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IDENTIFICATION OF CELL SURFACE ANTIGENS PRESENT ON MURINE HEMATOPOIETC STEM CELLS[1]

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Nine antigens found on murine bone marrow cells were examined to define their pattern of expression in murine hematopoietic differentiation. Lymphocyte function antigen (LFA-1), heat stable antigen (recognized by M1/69), common leukocyte antigen (CLA, T200, Ly-5) and Lgp100a (recognized by 30-C7) were present on early hematopoietic progenitors, BFU-E, CFU-E, CFU-GM, and CFU-M. All antigens found on progenitors were found on some immature precursor cells, myeloblasts, erythroblasts, or monoblasts, but their pattern of expression on identifiable hematopoietic cells varied. Three of these antigens, LFA-1, heat stable antigen recognized by M1/69, and CLA, were expressed on leukocytes of all stages of maturity but were lost from the erythroid lineage during differentiation. MAC-1, Forssman antigen, heat stable antigen (recognized by M1/75), anti-P-95 (recognized by M5/113), and Ia (recognized by M5/114) were found only on differentiated hematopoietic precursors or mature cells. The expression of these antigens was more lineage-specific. MAC-1 and heat stable antigen (recognized by M1/75) were restricted to either mature myeloid or erythroid cells, respectively. The marked differences in distribution of these antigens suggest that they may be useful in negative or positive selection experiments to enrich progenitors, and that some of them may have a functional role in differentiation.

Differentiation of hematopoietic stem cells has been studied intensively ever since in vitro culture systems for progenitors were described (1–3). However, the study of this differentiation has been hindered by the absence of purified populations of stem cells and the factors which stimulate them. Whereas antigens present on hematopoietic cells of specific lineage and maturity have been described in humans (4–6), few such antigens have been described in the mouse.

A series of monoclonal antibodies defining differentiation antigens on murine lymphohematopoietic cells have been generated recently by using the Kohler and Milstein hybrid technique (7–9). Identification of the distribution of these antigens during hematopoietic stem cell differentiation is the initial step in 1) understanding the function of these antigens and their role in stem cell differentiation and 2) using their distribution to further isolate specific progenitors.

In this report, the distribution of nine monoclonal antibody-defined antigens on murine hematopoietic progenitors is established by using sheep red blood cell (SRBC) rosetting, and the relevance of this expression pattern to murine hematopoietic stem cell differentiation is discussed. These nine antigens were chosen for study because their presence on some lymphohematopoietic cells was known and their molecular targets on certain lymphohematopoietic cells was well defined (8, 10–12).

MATERIALS AND METHODS

Animals. Mice used in bone marrow cultures were 5- to 12-wk-old BALB/cJ. C57BL/6J mice, 5 to 12 wk old, were used in the production of spleen cell supernatants. All of these mice were purchased from The Jackson Laboratory, Bar Harbor, ME.

Conditioned media. Pokeweed mitogen (PWM)-stimulated spleen cell supernatant (PWM-SCS)[3] was prepared as described previously and was used as a source of granulocyte/macrophage colony-stimulating activity (GM-CSF) and as a source of burst-promoting activity (BPA) (13). L cell conditioned media (LCM) was prepared from L929 cells as described (14), and was used as a source of macrophage colony-stimulating activity.

Bone marrow culture. Bone marrow cells were obtained by flushing the femurs of mice with Iscove's modified Dulbecco's media (IMDM; GIBCO, Grand Island, NY).

The methylcellulose culture technique described by Iscove and Sieber (2) was used with modifications. Nucleated marrow cells at 0.5 to 5 × 10^5 cells/ml were cultured in 35-mm Lux plastic tissue culture dishes (Miles Laboratories, Inc., Napenville, IL) in 1 ml of media containing 1.4% methylcellulose, 20% FCS, 1.3% BSA, 100 U penicillin, 100 μg streptomycin, 2 mM glutamine, and 1 × 10^{-4} M 2-mercaptoethanol. For BFU-E,[3] 2 U/ml erythropoietin (Connaught Laboratories, Ontario, Canada) were added to cultures along with 5% BPA. Colony-forming unit granulocyte/macrophages (CFU-GM) were cultured with the addition of 10% PWM-SCS, and CFU-M were incubated with the addition of 20% LCM. Petri dishes were incubated at 37°C in 5% CO_2 in air in a humidified atmosphere for 7 to 8 days. Colonies were scored by using an Olympus inverted microscope to identify pale aggregates of greater than 100 cells as myeloid colonies. Hemoglobinized aggregates of more than 100 cells were counted as BFU-E.

CFU-E were cultured in plasma clot as described (3). Clots were incubated in 5% CO_2 in high humidity, were removed from culture at 3 days, were fixed, and were stained, and CFU-E colonies of four or more nucleated hemoglobinized cells were scored.

Antibodies. The antibodies M1/70 (anti-MAC-1), M7/14 (anti-lymphocyte function antigen (anti-LFA-1)), M1/87 (anti-Forssman), M1/69 and M1/75 (anti-heat-stable antigens (anti-HSA)), M1/9.3 (anti-common leukocyte antigen (anti-CLA), Ly-5), M5/113 (anti-P-

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[3] Abbreviations used in this paper: BFU-E, burst-forming unit erythroid; CFU-E, colony-forming unit erythroid; CFU-GM, colony-forming unit granulocyte/macrophage; CFU-M, colony-forming unit macrophage; PWM-SCS, pokeweed mitogen-stimulated spleen cell supernatant; BPA, burst-promoting activity; LCM, L cell-conditioned media; HSA, heat-stable antigen; LFA-1, lymphocyte function antigen; CLA, common leukocyte antigen; IMDM, Iscove's modified Dulbecco's medium; CFU-S, colony-forming unit spleen.

95), and M5/114 (anti-Ia) were produced from xenogeneic rat/mouse hybridoma clones as described (7, 8). In addition, supernatant from the Lgp 100a 30-C7 clone described by Ledbetter and Herzenberg (9) and obtained from American Type Culture Collection (Rockville, MD) was used.

Positive selection of antigen-bearing cells by immunorosette formation. Affinity-purified goat anti-rat immunoglobulin at 0.5 mg/ml (Zymed Laboratories, Inc., South San Francisco, CA) was coupled to SRBC as described (15). Low density BALB/cJ marrow cells enriched for progenitors were separated from high density marrow cells by centrifugation of 5 ml of IMDM (GIBCO) containing 5×10^7 marrow cells atop 5 ml of a 51% Percoll layer at 2000 rpm for 20 min. Sixty to seventy percent of bone marrow cells remained at the top of the Percoll gradient. These cells were washed free of Percoll. Aliquots of recovered low density mononuclear cells were labeled separately with the antibodies M1/70, M7/14, M1/87, M1/69, M1/75, M1/9.3, M5/113, or 30-C7 used at a titer at which binding to cells was on a plateau. One milliliter of a 4% suspension of anti-rat immunoglobulin-coupled SRBC in IMDM was mixed with a 1-ml suspension of antibody-labeled marrow cells at 1×10^7 per ml and 0.5 ml of FCS, and was centrifuged at 1000 rpm for 10 min. The pellets were incubated on ice for 30 min and were then gently resuspended. The number of rosettes formed was assessed after staining cells with Acridine Orange. The rosetted cells were then separated from nonrosetted cells by layering the resuspended pellets atop 5 ml of 67% Percoll layer and centrifuging again at 2000 rpm for 20 min. Rosette-negative (antigen-negative) and rosette-positive (antigen-positive) cells rendered free of SRBC by ammonium chloride lysis were cultured in methylcellulose to assess growth of BFU-E, CFU-GM, and CFU-M and in plasma clot to assess growth of CFU-E.

Determination of purity of rosetted populations. Expression of antigens recognized by each of the nine antibodies on unseparated BALB/cJ bone marrow and on low density mononuclear cells enriched for progenitors on a 51% Percoll gradient was determined by using a fluorescence-activated analyzer. Cells were labeled with one of the nine antibodies, were washed, were then labeled with the $F(ab')_2$ fragment of rabbit anti-rat IgG heavy and light chain FITC-conjugated antibody (Zymed Laboratories, Inc.), were washed again, and were then analyzed. Aliquots of rosette-positive and rosette-negative cells for each of the antibodies were also labeled with that antibody and the $F(ab')_2$ fluoresceinated fragment of rabbit anti-rat IgG and were then analyzed to determine purity of the populations. Cytocentrifuge preparations of the rosette-positive and rosette-negative cells were also prepared, were stained with Wright-Giemsa, and were then analyzed to determine the distribution of antigen on recognizable progenitors.

Positive selection of M5/114 (Ia)-bearing cells by cell sorting. Ten million BALB/cJ marrow mononuclear cells were incubated with 0.5 ml of M5/114 antibody on ice for 30 min and were then washed. The same cells or unlabeled control cells were incubated with 100 μl of 0.5 mg/ml fluoresceinated $F(ab')_2$ rabbit anti-rat immunoglobulin antiserum (gift of Dr. K. Ault) and were washed. Cells stained with an irrelevant monoclonal antibody gave no higher background than tissue culture medium alone used in the first layer. The cells were sorted on a fluorescence-activated cell sorter (FACS II; Becton Dickinson Electronics, Mountain View, CA). Twenty-five to thirty percent of cells were included in the positive window. M5/114-labeled unsorted and M5/114-positive or -negative cells were cultured to determine the number of CFU-E, BFU-E, CFU-GM, and CFU-M in each fraction. Cells labeled with M5/114 were sorted and the distribution of antigen on progenitors was analyzed previously but not reported. These data are reported here along with data on the distribution of other antigens defined by rosetting.

RESULTS

Distribution of antigens on murine bone marrow. BALB/cJ whole bone marrow or low density mononuclear cells were labeled with anti-MAC-1, anti-LFA-1, anti-Forssman, anti-HSA (M1/69 and M1/75), anti-CLA (M1/9.3), anti-Lgp 100 (M5/113), anti-Lgp 100a (30-C7), and anti-Ia (M5/114) to determine the extent of expression of recognized antigens on murine bone marrow (Table I). Two antibodies, M1/69 and M1/9.3, recognized antigens present on nearly all bone marrow cells, whereas the rest of the antibodies recognized antigens with distributions varying from 19 to 66%. Low density mononuclear cells obtained after separation on a Percoll gradient differed only slightly in extent of antigen expression from whole bone marrow (Table I).

TABLE I

Distribution of antigens on bone marrow cells[a]

Antigen/Antibody	% Fluorescence Whole Bone Marrow	% Fluorescence Gradient-purified Bone Marrow	% Rosette-positive Gradient-purified Bone Marrow
MAC-1 (M1/70)	58.5	45.6	32.2 ± 8.5
LFA-1 (M7/14)	66.4	54.1	45.5 ± 4.9
Forssman (M1/87)	65.0	61.1	25.9 ± 9.1
HSA (M1/69)	91.8	90.6	73.9 ± 15.7
HSA (M1/75)	27.9	20.7	18.5 ± 8.0
CLA (M1/9.3)	89.1	80.1	61.6 ± 9.6
P-95 (M5/113)	50.3	50.5	31.5 ± 13.1
Lpg 100a (30-C7)	26.0	44.4	25.6 ± 6.1
Ia (M5/114)	25.7	—	—

[a] Percent fluorescence of whole or Percoll gradient-purified BALB/cJ bone marrow labeled with first antibody and fluorescent anti-rat antibody is expressed after subtraction of the percent cells labeled with fluorescent antibody alone. Percent bone marrow cells positive with immune rosette formation was assessed with Acridine Orange.

TABLE II

Enrichment of antigen-positive cells with immune sheep cell rosetting[a]

Antigen	% Fluorescence	
	rosette-positive fraction	rosette-negative fraction
MAC-1	96.3	10.6
LFA	82.2	<2
Forssman	70.4	6.8
HSA M1/69	98.1	9.35
HSA M1/75	68.4	11.1
CLA	98.7	<2
P-95	88.7	<2
Lgp 100a	74.1	6.8
Ia	75.3	5.3

[a] Purity of rosette-positive or -negative low density bone marrow cells separated with immune rosette formation expressed by relabeling positive and negative fractions with the appropriate antibody and fluorescent anti-rat antibody. Percent purity expressed as percent fluorescence detected by the FACS.

Distribution of antigens on hematopoietic progenitors. To assess the distribution of MAC-1, LFA-1, Forssman, HSA (recognized by M1/69 and M1/75), CLA, P-95, and Lgp 100a on hematopoietic progenitors, aliquots of Percoll gradient-purified bone marrow cells were labeled with each of the antibodies. The marrow samples were then immune-rosetted with anti-IgG-coupled sheep cells. The percentages of bone marrow cells which rosetted were assessed with Acridine Orange (Table I). The percentage of antigen-bearing cells assessed by rosette formation was routinely less than detected by fluorescence. Antigen expression at low density may be more likely to be detectable by fluorescence than by sheep cell rosetting. Recovery of cells in the rosette-positive and -negative fractions was 30 to 80% of the input cells.

The rosette-positive and -negative populations were assessed for purity by using the antibody with which they had been rosetted and $F(ab')_2$ fluorescent rabbit anti-rat antibody. The results are shown in Table II. Contamination of the rosette-negative (antigen-negative) population by antigen-positive cells detected by immunofluorescence ranged from <2 to 11%. Purity of the rosette-positive (antigen-positive) population ranged from 62.3 to 98.7%.

Rosette-positive and -negative cells were cultured for BFU-E, CFU-GM, and CFU-M in methylcellulose and for CFU-E in plasma clot. Results expressed as number of colonies per number of cells plated are shown in Figure

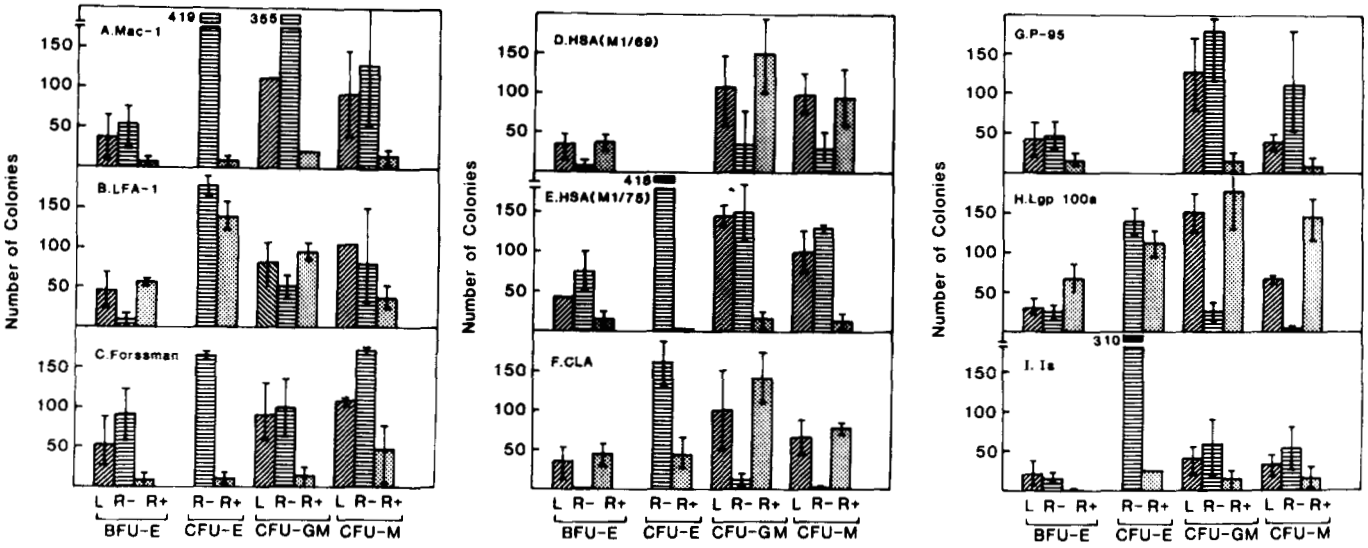

Figure 1. Number of colonies per 1×10^5 labeled unrosetted (L), rosette-negative (R^-), or rosette-positive (R^+) low density bone marrow cells cultured for BFU-E, CFU-GM, or CFU-M and per 5×10^4 cells cultured for CFU-E, BFU-E cultured with BPA, CFU-GM with PWM-SCS as a source of colony-stimulating activity, and CFU-M with LCM. Bone marrow cells rosetted with: *A*, anti-MAC-1; *B*, anti-LFA-1; *C*, anti-Forssman antigen; *D*, anti-HSA (M1/69); *E*, anti-HSA (M1/75); *F*, anti-CLA; *G*, P-95 (M5/113); or *H*, anti-Lgp 100a (30-C7). *I*, Number of colonies per 1×10^5 cells cultured from Ia (M5/114) antigen-positive or -negative cells separated on the FACS.

1*A* to *I*. Cytocentrifuge preparations of rosette-positive and -negative cells were also made to determine the distribution of antigens on recognizable precursors and mature cells, and these data are shown in Table III. MAC-1, an antigen found previously on granulocytes and monocytes, was not found on any hematopoietic progenitors (Fig. 1*A*), and on cytocentrifuge preparations, MAC-1-positive cells were mature polymorphs and monocytes. In contrast, LFA-1 antigen, which is expressed on 60 to 70% of bone marrow cells, was found on BFU-E, some CFU-E, CFU-GM, and some CFU-M (Fig. 1*B*). In the erythroid series, it was most strongly expressed in the earliest progenitor, the BFU-E, and its presence decreased during further differentiation, whereas in the myeloid series, it was strongly expressed throughout differentiation. Forssman antigen was not found on any hematopoietic progenitor (Fig. 1*C*), and its expression in bone marrow was limited to erythroblasts, polymorphs, and some less mature myeloid precursors.

BFU-E, CFU-GM, and CFU-M expressed HSA M1/69 (Fig. 1*D*), as did the majority of other marrow cells. In contrast, HSA M1/75 was not found on hematopoietic progenitors (Fig. 1*E*). Its expression in bone marrow appeared to be limited to erythroblasts and RBC, based on cytocentrifuge preparations. CLA (T200, Ly-5) antigen was present on CFU-GM, CFU-M, and BFU-E, but not most CFU-E (Fig. 1*F*). On cytocentrifuge preparations, cells of all stages of myeloid differentiation expressed this antigen, but only a few erythroblasts. This suggests that T200, although present on all immature progenitors, is lost during erythroid differentiation but maintained during myeloid. P-95 was found on no progenitor cells (Fig. 1*G*) and only on some polymorphs and monocytes. In contrast, Lgp 100a (30-C7) was found on all of the hematopoietic progenitors (Fig. 1*H*). It was expressed on only one-half of CFU-E, and only on a small population of erythroblasts, polymorphs, and monocytes. The expression of Ia (M5/114) on hematopoietic progenitors was studied on the FACS. Again, no progenitors were found to express the antigen (Fig. 1*I*), and only some macrophages and lymphocytes were found to express it.

TABLE III

Results of cytocentrifuge preparations[a]

Antigen	% Myeloblasts/ Myelocytes	% Polymorphs	% Monocytes	% Erythroid	% Lymphoid
MAC-1		93	7		
LFA-1	1	74	12	4	9
Forssman	9	50	3	31	6
HSA (M1/69)	8	40	11	37	4
HSA (M1/75)	1	8	—	87	1
CLA	7	69	8	12	2
P-95 (M5/113)	1	50	10	1	38
Lgp 100a (30-C7)	4	19	8	6	60

[a] Percent of myeloid, erythroid, and lymphoid cells found in cytocentrifuge preparations prepared from rosette-positive low density bone marrow cells sorted for the specific antigen.

Figure 2 summarizes the data on the expression of these lymphohematopoietic antigens on hematopoietic progenitors and their more differentiated progeny.

DISCUSSION

Although antibodies to specific stages of erythroid, myeloid, and lymphoid differentiation have been available in humans (4–6), this has not been so in the mouse. Purification of murine pluripotent progenitors has only been achieved through equilibrium density centrifugation followed by multi-step fluorescence-activated cell sorting (16), although an antibody distinguishing CFU-S from CFU-GM has been reported (17). Recently, murine spleen cells have been used to generate a variety of xenogeneic monoclonal antibodies in rats (8, 9). The expression of these antigens has been only partially characterized. It is noteworthy that Thy-1, which was initially thought to be specific for lymphocytes, has been found on hematopoietic progenitor cells (18, 19), either being lost or persisting as these cells differentiate. In this study, we determined the expression of nine antigens on hematopoietic progenitors in the mouse.

Four of these antigens were found on immature hematopoietic progenitors, BFU-E and CFU-GM. Three of these, LFA-1, HSA recognized by M1/69, and CLA are

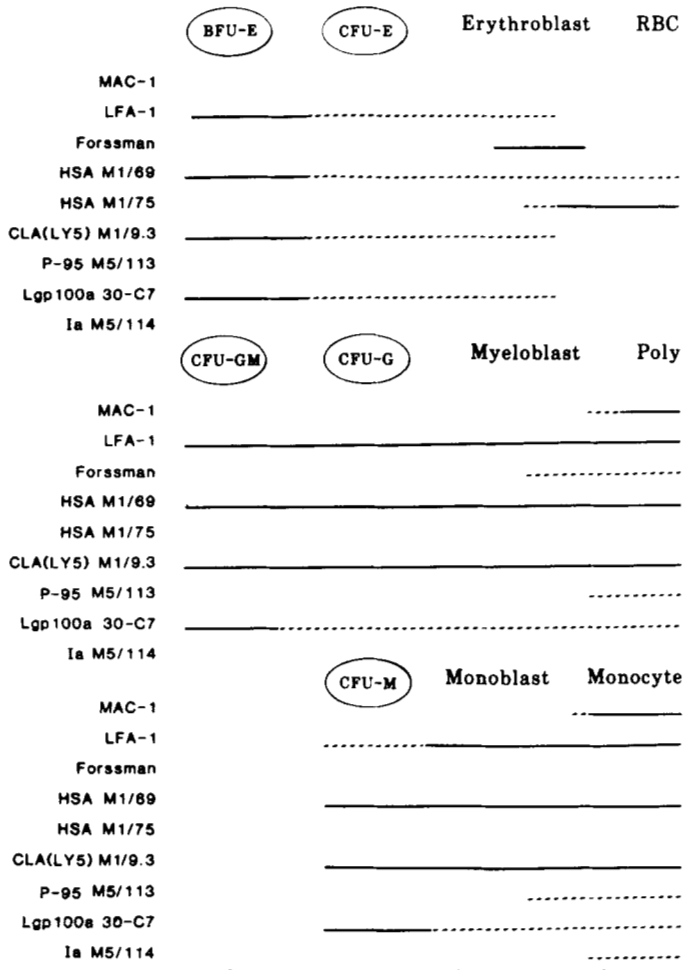

Figure 2. Schema of antigen expression on bone marrow of MAC-1, LFA-1, Forssman, HSA (M1/69), HSA (M1/75), CLA, P-95 (M5/113), Lgp 100a (30-27), and Ia (M5/114). The solid line indicates that a majority of cells express antigen, and the dotted line indicates <50% of cells expressed it.

leukocyte markers, widely expressed on myeloid cells of all stages of maturity, but lost from the erythroid lineage during differentiation. Lymphocyte function-associated antigen (LFA-1) is expressed on immature progenitors, on 60 to 70% of bone marrow cells, and on T and B lymphocytes. Anti-LFA-1 blocks T lymphocyte proliferative responses to antigens under Ir gene control, and it blocks cytotoxic T lymphocyte-mediated killing (10). Because LFA-1 appears to be involved in cells/cell adhesion, its expression on progenitors suggests that it may have a role in the cellular interactions controlling hematopoietic differentiation. HSA recognized by M1/69 is expressed on all hematopoietic progenitor cells and myeloid precursors, whereas HSA recognized by M1/75 recognizes all murine RBC and many erythroblasts, but not other hematopoietic cells. The two anti-HSA antibodies, M1/69 and M1/75, recognize the same antigen on RBC, which appears to be carbohydrate in nature. Their differing reactivities with white blood cells may reflect a difference in glycosylation or surface enrivonment on these cells (8). CLA has been found previously on T, B, and mature and immature bone marrow myeloid cells (8, 20); here, its expression on hematopoietic progenitors was also demonstrated. LGP 100a was initially described as present on only T and B cells, but in this study was found to be expressed on BFU-E, some CFU-E, CFU-GM, and CFU-M. Although it is lost during hematopoietic progenitor differentiation, its expression persists or is enhanced during lymphoid differentiation.

Five antigens, MAC-1, HSA (recognized by M1/75), Forssman antigen, anti-P-95 (recognized by M5/113), and Ia (recognized by M5/114) were found only on differentiated hematopoietic cells. Expression of these antigens was more specific for lineage and maturity than the antigens expressed on progenitors. MAC-1 was specific for polymorphs and macrophages, and was not found on CFU-GM or CFU-M, although LFA-1 and MAC-1 have identical β-subunits. In fact, MAC-1 antigen expression can be induced on the M1 myeloblast line by corticosteroids, lipopolysaccharides, and conditioned media, agents which also induce Fc and C receptors, phagocytosis, and lysosome production (21). MAC-1 is functionally associated with the macrophage/granulocyte complement receptor (11). HSA (recognized by M1/75) was specific for mature RBC, and Forssman antigen, found on early embryos and germinal tissues (8), was found only on more mature erythroid and myeloid cells. M5/113 (anti-P-95) recognizes a disulfide-linked dimer of 190,000 m.w. identical in structure to the transferrin receptor. Experiments are currently under way to determine whether this antibody does recognize this receptor. If so, the lack of reactivity of this antibody with early progenitors and heme-synthesizing cells is surprising, and may result from a low number of receptors being expressed on these cells, as compared to those of humans (4). Anti-Ia antibody was studied because Ia antigens have been found on hematopoietic progenitors (12, 22), but no expression of M5/114 on hematopoietic progenitors was found here. Ia was found only on some lymphocytes and macrophages.

The importance of identifying the cellular distribution of these antigens is to give a clearer understanding of cell surface changes during differentiation. The distribution of some of these antigens suggest that they may have an important role in hematopoiesis, being involved either in the differentiation of early progenitors or in the function of mature hematopoietic cells. Of equal importance, antibodies recognizing antigens not found on progenitors can be used to deplete marrow of mature cells and antibodies recognizing antigens found on progenitors, for positive selection in experiments designed to enrich and purify hematopoietic progenitor cells.

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