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Mac-1: a macrophage differentiation antigen identified by monoclonal antibody

We have previously described the derivation of M1/70, a hybrid myeloma line secreting monoclonal rat anti-mouse cell surface antibody (Springer, T., Galfré, G., Secher, D. S. and Milstein, C., *Eur. J. Immunol.* 1978. *8:* 539). We have now investigated the cellular distribution of this antigen using a ^{125}I-labeled anti-rat IgG indirect binding assay, the fluorescence-activated cell sorter, autoradiography and precipitation of cell surface molecules. Screening with a tumor cell panel showed strong reactivity with a macrophage-like line but no reactivity with B or T lymphoma lines. In normal tissues, M1/70 antigen was found to be present in small amounts on spleen and exudate granulocytes and a subpopulation of bone marrow cells, in moderate amounts on spleen and blood monocytes and expressed in much larger amounts on spleen histiocytes and peritoneal exudate macrophages. In contrast, M1/70 antigen was found to be absent from erythroid and lymphoid cells. M1/70 antibody precipitated two polypeptides of 190000 and 105000 mol. wt. which were present in much greater amounts on peritoneal exudate macrophages than on spleen cells. The expression on phagocytes of two other antigens identified by monoclonal antibodies M1/69 and M1/9.3 was also examined. Monocytes and granulocytes expressed large amounts of M1/69 and low amounts of M1/70 antigen, while in peritoneal exudate macrophages this pattern was dramatically reversed. M1/70 thus defines a differentiation antigen on mononuclear phagocytes and granulocytes, the expression of which is specifically increased during monocyte maturation. This antibody is the first to be described which recognizes a discrete molecule specific to phagocytes.

1 Introduction

In a previous report we described the derivation and purification of ten stable rat spleen cell-mouse myeloma hybrid clones which secrete antibodies to mouse cell surface antigens [1]. A study of their antigenic targets allowed us to divide these different monoclonal antibodies into groups: (a) M1/9.3 and M1/89.18 reacted with a 210000 mol. wt. protein. (b) M1/69, M1/22.54, M1/75, M1/9.47 and M1/89.1 recognized a heat-stable, putative glycolipid antigen, not seen as an iodinated band in gel electrophoresis. (c) M1/22.25 and M1/87 were specific for the Forssman glycosphingolipid antigen. (d) M1/70, the subject of the present report, precipitated two iodinated polypeptides of 190000 and 105000 mol. wt. present in low amounts on spleen cells. The two different carbohydrate antigens were found on various types of tissues [2, 3] while the 210000 mol. wt. protein antigen identified by M1/9.3 is a leukocyte-common antigen (LCA) found on essentially all leukocytes but not on nonlymphoid tissue [3, 4].

In this study, we examine the cellular distribution of the antigen recognized by M1/70. Immunoprecipitation, an indirect ^{125}I-labeled-anti-immunoglobulin (Ig) assay, autoradiography and the FACS (fluorescence-activated cell sorter) have been used to quantitatively study the binding of M1/70 monoclonal antibody to cell lines and to lymphoid tissue and peritoneal exudate cell (PEC) subpopulations. The results show that M1/70 recognizes a differentiation antigen (designated Mac-1) present on mononuclear phagocytes and to a much able to recognize the GT copolymer while we have shown that

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Abbreviations: **BBSS:** Balanced basal salt solution **LCA:** Leukocyte-common antigen **FACS:** Fluorescence-activated cell sorter **FITC:** Fluorescein isothiocyanate **PBS:** Phosphate-buffered saline **PE(C):** Peritoneal exudate (cells) **RBC:** Red blood cells **SDS-PAGE:** Sodium dodecyl sulfate polyacrylamide gel electrophoresis

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lesser extent on granulocytes, but absent from lymphoid cells. We also examine selective changes in the expression of the M1/70 antigen, compared to M1/69 and M1/9.3 antigens, during monocyte maturation.

2 Materials and methods

2.1 Cell lines

The T lymphomas S1A, BW5147 and TIMI [5], P1798 [6], from Litton Bionetics, Inc., Kensington, MD, and the Abelson line R8CL7 [7] were grown *in vitro* in Dulbecco's modified medium supplemented with 10% horse serum and were gifts of Dr. L. Sherman. P388D_1, a macrophage-like line [8], grown in suspension, was a gift of W. Wong.

2.2 Cell preparation

Cells were obtained as described [9], and with the exception of PEC, were further purified by Ficoll-Isopaque sedimentation [10]. The major effect of this purification was that dead and red cells were depleted. PEC were harvested 8–16 h (for polymorphonuclear cells) or 3–4 days (for macrophages) after injection of 1.5 ml of Brewer's thioglycollate medium (Difco, Detroit, MI). Clumped cells were mostly removed by sedimenting at 1 × g for 15 min. Wright-stained smears were counted to determine the percentage of polymorphonuclear cells and macrophages.

2.3 Binding assay

The anti-Ig binding assay, with ^{125}I-labeled-rabbit F(ab')$_2$ anti-rat IgG was performed as previously described [1]. All target cells were glutaraldehyde-fixed [11]. Adherent cells were fixed in plates with 0.125% glutaraldehyde in balanced basal salt solution (BBSS) for 5 min at 20 °C. An equal volume of 2% bovine serum albumin in BBSS was added, and cells were detached with a rubber policeman and suspended by sucking up and down in a pasteur pipette. For determination of antigen titers, 5-fold serial dilutions of target cells (5 µl) were incubated with spent clone supernatants (5 µl) and the assay carried out as described [1]. In antigen site and supernatant antibody excess, the amount of bound ^{125}I-labeled anti-rat Ig plateaued at a characteristic value for each clone.

2.4 Fluorescent antibodies

Rabbit F(ab')$_2$ anti-rat IgG was conjugated with fluorescein isothiocyanate (FITC) [12] and affinity-purified on rat serum-Sepharose CL-4B (Pharmacia, Uppsala, Sweden). Antibodies cross-reactive with mouse surface Ig were removed by passage through Sepharose CL-4B conjugated to $IgG_{2a}(\kappa)$, $IgG_1(\kappa)$ and IgM(λ) myelomas.

2.5 FACS analysis

Each labeling step was carried out for 30 min at 0–4 °C in medium containing 0.01 M NaN_3. Cells (5×10^7/ml) were incubated with an equal volume of 50 µl M1 monoclonal antibody or irrelevant monoclonal antibody, R4/18.2 [13], as control in the first step, washed, suspended in 50 µl of FITC-F(ab')$_2$ anti-rat IgG in the second step, and washed through a layer of fetal calf serum.

Analysis on the Becton Dickinson FACS II was carried out as described [14]. The fraction of labeled cells was corrected for spillover of a constant fraction of unlabeled cells by the following equation:

$$x = y + c(1 - y); \quad y = (x - c)/(1 - c)$$

where x = fraction of cells to the right of the threshold marker in the experiment, c = fraction of cells to the right of the marker in the control, and y = corrected fraction of labeled cells.

2.6 Immunoprecipitation

Lactoperoxidase radioiodinated cells were detergent-solubilized and antigens precipitated with myeloma-hybrid culture supernatants and rabbit anti-rat IgG, as previously described [1], except that supernatants were not concentrated by $(NH_4)_2SO_4$ precipitation.

3 Results

3.1 Identification by M1/70 of a specific antigen on macrophages

3.1.1 Binding of M1/70 supernatant to normal cells and cell lines

The distribution of the antigen recognized by M1/70 on a number of cell lines, lymphoid cells and red blood cells (RBC) was examined as follows. Serial cell dilutions were incubated with supernatants in the binding assay, and the reciprocal of the cell concentration (× 10^9) giving 50% maximal binding of ^{125}I-labeled-anti-Ig was called the antigen titer (Table 1). Antigen titer was taken as directly proportional to the average number of antigen sites/cell, if the avidity for antigen on different cells was equal, or sufficiently high to give near saturation binding. The antigen identified by M1/70 displayed a

Table 1. Mac-1 antigen titers on different types of cells

Cell types		Titer[a)]
Brain, kidney, liver, spleen[b)]		undetectable
RBC		< 0.02
Spleen Ficoll-Isopaque pellet		(5)
Spleen Ficoll-Isopaque band		(3)
Thymocytes		< 0.8
SIA	T lymphoma	< 4
BW5147	T lymphoma	< 0.8
TIMI	T lymphoma	(20)
P1798	T lymphoma	< 0.2
R8CL7	Abelson-induced lymphoma	< 4
NS-1	Myeloma	< 0.2
P388D_1	Macrophage-like line	480

a) Titer = 10^9 × (cells/ml giving half maximal ^{125}I-anti-IgG binding)$^{-1}$; ( ) = extrapolated, binding was positive but below half-maximal at highest concentrations tested; < = binding was negative at highest concentration tested.
b) Measured by absorption using spleen cells as targets.

striking distribution. It was present in very low amounts on spleen cells and not detected on RBC, thymocytes, Abelson lines and most T lymphomas. However, it was found in large amounts on the macrophage-like line, P388 D_1.

3.1.2 Binding of M1/70.15 to adherent PEC

Cells were taken from the peritoneum of mice, near the peak of their response to a challenge of H-2-incompatible cells. Following destruction of the challenging cells, many debris-laden macrophages as well as cytotoxic T lymphocytes were present. The population was separated into adherent and nonadherent fractions [15] and tested for antigen titer (Table 2). The adherent cells had high amounts of the antigen recognized by M1/70.15, and about ninefold more than the nonadherent cells. This correlated well with the percentage of macrophages in each, as judged by counting large, debris-laden cells. On the other hand, the antigen recognized by M1/69.16 was much lower on the adherent cells, and the two populations had similar amounts of the antigen recognized by M1/9.3.

3.1.3 Specific binding of M1/70 to PEC

Thioglycollate-induced PEC (85–90% macrophages) also contained large amounts of the antigen reactive with M1/70.15. The level of ^{125}I-labeled-anti-IgG binding was 36-fold above background (Table 3), in contrast to the barely detectable binding seen in earlier experiments with spleen cells [1].

Binding to macrophages did not occur through artifactual Fc receptor interactions. Binding through the Fc portion of M1/70.15 should have occurred equally well to rat or mouse PEC, while tritiated M1/70 IgG bound specifically to mouse PEC (see Fig. 5 of [1]). Furthermore, no inhibition of M1/70 binding by heat-aggregated or normal IgG preparations was seen (Table 3). It was concluded that M1/70 binding must occur through its Fab, rather than its Fc portion.

3.1.4 Molecular characterization of the antigen on macrophage surfaces

Equal quantities of radioactive spleen cell and macrophage antigens were analyzed by immunoprecipitation and sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Two different concentrations of antibodies used in each experiment demonstrated antibody excess. M1/70 precipitated large amounts of polypeptides of 190000 and 105000 mol.wt. from macrophage surfaces (Fig. 1a). Their precipitation was specific, since they were not precipitated by a control supernatant containing 50 μg/ml of normal rat IgG or by M1/9.3. M1/70 also specifically precipitated the 190000 and 105000 mol.wt. polypeptides from spleen cells, but in amounts only visible upon prolonged exposure of the autoradiogram (Fig. 1b). Electrophoresis under nonreducing conditions showed that the 190000 and 105000 mol.wt. polypeptides were not linked by interchain disulfides, and their faster mobility suggested intrachain disulfides (Fig. 1c). The

Table 2. M1/70.15 is expressed on peritoneal adherent cells[a]

	M1/70.15	M1/9.3	M1/69.16	Macrophages
	Antigen titers[b]			(%)
Adherent cells	400	80	(7)	61
Nonadherent cells	44	167	50	5

a) From mice which had just rejected an i.p. challenge of tumor cells.
b) See legend to Table 1 for details of assay.

Table 3. Binding of M1/70.15 to PEC[a]

Pretreatment	^{125}I-labeled IgG bound (cpm)	
	M1/10.15	R4/18.2[b] (control)
None	22064	668
Heat-agg. human IgG[c] (10 mg/ml)	23512	600
Normal human IgG (10 mg/ml)	22622	543
Normal mouse serum (50%)	20897	645

a) Macrophages were prepared from PEC 4 days after thioglycollate treatment.
b) An IgG, anti-rat histocompatibility antigen supernatant.
c) Heat-aggregated at 20 mg/ml in saline at 63 °C for 1 h.

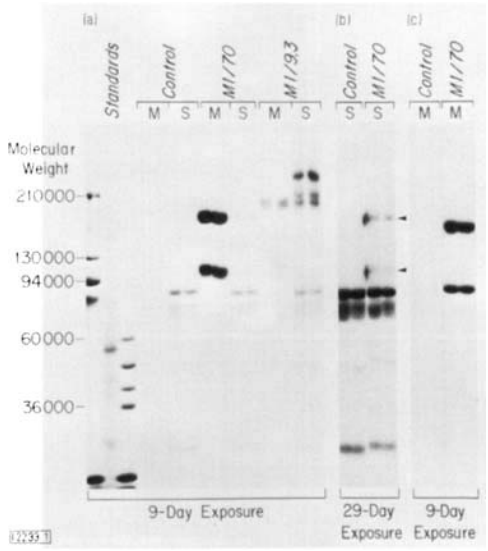

Figure 1. SDS-PAGE of immunoprecipitates from ^{125}I-labeled spleen cells and 4-day thioglycollate-induced macrophages. Equal quantities (240000 cpm) of spleen (S) and macrophage (M) antigens were indirectly precipitated using supernatants from M1/70.15, M1/9.3.4, or NS-I + 50 μg rat IgG/ml as control. In each experimental group, two different concentrations of antibodies were employed: 30 or 100 μl of clonal supernatant and 10 or 33 μl of rabbit anti-rat IgG, electrophoresed in left or right gel lanes, respectively. SDS-PAGE was performed in 5–12% polyacrylamide gradient gels [1]. (a) Reduced sample buffer (plus 5% 2-mercaptoethanol). In addition to bands specifically precipitated from spleen cells, μ, δ and light chains were precipitated by cross-reaction with the rabbit anti-rat IgG. (b) As above, except that autoradiogram was exposed three times as long. Arrows show positions of Mac-1 bands. Radioactivity on the edges of the lanes is from intervening lanes containing M1/70 precipitates from macrophages. (c) Nonreduced sample buffer (plus 50 mM iodoacetamide). Mol. wt. standards were as in [1], Fig. 4.

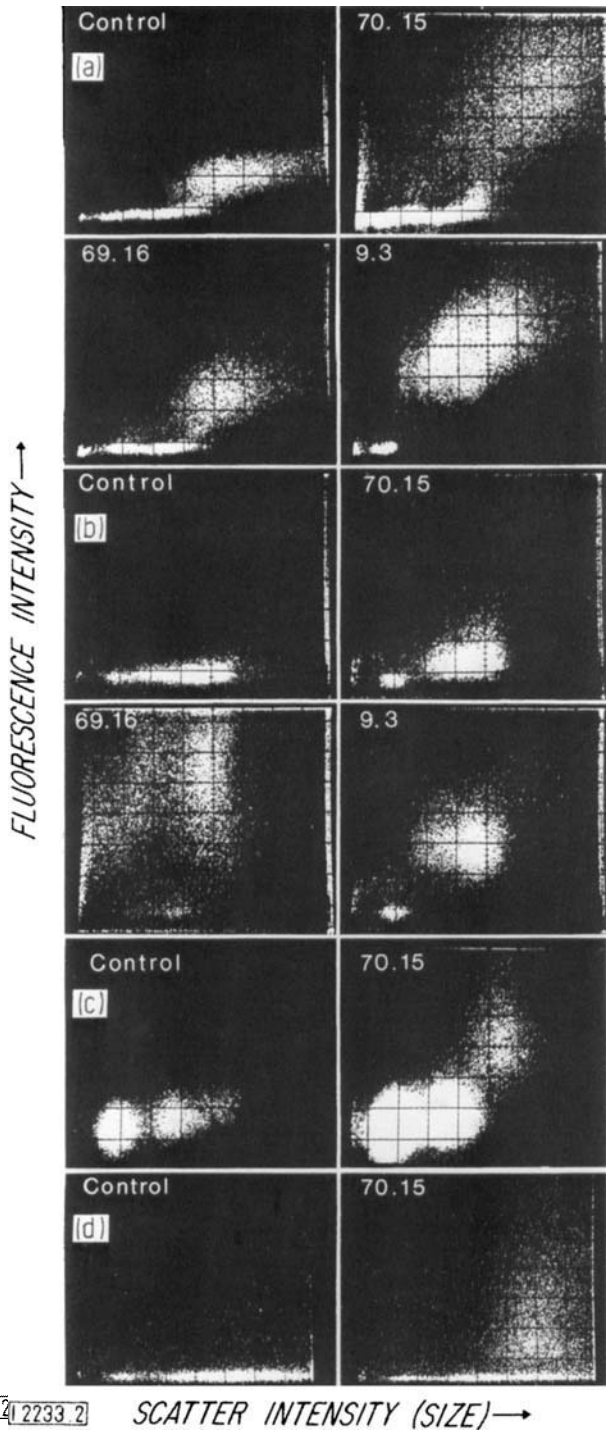

Figure 2. FACS two-dimensional maps of white cells from different tissues labeled by monoclonal antibodies. Dots represent fluorescence and scatter intensities of individual cells. Cell suspensions were first labeled with clonal supernatants, then with FITC-rabbit anti-rat IgG. Fluorescence and scatter gains differed between tissues but were identical within each tissue. (a) Four-day PEC, 120000 cells counted. Macrophages constituted 87% of the PEC by Wright's staining and were the cells with the largest scatter in the dot plots. However, they seemed to constitute a smaller percentage of the analyzed population because they tended to aggregate. Aggregates were counted as one cell and were off-scale. (b) Eighteen-hour PEC, 40000 cells counted. Polymorphs (almost all neutrophils) constituted 95% of the population by Wright's staining. The polymorphs which were the largest cells in these plots, had a scatter identical to the cells with intermediate scatter which were dimly labeled by M1/70 in Fig. 2a. 210000 mol.wt. LCA antigen precipitated by M1/9.3 was present in somewhat higher amounts on spleen cells than on macrophages. Several different mol. wt. species of LCA were found in spleen cells, while only one was found in macrophages. Differences in the mol. wt. of this antigen on B and T lymphocytes have been reported [16].

3.2 Antigen expression on white blood cell subpopulations and during monocyte differentiation

3.2.1 General remarks

Cells in lymphoid tissue and PE were labeled with clonal supernatants and then with fluorescein-labeled rabbit $F(ab')_2$ anti-rat IgG which had been absorbed with mouse IgG and IgM.

3.2.2 Labeling of PEC

M1/70-positive cells in 4-day peritoneal exudates were visualized in dot plots on the FACS (Fig. 2a). Macrophages were the largest cells in the 4-day PE. This population showed moderate fluorescence in the control even in the absence of added FITC-anti-rat IgG: *i.e.* autofluorescence. Upon addition of M1/70, the fluorescence of this population was dramatically increased. Smaller cells, such as lymphocytes, were not labeled. The specificity of M1/70 for macrophages was demonstrated by comparison with M1/9.3 which labeled the smaller white blood cells present in the exudate as well as the macrophages (Fig. 2a). M1/69.16 labeling of macrophages was very weak, in agreement with the antigen titer study (Table 2).

The polymorphonuclear cells of the 18-h thioglycollate-induced PE were only weakly labeled by M1/70.15 (Fig. 2b), and only 53% of these cells were above background (Fig. 3, inset). While polymorphs only weakly express M1/70 antigen, they strongly express M1/69 antigen (Fig. 2b), the converse of the pattern seen with macrophages (Fig. 2a).

3.2.3 M1/70 labeling of cells in different tissues and different stages of monocyte differentiation

Blood monocytes, but not lymphocytes or RBC, were labeled by M1/70 (Fig. 2c). M1/70 was expressed also on a subpopulation of bone marrow cells (Figs. 2d and 3a). These cells appeared to be among the largest of the tissue. The nature of these cells remains to be established, but they may contain granulocyte precursor cells.

M1/70 antibody labeled almost no cells in the thymus, a few cells in spleen, higher numbers in blood leukocytes, and a very

(c) Blood cells, Ficoll-Isopaque-purified to deplete RBC and granulocytes, 200000 cells counted. RBC were the population with the smallest scatter, lymphocytes were the cells with intermediate scatter, and the less numerous population of cells, with the largest scatter, were monocytes [17]. In the control, fewer cells were counted, but the center of each population could be clearly seen. (d) Bone marrow cells, Ficoll-Isopaque-purified.

high proportion of 4-day PEC macrophages (Fig. 3). This was proportional to the content of monocytes and macrophages in all these tissues. The labeling of 18-h PEC and bone marrow cells suggested the presence of M1/70 on polymorphonuclear leukocytes and their precursors, although in very low amounts (Fig. 3). Autoradiography of spleen cells showed that monocytes, and to a lesser extent granulocytes, were labeled by M1/70. Histiocytes, which were much rarer cells in these preparations, were very heavily labeled. Erythroid and lymphoid cells were unlabeled.

PE macrophages expressed more M1/70 antigen than blood or spleen monocytes (Fig. 3). This was confirmed by determining the number of cells giving 50% binding in the indirect

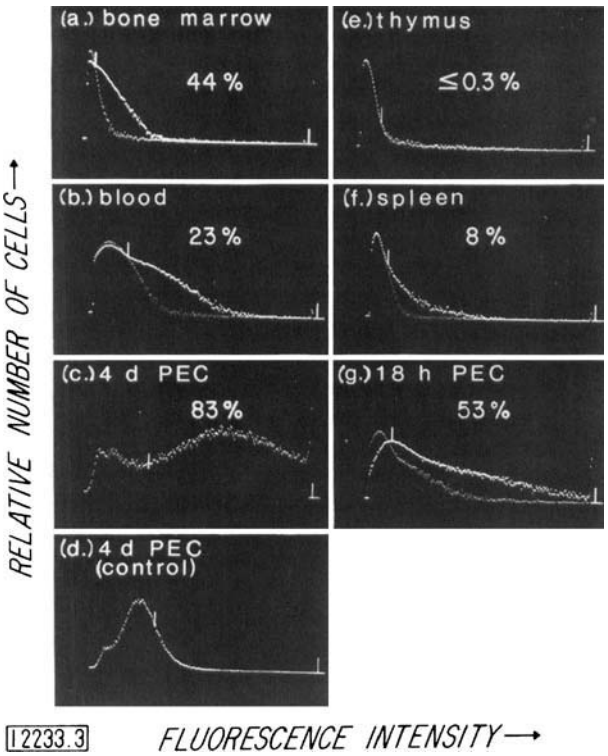

Figure 3. Fluorescence labeling of white cell tissues by M1/70. Cells were labeled with M1/70.15.1 (bright dots) or with R4/18.2, an irrelevant rat monoclonal antibody, as control (dim dots). The scatter gate was set to exclude RBC and dead cells, and for 4-day PEC, to include macrophages but exclude most polymorphs and smaller cells. Relative cell number was plotted on a logarithmic scale for all cells, except 4-day PEC which were plotted on a linear scale. Fluorescence gain was 2 for 4-day PEC and 4–8 for other analyses.

Table 4. M1/70.15 expression on spleen cells, PEC, and P388 D_1 cells

	M1/70.15 Titer	M1/70.15 positive cells (%)	Titer/fraction positive cells
Spleen cells	4.6	8	60
PEC[a)]	430	89	480
P388 D_1 cells	500	100	500

a) Three-day thioglycollate-induced PEC.

radioimmunoassay (antigen titer, Table 4). Thioglycollate-stimulated cells had a 100-fold greater amount of the antigen recognized by M1/70.15 than spleen cells. After correction for the number of positive cells in each preparation, positive 4-day PEC still showed 8-fold more antigen on their surface than positive spleen cells.

4 Discussion

The monospecificity of monoclonal antibodies is of great value for the analysis of the quantitative expression of an antigen in different cell populations. Ordinary antisera, even when extensively absorbed, often contain different antibody specificities. Studies of antigen expression in different tissues may thus reflect qualitative changes in the types of antigenic specificities expressed, as well as quantitative changes in the individual specificities. The use of monoclonal antibodies allows us to draw meaningful conclusions from the differences in quantitative expression of a given specificity on different cell types, in a similar way as Ig or terminal transferase [18] quantitative expression is used to define different stages of maturation. This is well illustrated by the expression of the Mac-1 antigen during monocyte maturation.

Macrophages and monocytes are derived from monocytes and promonocytes, while more primitive precursors are less well defined. From the bone marrow, monocytes enter the blood and with a half-life of 22 h, enter the tissues where they differentiate into tissue macrophages (peritoneal macrophages, Kupffer cells, alveolar macrophages, histiocytes, etc.) [19]. Differentiation from blood or spleen monocytes to PE macrophages is accompanied by a dramatic increase in the amount of Mac-1 antigen. This increase is not due to a non-specific general increase in surface molecules, since it is paralleled by a decrease in M1/69.16 labeling.

Small amounts of the antigen identified by M1/70 are also found on neutrophils in 18-h PE, on 44% of bone marrow cells and on granulocytes and monocytes in spleen. This expression is weak, but the same antigen is precipitated by M1/70 from the surface of both spleen cells and 4-day PEC. M1/70-positive and negative subpopulations in bone marrow are clearly separated by the FACS (Fig. 2d). The positive cells are likely to be the myelocyte and metamyelocyte precursors of granulocytes, since these cells are thought to make up a substantial amount of bone marrow and to greatly outnumber promonocytes. This pattern of expression is in agreement with the idea that the granulocytic and monocytic lines are closely related [20]. Mac-1 is absent from lymphocytes and RBC and also appears to be absent from kidney, liver and brain.

The M1 hybridization was not designed to produce anti-macrophage antibodies. Nylon wool-purified and concanavalin A-stimulated spleen lymphocytes were the immunogens. Non-specific esterase-positive macrophages have been found to constitute about 3% of nylon wool-passed spleen cells [21]. The derivation of M1/70 illustrates the usefulness of the myeloma hybrid technique for the identification of antigens on minor cell subpopulations. M1/70 was first recognized as a macrophage-specific antibody by its reactivity with the P388 D_1 tumor line. We have proposed screening of tumor cell panels as a sensitive method for detecting antibodies reacting with cells present as small subpopulations in normal tissues

[2, 4]. P388 D_1 has been identified as a macrophage-like line by its adherence to surfaces, phagocytosis and Fc and C3 receptors [22]. The finding that this line bears a macrophage differentiation antigen in amounts similar to stimulated macrophages lends further support to this conclusion.

We believe that M1/70 antigen (Mac-1) is the first individual phagocyte-specific differentiation antigen to be described. Previously obtained macrophage antibodies were xenoantisera rendered specific by absorption [23–27] and appear to have recognized a complex of antigens. The M1/70 antibody precipitates a discrete molecular species with polypeptide chains of 190000 and 105000 mol. wt. from macrophages. These polypeptides appear to be major components of the macrophage plasma membrane. The polypeptides precipitated from cells in the spleen are of identical mol. wt. The two chains are not linked by disulfide bonds. Our working hypothesis is that the two chains are noncovalently associated in the membrane, but we cannot presently rule out a proteolytic derivation of the 105000 mol. wt. chain. The M1/70 antigenic determinant is heat-labile and not allospecific [1].

M1/70 appears to be an excellent monospecific reagent, and we hope its use will further our understanding of phagocyte differentiation, function and interaction with lymphocytes. In an attempt to elucidate the role of Mac-1 on the cell surface, M1/70 is currently being tested for inhibition of specific macrophage functions. Analysis of macrophage interactions with other cells has often required macrophage depletion, a difficult operation by conventional means [28]. Preliminary experiments suggest that M1/70 is only weakly lytic for PE macrophages in the presence of rabbit complement. The antibody is of the IgG subclass [1]. We are now pursuing other means of depleting macrophage functional activity from lymphoid populations, such as panning [29] and the FACS.

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