 (lane 15), also failed to synthesize Mac-1. PU5-1R (lane 4) was also Mac-1-negative, and parallel immunoprecipitation using anti-mouse Ig reagents showed large amounts of Ig light chains being synthesized (not shown). Because both lymphoid and macrophage culture lines were derived from mice bearing the Ig$^+$ tumor PU5-1 (26), this line from the Cell Distribution Center was evidently a B lymphoma.

When the synthesis of Mac-2 in this panel of tumor lines was examined, similar results were obtained (Fig. 2*A*). Thus, cells that synthesized Mac-1, such as J774 and its variants (Fig. 2*A*, lanes 6–8), RAW264.7 (lane 9), WEHI-3A (lane 10), P388D_1 and its subclones (lanes 11–13), WEHI-3 (lane 14), and 426 C (lane 17) all synthesized the 32,000 M_r Mac-2 antigen. Another macrophage cell line, PU5-1.8, not examined for Mac-1 synthesis, was also found to be Mac-2$^+$ (lane 15). The M1 myeloid progenitor line (lane 16), T lymphomas YAC and EL-4 (lanes 1 and 2), P815 (lane 3), PU5-1R (lane 4), and C1498.3 (lane 5) failed to show any synthesis of Mac-2.

A slightly different distribution was found for Mac-3 than Mac-1 and Mac-2. All cell lines positive for Mac-1 and Mac-2, including J774 (Fig. 2*C*, lanes 6–8), RAW267.7 (lane 9), WEHI-3A (lane 10), P388D_1 (lanes 11–13), WEHI-3 (lane 14), PU5-

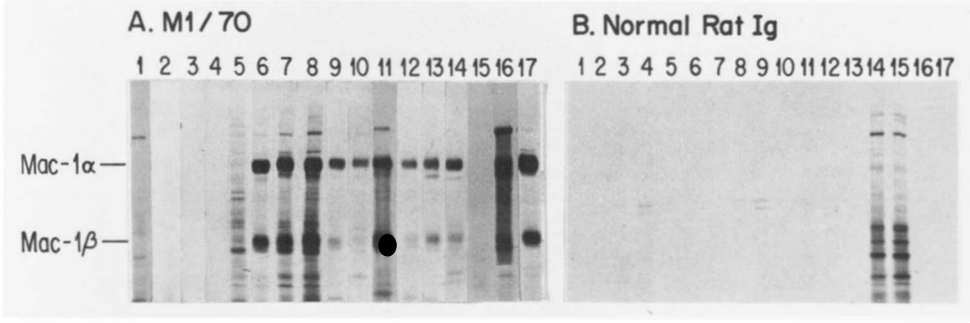

Figure 1. SDS-PAGE of ^{35}S-methionine-labeled Mac-1 from thioglycollate-elicited macrophages and tumor cell lines. Cells were labeled with ^{35}S-methionine as described in *Materials and Methods.* Equal quantities of Triton X-100 lysates (1.8 × 10^6 cpm) were shaken with 8.7 μg M1/70 or 8 μg normal rat IgG coupled to Sepharose CL-4B for 90 min at 4°C. Immunoprecipitates were washed and subjected to SDS-PAGE and fluorography. The lysates used were from the following cell types: lane 1, YAC; lane 2, EL-4; lane 3, P815; lane 4, PU5-1R: lane 5, C1498.3; lane 6, J774; lane 7, J774N$^+$; lane 8, J774N$^-$; lane 9, RAW264.7; lane 10, WEHI-3A; lane 11, P388D_1; lane 12, P388D_1-E2; lane 13, P388D_1-E8; lane 14, WEHI-3; lane 15, M1; lane 16, 424 C: lane 17, thioglycollate-elicited peritoneal macrophages.

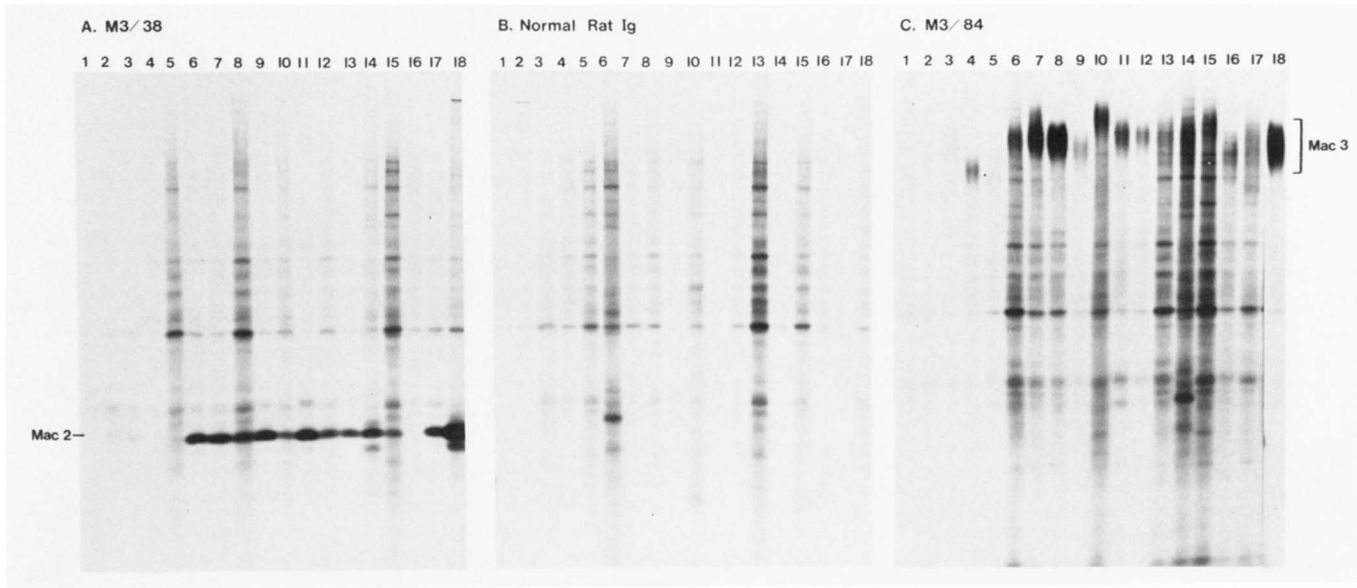

Figure 2. SDS-PAGE of ^{35}S-methionine-labeled Mac-2 and Mac-3 from thioglycolate-elicited macrophages and tumor cell lines. First, 1.8×10^6 cpm of each cell lysate were shaken with Sepharose CL-4B coupled with 6.8 μg of M3/38, 8.5 μg M3/84, or 8 μg of normal rat IgG for 90 min at 4°C. The beads were then washed and immunoprecipitates were eluted and analyzed by SDS-PAGE as described in *Materials and Methods.* The lysates used for immunoprecipitation were from the following cells: lane 1, YAC; lane 2, EL-4; lane 3, P815; lane 4, PU5-1R; lane 5, C1498.3; lane 6, J774; lane 7, J774N$^+$; lane 8, J774N$^-$; lane 9, RAW264.7; lane 10, WEHI-3A; lane 11, P388D$_1$; lane 12, P388D$_1$-E2; lane 13, P388D$_1$-E8; lane 14, WEHI-3; lane 15, PU5-1.8; lane 16, M1; lane 17, 426 C; lane 18, thioglycollate-elicited peritoneal macrophages.

1.8 (lane 15), and 426 C (lane 17) also synthesized Mac-3; however, P815 (lane 3), PU5-1R (lane 4), and M1 (lane 16) were Mac-3$^+$ even though no Mac-1 or Mac-2 could be precipitated from these three lines. No Mac-3 polypeptides were detected in lysates of YAC (lane 1), EL-4 (lane 2), and C1498.3 (lane 5). The expression of Mac-3, but not the other two antigens by M1, a poorly differentiated myeloid line, suggests the appearance of Mac-3 in myeloid differentiation precedes that of Mac-1 and Mac-2.

The M_r of Mac-3 antigens precipitated from various cell lines ranged from 100,000 (for PU5-IR) to 170,000 (for WEHI-3A). Similar variations in the M_r of Mac-3 have been noted in peritoneal macrophages elicited by different agents (9). These variations may result from differential glycosylation of Mac-3 in different cell types.

Immunofluorescent flow cytometry analysis of Mac-1 and Mac-2 on tumor cells. To determine the expression of Mac-1 and Mac-2 on cell surfaces, tumor cells were stained by M1/70 or M3/38, followed by fluorescein-conjugated F(ab′)$_2$ fragments of rabbit anti-rat IgG. Cells were then analyzed by flow cytometry and a part of the results are shown in Figure 3. Variants of P388D$_1$, E2 (not shown), and E8 (Fig. 3*a* and *b*), RAW264.7 (Fig. 3*c* and *d*), 426 C (Fig. 3*e* and *f*), PU5-1.8 (Fig. 3*k* and *l*), WEHI-3A (Fig. 3*m* and *n*), and J774 (not shown) all expressed Mac-1 and Mac-2 on their surfaces. In contrast, C1498.3 (Fig. 3*o* and *p*) was negative for both antigens. Data obtained by flow cytometry agree well with those from immunoprecipitation experiments, with two exceptions. Low levels of Mac-1, but not Mac-2, were found on the surface of M1 cells, whereas small amounts of Mac-2 but not Mac-1 were detected on P815. Neither antigen could be precipitated from these cells, which could be due to low rates of synthesis by the lines or to variable expression of these antigens during culture. Based on binding assays (see below), the expression of Mac-1 on M1 cells varies from undetectable to low amounts of antigen.

Screening of cell lines by ^{125}I-MAb binding. For screening purposes, a labeled antibody binding assay was used to detect differentiation antigens on cell lines. Table I shows that Mac-1, Mac-2, and Mac-3 antigens were all found on six macrophage-related cell lines. These lines included PU5-1.8 and RAW264.10, highly active, and J774.1, moderately active in antibody-dependent and nonspecifically induced cytolysis of

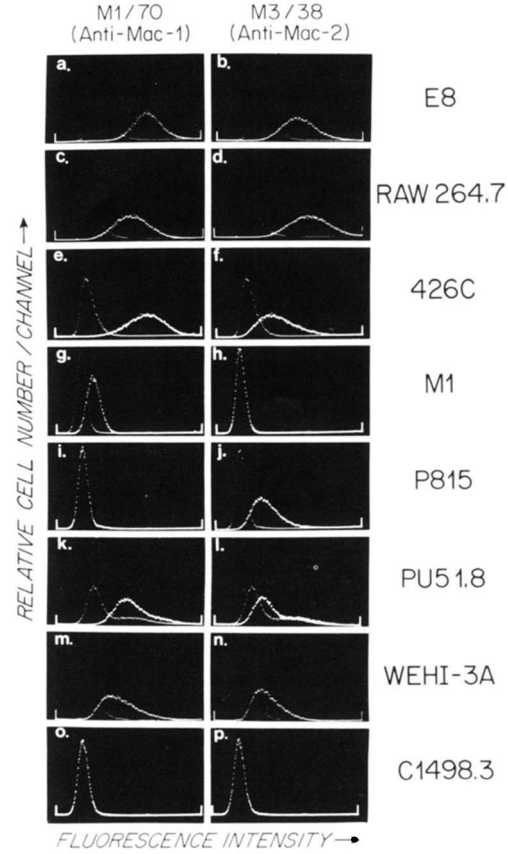

Figure 3. Immunofluorescent flow cytometry analysis of tumor cells. Tumor cells were recovered from tissue culture flasks and were labeled with M1/70 or M3/38 (dark curves) as described in *Materials and Methods.* Controls were stained with NSI supernatants plus 50 μg/ml rat IgG (light curves). The scatter gates were set to exclude dead cells. The histograms shown are plotted with the relative cell number on a linear scale and fluorescence on a logarithmic scale.

tumor targets (13), clone J774.16C3C, deficient in hydrogen peroxide and superoxide anion production (27), and WEHI-3, maintaining lysozyme production and nonspecific phagocytosis but lacking macrophage properties of immune phagocytosis and various cytotoxic reactions (13). Myeloblast lines M1 and RFM had no or variably low expression of Mac-1 and Mac-3 antigens and lacked Mac-2, as seen by immunofluorescence. Erythroleukemia line GM-86 and B cell and myeloma lines had a low amount of surface Mac-3.

Induction of Mac-1 and Mac-3 expression on myeloblast leukemia M1 cells. The M1 line has been extensively used as a model for the induction of granulocyte/macrophage differentiation (14). Table II shows that incubation of M1 cells with a variety of inducing agents greatly increased the expression of Mac-1 and Mac-3 antigens. Mac-2 antigen was never observed on M1 in these experiments. As a control, line GM-86, which is inducible for erythroid properties, was treated with the same agents. Mac-1 and Mac-2 antigens were not detected; Mac-3 expression on GM-86 was stimulated slightly (less than three-fold) but remained low (not shown).

The expression of macrophage antigens on M1 cells was apparent by day 2 of culture with inducing agents and increased up to 6 days of treatment (Fig. 4), approaching the levels found on the mature macrophage cell lines. Mac-1 and Mac-3 antigens were attained by the induced cells in parallel with induction of C receptors (Table II), Fc receptors, and latex bead phagocytosis (not shown).

Inhibition of antigen induction in M1 cells by antibody to Mac-1. Because the three Mac antigens are present on mature phagocytic cells but not in appreciable amounts on immature cells, we tested if the antigens had any role in the process of differentiation of these cell types. M1 cells were incubated with inducing agents in the presence of antibody to Mac-1 or Mac-3. Table III shows that M1/70-anti Mac-1 blocked the *induction* by dexamethasone or WEHI-3 CM of Mac-3 antigen on M1 cells by 64 to 84%. Anti-Mac-1 did not block the *detection* of Mac-3 on already induced M1 cells. LPS induction of Mac-3 antigen was also blocked by M1/70 (74% inhibition, not shown). In contrast, M3/84 anti-Mac-3 did not affect the induction of Mac-1 antigen. An independently derived monoclonal anti-Mac-1, M19/24, also strongly inhibited induction of Mac-3 in M1 cells, whereas five other MAb had weaker or no effect on the development of Mac-1 or Mac-3 expression (Table III).

DISCUSSION

The Mac-1, 2, and 3 antigens have been characterized for their distribution in normal tissues (3–9). This study examined their expression on tumor cells that had been classified as macrophage-like on the basis of functional properties, on other hematopoietic tumor lines, and on an immature myeloid line that can be induced to acquire some properties of mature macrophages.

The Mac-1 antigen was found on eight of eight macrophage lines of independent origin. These lines all expressed macrophage markers such as C and Fc receptors, but differed in other properties such as antibody-dependent and nonspecific cytolysis (11–26). Among normal cells, Mac-1 has similarly been found to be a general macrophage marker present on 95% of resident macrophages and macrophages elicited by sterile inflammatory agents, intracellular facultative bacteria, and immune modulators (4). It is on macrophages present in

TABLE I

Detection of antigens on cell lines by ^{125}I-antibody binding assay[a]

Line	Type	Mac-1	Mac-2	Mac-3
PU5-1.8	Macrophage	9,867 ± 4,121 (4,700–18,000)	6,077 ± 2,283 (2,100–10,010)	7,373 ± 1,839 (5,120–11,000)
RAW 264.10	Macrophage	15,110 ± 6,009 (3,330–23,000)	7,870 ± 2,348 (3,310–11,210)	4,570 ± 1,088 (3,100–8,500)
J774.1	Macrophage	23,100 ± 3,040 (18,600–28,900)	18,600 ± 3,700 (14,800–26,000)	18,280 ± 1,960 (16,080–22,200)
J774.16C3C	Macrophage	26,640 ± 9,240 (8,930–40,000)	21,400 ± 7,320 (12,000–43,000)	17,720 ± 5,000 (4,470–27,400)
NCTC 1469	Macrophage	9,670 ± 1,650 (7,200–12,800)	12,870 ± 5,210 (6,540–23,200)	11,270 ± 4,270 (5,900–19,700)
A2	Macrophage	9,360 ± 2,640 (4,070–12,000)	22,370 ± 2,990 (17,800–28,000)	4,300 ± 1,310 (1,740–6,070)
WEHI-3	Immature macrophage	6,750 ± 1,187 (4,385–8,020)	5,430 ± 1,480 (2,300–7,520)	4,400 ± 1,300 (2,201–6,710)
M1	Myeloblast	—[b] (0–900)	— (0–263)	— (0–550)
RFM	Myeloblast	— (0–200)	— (0–1,120)	1,533 ± 508 (520–2,080)
GM-86	Erythroleukemia	— (0–191)	— (0–430)	— (160–1,400)
18-8	Pre-B cell	— (0–170)	— (0–290)	— (90–590)
WEHI 231	B cell	— (0–1,200)	— (110–316)	1,707 ± 448 (920–2,600)
X-63	Myeloma	— (0–310)	— (44–700)	754 ± 223 (140–1,400)
EL4	T lymphoma	— (0–100)	— (37–420)	— (0–423)
R1	T lymphoma	— (54–300)	— (0–26)	— (69–245)

[a] Cpm bound to 10^6 cells using 70 ng ^{125}I-labeled antibody (about 150,000 cpm) as described in *Materials and Methods*. Mean of at least three determinations ± SE and range in parentheses. Binding of each ^{125}I-MAb was blocked over 90% of 1 μg unlabeled homologous MAb but not by heterologous MAb.

[b] — = Mean not significantly different from 0.

TABLE II

Induction of Mac-1 and Mac-3 antigens in M1 myeloblast line[a]

Inducer	Mac-1 (cpm)	Mac-2 (cpm)	Mac-3 (cpm)	CR (%)
0	720	—[b]	460	3
WEHI-3 CM				
5%	7,800	—	3,190	
20%	9,100	—	9,300	25
50%	10,100	—	7,900	
Lymphokine				
5%	2,900	—	1,470	
20%	10,700	—	8,800	31
50%	12,000	—	7,200	
DM[c]				
10^{-8} M	1,900	—	900	
10^{-7} M	11,600	—	1,800	
10^{-6} M	9,900	—	9,800	25
10^{-5} M	12,300	—	10,800	28
LPS				
0.01 μg/ml	4,200	—	2,130	
0.1 μg/ml	11,500	—	6,030	22

[a] M1 cells cultured with agents as shown, diluted 1/2 with growth medium at day 3, and on day 6 washed and assayed for binding ^{125}I-MAb as in Table I. Cells positive for C receptors (CR) were detected by EAC rosettes (24). Control cultures increased six- to 10-fold in cell numbers. Cells recovered in treated cultures were within 20% of control cultures except for 0.1 μg/ml LPS, which inhibited growth over 80%.

[b] Less than 250 cpm.

[c] DM, dexamethasone.

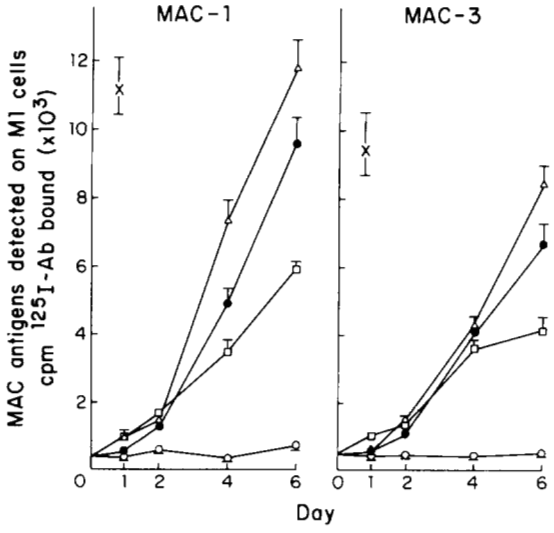

Figure 4. Time course of induction of antigens on M1 cells. M1 cells were assayed for antigen expression at various days of incubation with 20% lymphokine (△), 20% WEHI-3 CM (●), or 10^{-5} M dexamethasone (DM) (□) compared to control cultures (○), as described in Table II. Levels of Mac-1 and Mac-3 antigens on positive control line PU5-1.8 are also shown (X).

the spleen marginal zone and red pulp, and on lymph node medullary cords, on tissue macrophages, but not on "fixed" macrophages such as Kupffer cells (Reference 4).[6] Mac-1 is also present on 50% of bone marrow-nucleated cells, including granulocytic precursors, and on natural killer cells (3, 5), but is absent from lymphocytes and nonlymphoid tissues. This correlates with the absence of Mac-1 from the T and B lymphoid and erythroleukemia lines studied here. The mastocytoma P815, the myeloid leukemia C1498.3, and the M1 and RFM myeloblast lines were also Mac-1$^-$. Recently, Mac-1 has been found to be identical to or closely associated with the function of the C receptor type three, which is specific for C3bi or C3d,g (reviewed in Reference 7).[4] The expression of C receptors on macrophage lines correlates with Mac-1 expression. Furthermore, the M1 line became positive for both Mac-1 and C receptors after induction of maturation. It should be pointed out, however, that monocytes/macrophages express two distinct types of C receptors: type one for C3b and type three for C3bi or C3d,g (reviewed in References 28 and 29). Although it would be expected that macrophage cell lines would express both of these types of receptors, this has not been definitively established.

Mac-2 was expressed on constitutively mature macrophage lines and was absent from nonmacrophage lines, having a distribution essentially identical to that of Mac-1. The interesting exception was the induced M1 line, which expressed Mac-1 but not Mac-2. *In vivo,* all types of macrophages localized in lymphoid and nonlymphoid tissues are Mac-2$^+$.[6] This expression is at a low level, which is detectable by ^{35}S-methionine labeling and immunoperoxidase, but not by immunofluorescent flow cytometry (8). In response to thioglycollate but not to a number of other agents, a 10- to 30-fold higher level of Mac-2 expression is induced (8). This induction of Mac-2 is independent of Mac-1 and Mac-3, which remain unchanged within a factor of 2 (4, 9). It is interesting that this lack of coordination between Mac-2 and the other markers is also reflected in the M1 cell line, in which Mac-1 and Mac-3 but not Mac-2 were induced by several different agents.

In contrast to Mac-1, both Mac-2 and Mac-3 antigens have been found among normal cells on nonhematopoietic tissues (Reference 8).[5, 6] Mac-2 is present on epithelial cells, and Mac-3 is present on both epithelial and endothelial cells. Both antigens have reticular distributions in lymphoid tissues, and are absent from lymphocytes (References 8 and 9).[5, 6]

Despite its presence on nonhematopoietic tissues, Mac-2 delineated similar subsets of hematopoietic normal and tumor cells. Mac-3 had a broader distribution among tumor cells. It

TABLE III

Inhibition of induction of Mac-3 antigen on M1 cells by anti-Mac-1[a]

Inducer	Addition	Addition after Induction	Mac-1 (cpm)	Percent Control[b]	Mac-3 (cpm)	Percent Control[b]
0	0		0		0	
DM	0		8,700		9,300	
DM	M1/70		140[c]		2,900	36 ± 6
DM	M3/84		8,210	102 ± 8	330[c]	
DM		M1/70	0	1 ± 1	9,660	99 ± 5
DM		M3/84	8,800	98 ± 2	720	4 ± 2
0	0		480		580	
WEHI-3	0		4,800		2,040	
WEHI-3	M1/70		0[c]		816	21 ± 5
WEHI-3	M3/84		5,150	104 ± 4	610[c]	
0	0		400		320	
WEHI-3	0		4,625		3,765	
WEHI-3	M1/70 (G2b, Mac-1)		460[c]	1 ± 1	990	16 ± 4
WEHI-3	M19/24 (?, Mac-1)		505[c]	1 ± 1	1,150	30 ± 5
WEHI-3	M1/42 (G2a, H-2)		5,035	94 ± 16	3,480	91 ± 3
WEHI-3	M1/84 (2a, pan)		4,035	106 ± 20	2,640	72 ± 1
WEHI-3	M1/69 (G2b, HSA)		5,055	82 ± 27	2,940	65 ± 13
WEHI-3	M5/113 (G2b, Lgp100)		4,635	98 ± 3	3,530	86 ± 10
WEHI-3	M17/4 (G2a, LFA-1)		4,620	96 ± 4	3,195	79 ± 8

[a] M1 cells were incubated 6 days with 10^{-6} M dexamethasone (DM) or 20% WEHI-3 CM, plus MAb at 1 μg/ml as indicated, washed, and assayed for Mac-1 and Mac-3 antigens as in Table II. IgG class and antigen specificity of each MAb are given in parentheses. Normal rat IgG (U. S. Biochemical Corp., Cleveland, OH) at 1 and 10 μg/ml did not affect induction of the antigens (not shown).

[b] Percent of induced antigen level, mean ± SE for two experiments.

[c] Detection of antigen by ^{125}I-MAb at day 6 blocked by the presence of unlabeled MAb of the same specificity added at day 0 of culture.

was present not only on macrophages, but also in three to 20-fold lower quantities on the RFM myeloblast line, the PU5-1R and WEHI 231 B cell lines, and the X-63 myeloma. Mac-3 was clearly absent from T lymphoma lines.

Mac-3 ran as a diffuse band in SDS-PAGE and its M_r varied from 100,000 to 170,000 depending on the macrophage cell line. Similar variation in M_r was found among peritoneal macrophages elicited by different agents (Reference 9).[6] This variation appears due to carbohydrate, because Mac-3 is synthesized from a 76,000 M_r precursor.[6]

Expression of Mac-2 and Mac-3 antigens did not correlate with known macrophage cell line functional activities. The high levels of Mac-2 and Mac-3 in 5′ nucleotidase-deficient J774 cells and in H_2O_2, O_2^--deficient J774.16C3C cells suggest they are not involved in these properties. Similarly, the lack of all antibody-dependent phagocytosis and lysis and nonspecific killing of tumor or erythrocyte targets by WEHI-3 despite levels of Mac-1, 2, and 3 similar to that of the highly active RAW 264 line (13) suggests the antigens are not related to expression of these effector functions.

The myeloblast M1 line is a model for differentiation in the myeloid macrophage pathways (14). Mac-1 and 3 antigens are undetected or are in variable but low amounts in undifferentiated M1 cells. These two antigens, however, could be induced in M1 by incubation with a variety of stimulating agents—dexamethasone, LPS, lymphokine, or WEHI-3 conditioned medium (CM). Mac-1 expression on induced M1 cells is in agreement with a previous report (30). Because Mac-2 is not detected on uninduced or induced M1, this antigen is not necessary for the expression of Fc and C receptors, nonspecific phagocytosis, and lysozyme production, which are induced in these cells.

Surprisingly, the presence of anti-Mac-1 blocked the appearance of Mac-3 antigen during induction of M1 cells. A number of other rat MAb did not inhibit induction of Mac-3, and no MAb was found to block induction of Mac-1 in M cells. Inhibition of Mac-3 induction by anti-Mac-1 was seen when LPS, lymphokine, dexamethasone, or WEHI-3 CM were used as inducing agents. It is unlikely that Mac-1 is a receptor site for all these inducing agents, and this is confirmed by the fact these inducers do not block binding of ^{125}I-anti-Mac-1 to macrophages (unpublished). Therefore, we suggest the antibody binds to Mac-1 sites as they appear on M1 cells during induction, and this binding modulates the expression of Mac-3. Because Mac-1 is associated with the C receptor type three (Reference 7),[4] this phenomenon may be related to a regulatory effect of the C system on macrophage differentiation.

Mac-1 is structurally related (7, 30, 31) to an antigen on lymphoid and some myeloid cells called LFA-1 (lymphocyte function-associated antigen 1). These antigens contain distinct α subunits of 170,000 or 180,000 M_r noncovalently associated with highly homologous or identical β subunits of 95,000 M_r (31, 32). MAb to LFA-1 block T cell-mediated antigen-specific killing and the induction of T helper cell proliferative responses (7). The expression on tumor cells of LFA-1 and Mac-1 was compared (32). LFA-1 is present in largest quantities on T lymphoid cell lines. Trace quantities of LFA-1 were found on some but not all macrophage-like tumor lines. When co-expressed, as in J774, Mac-1 and LFA-1 were found to be separate molecular moieties as shown by independent immunoprecipitation. Because anti-LFA-1 blocks T cell functions and anti-Mac-1 blocks C type three receptors and the induction of Mac-3, the LFA-1 and Mac-1 antigen family has a number of important functional and regulatory roles in different cell types.

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