MEMBRANE RECEPTORS OF MOUSE LEUKOCYTES

II. Sequential Expression of Membrane Receptors and Phagocytic Capacity during Leukocyte Differentiation*

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The maturation sequence of hematopoietic cells has been traditionally characterized by the distinct changes that are noted in the morphologic appearance of cells. By contrast, little has been done to define leukocyte differentiation on the basis of the acquisition of mature cell membrane receptors and functional properties. Surface markers have proved to be of great value in the distinction of morphologically identical differentiation phases of mouse B and T lymphocytes (1, 2). This suggests that standard morphological criteria may be inadequate to identify certain essential phases of terminal maturation. Also, previous studies have suggested that the appearance of cell markers on cultured myeloid cells may be closely associated with cellular differentiation (3, 4).

In the current study, murine leukocytes from both normal tissues and bone marrow cultures were examined during the process of differentiation for the appearance of surface receptors for IgG (Fc receptors or FcR),[1] complement (C) receptors (CR_1 and CR_2) and phagocytic capacity. Neutrophil maturation was

* Supported by the Swiss National Foundation for Scientific Research, grants from the National Cancer Institute (CA-1690, and CA-17805) Departments of Health, Education, and Welfare and the American Cancer Society (ACS-CH3).

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[1] *Abbreviations used in this paper:* B lymphocytes, bone marrow-derived lymphocytes; C, complement; CR_1, complement receptor type one, the immune adherence (C4b-C3b) receptor; CR_2 complement receptor type two, the C3d receptor; CSF, colony-stimulating factor; DMEM, Dulbecco's modified Eagle's medium; EA_{IgG}, sheep erythrocytes sensitized with rabbit IgG antibody to sheep erythrocytes; EA_{IgM}, sheep erythrocytes sensitized with rabbit IgM antibody to sheep erythrocytes; EAC, sheep erythrocyte-rabbit IgM antibody-complement complexes; EAC1-$3b^{mo}$, EAC complexes containing mouse C3 only in its C3b form; EAC 1-$3d^{mo}$, EAC complexes containing mouse C3 only in its C3d form; $EAC14^{oxy}2^{hu}$, EAC prepared with human C1, C4, and oxidized C2; FcR, Fc receptor, cell membrane receptor for the Fc portion of IgG; FCS, fetal calf serum; FITC, fluorescein isothiocyanate; GVB, gelatin veronal buffer; GVB-S, gelatin veronal buffer containing sodium suramin; hu, human; KLH, keyhole limpet hemocyanin; mo, mouse; PBS-BSA, phosphate-buffered saline containing 1% bovine serum albumin and 0.2% sodium azide; Pi, particle ingestion; T lymphocytes, thymus-derived lymphocytes.

shown to be characterized by a distinct order of appearance of these four mature cell markers.

Materials and Methods

Mouse Leukocytes. Suspensions of spleen, bone marrow, peritoneal cells, and 4% starch induced peritoneal exudate cells were obtained from 2- to 4-mo-old C57BL/6 or AKR mice as previously described (4).[2] Pure preparations of neutrophils (90–98%) and enriched preparations of eosinophils (40–70%) were isolated by discontinuous density gradient centrifugation in Ficoll-Hypaque. A stock solution of 20% Hypaque-M (Winthrop Laboratories, New York) and 15% Ficoll 400 (Pharmacia Fine Chemicals, Piscataway, N. J.) in water (d = 1.15–1.16 g/ml, 20°C) was prepared and diluted with water to make densities 1.070 and 1.12 g/ml for cell separation. Spleen, bone marrow, and 3% dextran T500 (Pharmacia Fine Chemicals) sedimented blood cells were resuspended in 6 ml phosphate-buffered saline (PBS) in 24 × 107 mm siliconized glass tubes (Corex no. 8445, Corning Glass Works, Corning, N. Y.). Two 6-ml layers of Ficoll-Hypaque of density 1.070 and 1.12 g/ml were then layered beneath the cell suspension with a thin capillary tubing and a syringe pump (model 351, Sage Instruments, Cambridge, Mass.). After centrifugation at 1,200 g for 45 min at 20°C, cells were harvested from the two density interfaces and the pellet. After two washes with PBS containing 1% bovine serum albumin and 0.2% sodium azide (PBS-BSA), the cells were examined for differential morphology in Wright-Giemsa stained smears. Neutrophils were contained in the 1.12 g/ml density interface fraction, whereas eosinophils were found in the pellet fraction. To lyse contaminating erythrocytes, pellet fractions were further treated with Tris-buffered isotonic 0.92% ammonium chloride pH 7.2 at room temperature for 3 min and then washed three times with PBS-BSA. Spleen and blood mononuclear cells were obtained from the 1.070 g/ml interface fractions.

Soft Agar Culture of Leukocyte Colonies. Colony macrophages, neutrophils, and eosinophils were grown in semisolid medium by the bone marrow agar culture technique as described previously (4, 6). The agar-medium used was a mixture of four parts of double strength modified Eagle's medium, one part of 3% trypticase soy broth, and five parts of 0.6% Difco bacto-agar (the last boiled for 2 min and held at 37°C). The composition of the Eagle's medium was: Dulbecco's modified Eagle's medium HG instant tissue culture powder H-21 (13.47 g) (Grand Island Biological Co., Grand Island, N. Y.); double glass distilled water, 215 ml; 3 ml L-asparagine (20 μg/ml); 1.5 ml DEAE-dextran (75 μg/ml, Pharmacia, Uppsala, Sweden, mol wt = $2 \times 10^6/n/ = 0.70$); 0.575 ml penicillin (200 U/ml); 0.375 ml streptomycin (200 U/ml); 175 ml $NaHCO_3$ (2.8% wt/vol); 250 ml unheated fetal calf serum. Marrow cells were resuspended in this soft agar media at 7.5×10^4/ml with colony-stimulating factor and cultured in Petri dishes. The formation of macrophage colonies was stimulated by inclusion in each culture of lung conditioned medium (7). Neutrophil and eosinophil colony formation was stimulated by adding medium harvested from mitogen stimulated 7 day C57BL/6 spleen cell cultures (8). Colonies were individually harvested under microscopic visualization with a Pasteur pipette, and placed into 400-μl polystyrene tubes (Milian Instruments S.A., Geneva, Switzerland) containing Dulbecco's modified Eagle's medium (DMEM). Colony cells were freed from the agar by repeated gentle pipetting and washed twice with DMEM with centrifugation at 700 g for 5 min. The colony type was initially identified in the agar by the characteristic growth appearance of each colony type and subsequently by examination of the harvested cells in Wright-Giemsa stained smears.

Continuous Liquid Cultures of Bone Marrow Leukocytes. Cultures were established by the technique of Dexter et al. (9) with modifications introduced by Williams et al. (10). Briefly, proliferation of granulocyte-macrophage progenitor cells was sustained in cultures where a previously established feeder layer of bone marrow adherent cells was cocultivated with freshly isolated marrow cells. Bone marrow cells from a single femur of a $B_6D_2F_1$ mouse (Cumberland Farms, Inc., Cointown, Tenn.) were resuspended in 10 ml of Fisher's medium containing 200 mM glutamine, 10^{-4} M 2-mercaptoethanol, 100 U/ml penicillin, 100 μg/ml streptomycin, and 20% horse serum, and placed into 25-ml plastic flasks (Corning Glass Works). The adherent cell layer

[2] E. M. Rabellino, G. D. Ross, and M. J. Polley. 1977. Membrane receptors of mouse leukocytes. I. Two types of complement receptors for different regions of C3. Manuscript submitted for publication.

was then generated from this marrow cell culture over 7 days at 33°C after which all remaining nonadherent cells were removed and the cultures refed with medium containing 4×10^6 freshly isolated bone marrow cells. Each week thereafter half the cells and medium were removed and the cultures were fed with fresh medium. The cells harvested were routinely assessed for cell viability and differential morphology in standard Wright-Giemsa stained smears.

Assays for the Two Types of C Receptors. CR_1 and CR_2 on leukocytes were detected by rosette formation with EAC1-3b^{mo} and EAC1-3d^{mo}, respectively, as described in more detail elsewhere.[2] EA_{IgM} were prepared by sensitization of sheep erythrocytes (E) (Behring Werke A.G., Germany) with rabbit IgM anti-sheep erythrocyte antibody (A) (Cordis Laboratories, Miami, Fla.). EAC14^{oxy}2^{hu} were prepared from EA_{IgM} by sequential addition of purified human C components as previously described (11). EAC containing mouse C3b were prepared by incubating EAC14^{oxy}2^{hu}, in mouse serum containing sodium suramin (Bayer, Leverkunsen, West Germany). 10 ml of EAC14^{oxy}2^{hu}, suspended at 2×10^8/ml in gelatin veronal buffer (GVB) containing 1.5 mg/ml of suramin (GVB-S), were mixed together with 10 ml of mouse serum diluted 1:5 with GVB-S. After 8 min at 37°C, the EAC14^{oxy}2^{hu}3b^{mo} (EAC1-3b^{mo}) were washed once with ice-cold GVB-S and twice with ice-cold GVB. EAC containing mouse C3d were formed by incubating at 37°C for 30 min an equal volume of EA_{IgM} at 2×10^8/ml in GVB and whole C5-deficient mouse serum diluted 1:5 with GVB. To assure complete cleavage of cell bound C3b into C3d, EAC1-3d^{mo} preparations were further treated with mouse serum containing EDTA. To prevent additional uptake of C4b and C3b during this treatment, cell-bound C1 was removed by first washing the EAC1-3d^{mo} twice with warm 0.04 M EDTA-GVB and then incubating them in 0.04 M EDTA-GVB at 37°C for 30 min. For the final mouse serum treatment, the cells were resuspended at 2×10^8/ml in 0.04 M EDTA-GVB and mixed with an equal volume of mouse serum diluted 1:5 with 0.04 M EDTA-GVB and incubated at 37°C for 60 min. If the immune-adherence activity of the EAC1-3d^{mo} with human erythrocytes was more than 5% of that obtained with EAC1-3b^{mo}, the EDTA-mouse serum treatment was repeated. The assay for sheep erythrocyte-rabbit IgM antibody-complement complexes (EAC) rosette formation with leukocytes was performed as previously described (4). Colony macrophages and neutrophils were rosetted under the same conditions with a microtechnique specially developed because of the low number of colony cells available (4). Rosetted preparations were examined in cell suspensions and on fixed stained smears. For smearing the rosette mixture, cells were pelleted down at 100 *g* for 2 min, resuspended in 5–20 μl of heat inactivated whole fetal calf serum (FCS) and gently smeared on a microscope slide with a 22×11 mm coverslip. Smears were immediately air dried and stained with Wright-Giemsa. The smear technique allowed differential counts among rosetted cells. To assure accuracy in rosette enumeration, smears were scanned both horizontally and vertically and over 1,000 cells were counted. Appropriate controls were used to exclude the possibility of either rosette disruption or the production of false rosettes during smearing.

Detection of FcR. FcR were detected by either rosette formation with EA_{IgG} (sheep erythrocytes sensitized with rabbit IgG antibody) or by fluorescence assay with soluble immune complexes conjugated to fluorescein isothiocyanate (FITC) (12).[2] For the rosette assay, $0.05–5.0 \times 10^6$ leukocytes were mixed with an equal volume of EA_{IgG} at a 40–80:1 ratio of EA_{IgG} to leukocytes, centrifuged at 200 *g* for 5 min, and then incubated at room temperature for 30 min. After gently resuspending the pellet, rosettes were enumerated either in suspension by phase contrast microscopy or in Wright-Giemsa stained smears by bright field microscopy. For the fluorescence assay, $0.1–5.0 \times 10^6$ leukocytes were incubated for 30 min at room temperature with a mixture of 25 μl of rabbit IgG anti-keyhole limpet hemocyanin (KLH) conjugated to FITC (3 mg/ml) and 25 μl of KLH-conjugated to FITC (100 μg/ml). After three washes with PBS/BSA, the cells were examined for fluorescence with a Leitz Ortholux II microscope equipped with Ploem illuminator (E. Leitz, Inc., Rockleigh, N.J.).

Phagocytosis. The phagocytic capacity of normal and cultured leukocytes was measured by their ability to ingest 1.1-μm latex particles (Dow Diagnostics, Indianapolis, Ind.) or heat killed *Staphylococcus aureus*. These particles were mixed with the cells at a ratio of 50 or more particles per cell in the presence of 20% FCS (heat inactivated at 56°C for 30 min). Before use, latex and bacteria suspensions were freed of large aggregates by centrifugation of 400 *g* for 30 min. After incubation at 37°C for 60 min the cells were centrifuged through a layer of undiluted FCS and then washed twice with PBS/BSA. Particle ingestion was visualized in suspension

TABLE I
Membrane Receptors and Phagocytosis in Normal Mouse Leukocytes

Leukocyte type	Proportions of cells expressing			
	CR_1	CR_2	FcR	Phagocytosis*
	%	%	%	%
Neutrophils				
Blood	80 ± 5‡	93 ± 4	95 ± 3	97 ± 3
Spleen	60 ± 12	75 ± 10	80 ± 11	80 ± 2
Bone marrow	4 ± 3	6 ± 2	50 ± 14	6 ± 4
Monocyte-macrophages				
Blood	80 ± 4	85 ± 4	90 ± 4	90 ± 3
Spleen	85 ± 5	90 ± 3	95 ± 3	92 ± 2
Peritoneal resident	88 ± 3	90 ± 2	95 ± 2	90 ± 1
Peritoneal exudate	80 ± 5	28 ± 25	90 ± 2	87 ± 3
Eosinophils				
Blood	0	0	90 ± 2	0
Spleen	0	0	90 ± 3	0

* Phagocytosis–ingestion of latex particles or bacteria.
‡ Mean and 1 SD of cells bearing markers from a minimum of 10 experiments.

under phase contrast microscopy or in Wright-Giemsa stained smears. Cells that had been labeled with latex were frequently also tested for other markers.

Results

Membrane Markers on Normal Tissue Leukocytes. Mouse leukocytes obtained from various normal tissues were studied for the presence of membrane CR_1, CR_2, FcR, and phagocytosis (Table I). The proportion of receptor bearing cells of each type of leukocyte was determined either by testing pure populations of each leukocyte type or by examining Wright-Giemsa stained unseparated leukocyte preparations after formation of EAC or EA_{IgG} rosettes. When FcR were assayed by the immunofluorescence technique, the morphology of FcR bearing cells was assessed by phase contrast microscopy. The majority of blood and spleen neutrophils were actively phagocytic and expressed CR_1, CR_2, and FcR. In contrast, only a small proportion of the marrow neutrophilic cells contained these same markers. Differential analysis revealed that all of the blood and about 90% of the spleen neutrophils were morphologically mature cells containing either segmented or O-ring shaped nuclei. Among marrow neutrophils, however, only 60% were mature forms. Multilabel analysis of morphologically mature neutrophils disclosed that the four markers were present on all blood neutrophils and over half of the spleen neutrophils. By contrast however, only a small proportion of morphologically mature bone marrow neutrophils contained all four markers. Of the mature neutrophilic cells that did not express all four markers, most had FcR, fewer had CR_2, and phagocytosis was variable. Also, simultaneous rosette formation with both types of EAC disclosed that approximately one third of the mature spleen and marrow neutrophils had CR_2 but not CR_1. Myelocytes and metamyelocytes

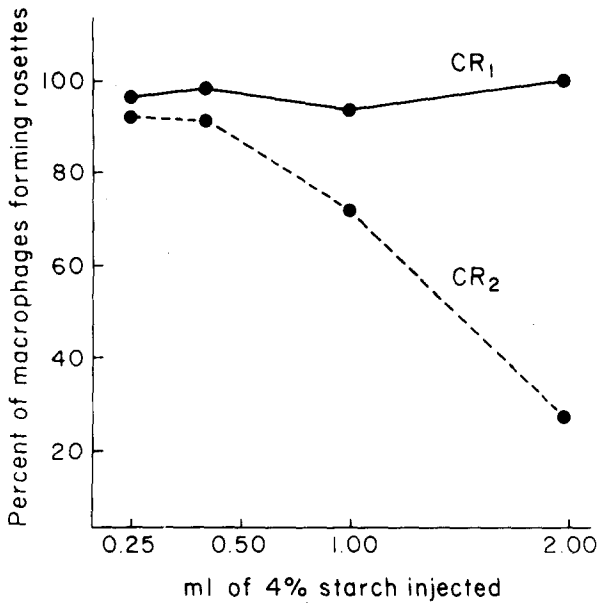

FIG. 1. Effect of starch injection on the expression of CR_1 and CR_2 by peritoneal exudate macrophages.

from bone marrow contained only FcR and lacked C receptors. C receptors and phagocytosis were associated exclusively with more mature neutrophils having either segmented or O-ring shaped nuclei.

Peritoneal exudate macrophages differed from the majority of normal tissue monocyte-macrophages in that variable proportions of cells did not express CR_2 (Table 1). This variation in CR_2 exppression among different exudate preparations may have been related to the degree of macrophage activation, since CR_2 expression was inversely proportional to the amount of starch injected (Fig. 1). Furthermore, kinetic studies of phagocytosis disclosed that exudate macrophages ingested EAC1-$3b^{mo}$ more efficiently than EAC1-$3d^{mo}$, and that EAC ingestion was directly correlated with EAC rosette formation (Table II).

Eosinophils isolated from the blood and spleen differed from neutrophils and macrophages in that they contained only FcR, and the other three markers were not detectable (Table I).

Membrane Markers on Bone Marrow Cells Grown in Agar Culture. Colony leukocytes harvested from agar cultures at various times were tested for membrane markers (Table III). Over 75% of the colony macrophages obtained from 3 to 11-day cultures had all four markers. Morphological examination of Wright-Giemsa stained smears demonstrated that all receptor bearing cells were morphologically mature macrophages that resembled those isolated from normal tissues. Colony neutrophils harvested from 4, 7, and 9-day cultures were also morphologically mature, however they expressed only CR_2 and FcR, and totally lacked CR_1 and the ability to phagocytize latex or bacteria. Colony eosinophils were similar to tissue eosinophils in that all markers except FcR were undetectable.

Membrane Receptors in Continuous Bone Marrow Cultures. Leukocytic

TABLE II

Rosette Formation and Ingestion of EAC by Macrophages from a Starch-Induced Peritoneal Exudate

Time of incubation	Rosette formation with		Ingestion of	
	EAC1-3b^{mo}	EAC1-3d^{mo}	EAC1-3b^{mo}	EAC1-3d^{mo}
Min	%	%	%	%
1	70	20	0	0
3	90	25	50	5
5	100	30	70	8
10	100	50	80	9
15	100	55	82	11
30	100	70	90	40

TABLE III

Membrane Receptors and Phagocytosis in Mouse Colony Leukocytes Grown in Agar Culture

Colony type	Days in culture	Proportion of colony cells expressing			
		CR_1	CR_2	FcR	Phagocytosis*
		%	%	%	%
Macrophages	3	75‡	76	75	80
	7	80	83	85	83
	11	90	88	79	80
Neutrophils	4	0	4	70	0
	7	0	56	80	0
	9	0	59	70	0
Eosinophils	5	0	0	34	0
	7	0	0	50	0
	11	0	0	50	0

* Ingestion of latex or bacteria.

‡ Averages from 6–8 sets of cultures.

cells from long-term liquid cultures of bone marrow were analyzed for differential morphology, production of pluripotent stem cells, and granulocytic progenitor cells and the presence of surface markers. Each week half of the cell suspension from six cultures was pooled for assay. Pluripotent stem cells and granulocyte-macrophage progenitor cells were produced over the time of culture as described elsewhere (10). Cell numbers per culture ranged from 0.8×10^6 at wk 1, and 24×10^6 at wk 2 to 1.0×10^6 at wk 5. Cells representing all morphologic stages of both neutrophilic and monocytic maturation were continuously present in these cultures. The differential morphology was similar at all times with 15–20% blast cells, 5–12% mononuclear cells and macrophages, with the remainder of cells being immature and mature granulocytes. Even though macrophages expressed all four markers, neutrophilic cells contained in the same cultures exhibited only some of the mature cell markers (Table IV). After the 2nd and 3rd wk of culture, variable proportions of neutrophilic cells had CR_2, FcR, and phagocytic activity, but none contained CR_1. CR_1 did not appear on neutrophilic cells until the 4th wk of culture.

TABLE IV

Membrane Receptors and Phagocytosis in Leukocytes Grown in Long-Term Bone Marrow Cultures

Leukocyte type	Week of culture	Proportion of cells expressing			
		CR_1	CR_2	FcR	phagocytosis*
		%	%	%	%
Monocyte-macrophages	2	85	90	85	90
	3	80	85	90	89
	4	90	77	90	92
	5	76	67	85	95
Neutrophils	2	0	20	70	48
	3	0	14	65	63
	4	62	14	80	59
	5	56	26	70	62

* Ingestion of latex particles or bacteria.

TABLE V

Expression of Markers on Cells of the Myelocytic Series Grown in Long-Term Bone Marrow Cultures

	Expression of markers at different phases of maturation											
	CR_1			CR_2			FcR			Phagocytosis		
Week of culture	Pro-meta* myelocyte	O-ring	Seg.	Pro-meta myelocyte	O-ring	Seg.	Pro-meta myelocyte	O-ring	Seg.	Pro-meta myelocyte	O-ring	Seg.
		%			%			%			%	
2	0	0	0	0	26	17	20	30	80	0	13	70
3	0	0	0	0	10	16	15	25	70	0	20	65
4	0	45	87	0	8	17	18	30	74	0	30	70
5	1	68	80	0	28	43	25	35	76	0	38	70

* Abbreviations used: pro-metamyelocyte, promyelocytes, myelocytes, and metamyelocytes; O-ring, neutrophils with O-ring shaped nucleus; seg., neutrophils with segmented nucleus (polymorphs).

To investigate the relationship between the appearance of markers and the degree of cell maturation, the morphology of rosetted cells was examined in Wright-Giemsa stained smears. These studies disclosed that myelocytes and metamyelocytes bore only FcR, and that CR_2, CR_1, and phagocytic activity were exclusively associated with more mature neutrophils (Table V). The acquisition of C receptors by morphologically mature cells was apparently a terminal phase of neutrophilic maturation in culture, because many morphologically mature neutrophils with O-ring and segmented nuclei did not contain either type of C receptor. Furthermore, even though FcR appeared at an early stage of maturation, 20% of more of segmented neutrophils lacked detectable FcR.

Discussion

The bone marrow is the source of a wide variety of leukocyte types that are all believed to develop from a single primitive pluripotent stem cell. Much

attention has recently been focused on this process because several types of disease are believed to involve abnormal or suppressed leukocyte differentiation. The value of surface marker analysis for the distinction of morphologically identical lymphocyte subsets has been clearly established, but there have been relatively few studies that have been able to relate the presence of particular membrane markers to the differentiation process of other types of leukocytes. Komuro and Boyse (1), and Hämmerling et al. (2), who pioneered in this field, have shown that there is a distinct order of appearance of membrane markers during the differentiation of B and T lymphocytes (1, 2). Also studies performed on cultured leukemic and normal bone-marrow derived leukocytes have suggested that the appearance of C receptors and FcR may be associated with cell differentiation (3, 4). In the present study, murine leukocytes from both normal tissue and bone-marrow cultures were examined for the sequential appearance of three different membrane receptors and one functional marker during the process of cell maturation. Among differentiating neutrophils, a distinct sequence was observed in the appearance of FcR, C receptors (CR_1 and CR_2), and the functional capacity to ingest various small particles (Pi). These studies were facilitated by the finding of an apparent differentiation blockade that was present in certain types of neutrophil cultures, and by the development of specialized techniques for the simultaneous examination of individual cells for membrane receptors, phagocytosis, and cell morphology.

In normal tissue, there was considerable variation in the expression of mature cell markers on neutrophils that were morphologically mature polymorphonuclear cells. Whereas over 95% of blood polymorphs expressed all four markers, the same was true of only half of spleen polymorphs and only 20% of bone marrow polymorphs. This latter finding indicated that a heterogeneity existed among morphologically identical polymorphs that was only detectable by means of surface marker and functional analysis.

A similar type of heterogeneity in mature-appearing polymorphs was observed in bone marrow cultures. Even though cultures of bone marrow myeloid cells developed into morphologically mature polymorphs, they differed from normal blood neutrophils in that they lacked some of the four mature cell markers. Colony neutrophils from soft agar cultures contained FcR and CR_2, but lacked CR_1 and Pi. This apparently represented a major functional deficiency in the agar-grown neutrophils, since they not only failed to ingest latex but also failed to ingest carbonyl-iron, heat killed bacteria, and membrane receptor-bound EAC1-3d, EA_{IgG}, and $EA_{IgG}C1$-3d. Neutrophils grown from marrow in a long-term liquid culture system differed from agar colony neutrophils in that Pi was noted in 2- to 3-wk-old cultures in addition to FcR and CR_2. However, similar to agar cultures, neutrophils from these 2 and 3 wk continuous liquid cultures did not express CR_1. CR_1 appeared abruptly in these continuous cultures after 4 wk of incubation, completing the marker profile of normal blood neutrophils. The incomplete marker expression of cultured neutrophils apparently represented a selective blockade of neutrophil differentiation rather than any kind of dedifferentiation process, since both the agar and liquid cultures sustained the proliferation and full differentiation of macrophages. This selective blockade of neutrophil maturation may have been due to insufficient

concentrations of neutrophil-specific differentiation-inducing factors. Colony-stimulating factor (CSF), the stimulus for neutrophil and macrophage colony formation in vitro, is thought to be a regulator of granulopoiesis, and it is of note that colony stimulating factor can not be detected in the suspension medium of those long term cultures (N. Williams, unpublished observations) (9). It is therefore possible that CSF is rate limiting over the first weeks of culture, preventing full expression of membrane components.

While myeloblasts obtained from bone marrow or bone marrow cultures did not contain any of the four mature cell markers, a significant proportion of myelocytes and metamyelocytes expressed FcR and occasionally also CR_2. Most mature neutrophils had FcR, while lower proportions had CR_2 and CR_1. The acquisition of these markers was directly correlated with cell maturation, since the proportions of marker-bearing cells increased progressively with morphologic maturation. In particular, terminal morphologic maturation from an O-ring shaped nucleus to a segmented nucleus was directly associated with an increase in the expression of all four markers. Thus, undifferentiated myeloblasts underwent a progressive process of proliferation and differentiations that was accompanied by the sequential expression of mature cell markers. Myelocytes and metamyelocytes expressed FcR and occasionally also CR_2. As neutrophilic cells matured beyond this stage, the proportions of cells containing FcR and CR_2 increased, and then finally CR_1 appeared along with FcR and CR_2 as a terminal maturation event. Thus, by correlating morphologic maturation with cell marker expression, a sequence of marker appearance was determined that can be used to identify important terminal maturation stages that are not obvious by changes in morphologic appearance: FcR $\rightarrow$ CR_2 $\rightarrow$ Pi $\rightarrow$ CR_1.

The vast majority of both tissue and cultured macrophages contained all four mature cell markers. Studies of the order of appearance of these markers during monocyte differentiation were not possible because of the lack of morphologically distinct maturation stages. Furthermore, among morphologically mature cells in culture there were no intermediate stages of marker appearance. More than 90% of macrophages obtained from 2 and 3-day agar colony clusters already contained all four mature cell markers.

Even though it was not possible to correlate marker appearance with the early phases of monocyte maturation, the terminal phase of differentiation from mature monocytes into activated macrophages was shown to be associated with the loss of membrane CR_2. Mature monocyte-macrophages obtained from either normal tissue or bone marrow cultures contained all four markers, whereas starch induced peritoneal exudate macrophages were missing CR_2. The loss of CR_2 was directly proportional to the amount of starch that was injected to induce the exudate. Furthermore, since starch injection was also correlated with an increase in EAC1-3b phagocytosis, then the loss of CR_2 occurred simultaneously with macrophage activation (12a). This conclusion is in complete agreement with the findings of Griffin et al. (13) that activated peritoneal exudate macrophages contain only CR_1 and lack CR_2.

Tissue and colony eosinophils contained only FcR and lacked the other markers of mature neutrophils and macrophages. In this regard, murine eosinophils are distinctly different from human eosinophils that contain both CR_1 and CR_2 and are actively phagocytic (14).

These data suggest that six stages of granulocytic cell differentiation can be distinguished by cell marker analysis.

Stage 1. No markers (myeloblasts, promyelocytes); Stage 2. FcR (myelocytes → polymorphs); Stage 3. FcR, CR_2; Stage 4. FcR, CR_2, Pi; Stage 5. FcR, CR_2, Pi, CR_1 (mature neutrophils and monocytes); Stage 6. FcR, Pi, CR_1 (activated macrophages).

Parallel studies of human neutrophil maturation markers have shown a similar sequence of appearance of membrane CR_1 and CR_2. Human blood neutrophils differed from murine blood neutrophils in that fully mature human polymorphonuclear cells contained only CR_1 and lacked CR_2.[3] Even though a similar loss of CR_2 was not observed with murine neutrophils, the loss of CR_2 was a marker of murine macrophage terminal differentiation into activated cells.

Although previous studies have demonstrated that a variety of neutrophil and macrophage functions were closely associated with the expression of FcR and C receptors (15–17), there have been no previous attempts to relate the appearance of these mature cell properties with the degree of cell differentiation. Phagocytosis, for example, was thought to be directly dependent upon the interaction of particles with either FcR of C receptors (12, 18). However, it is also recognized that the ingestion induced by C or Fc receptors does not necessarily trigger the ingestion of particles whose phagocytosis is mediated by other signals (13). Thus, it is probable that mature cell phagocytic activity is independent of FcR and C receptors. The present finding of independent expression of membrane receptors and phagocytic activity in cultured neutrophils lends further support to this conclusion. In particular, it was striking that simultaneous attachment of $EA_{IgG}C1\text{-}3d$ to both FcR and CR_2 did not trigger ingestion of these complexes into agar colony neutrophils that were deficient in particle ingestion capacity.

The findings of these studies have provided a new way to analyze the terminal phases of neutrophil and macrophage maturation. Surface marker expression seems to be closely correlated with the acquisition of mature cell functions and independent of the standard morphologic criteria for analyzing leukocyte maturation. Thus, cell marker analysis may be useful for the diagnosis of abnormal or suppressed leukocyte maturation, and it may provide additional means to distinguish important neutrophil and macrophage subsets that may be characteristic of specific inflammatory, leukemic, or degenerative disease processes.

Summary

Analysis of four mature cell markers on mouse bone marrow leukocytes grown in vitro, demonstrated a distinct sequence of marker appearance during the terminal phases of granulocytic cell differentiation. A similar pattern of marker expression was also suggested by analysis of mature neutrophils and macrophages isolated from normal tissues. Among cultured neutrophils, recep-

[3] G. D. Ross, C. I. Jarowski, E. M. Rabellino, and R. J. Winchester. 1977. The sequential appearance of Ia-like antigens and two different complement receptors during the maturation of human neutrophils. Manuscript submitted for publication.

tors for the Fc portion of IgG (FcR) were first expressed on myelocytes and metamyelocytes, and then subsequently on more mature cells. Morphologically mature colony neutrophils (polymorphs) from agar cultures contained only FcR and complement receptor type two (CR_2) (C3d receptor), and lacked both complement receptor type one (CR_1) (C3b receptor) and the capacity to ingest latex, bacteria, or iron particles. Neutrophils from 2 and 3 wk liquid media cultures of marrow cells differed from agar grown neutrophils in that they had phagocytic capacity (particle ingestion) [Pi] in addition to FcR and CR_2. Furthermore, in the 4th and 5th wk of these continuous liquid cultures, CR_1 was also expressed, completing the surface marker profile of normal blood neutrophils. Based on these studies, the following order of appearance of these four markers on cells from the myelocytic series was proposed:

$$\text{FcR} \rightarrow \text{FcR CR}_2 \rightarrow \text{FcR CR}_2 \text{ Pi} \rightarrow \text{FcR CR}_2 \text{ Pi CR}_1$$

Differential studies of tissue leukocytes containing these same markers revealed that a heterogeneity existed among morphologically mature neutrophils. Even though 95% of blood polymorphs contained all four markers, the same was true of only half of spleen polymorphs and only 20% of bone marrow polymorphs. Cells of the monocyte-macrophage series were studies in parallel with neutrophils. Cultured marrow monocytes acquired the four mature cell markers so rapidly that the order of receptor appearance could not be determined. However, it was found that CR_2 was lost during the terminal phase of monocyte maturation into activated macrophages.

The authors thank Dr. Jean-Charles Cerottini and Dr. Nicholas Odartchenko for their helpful advice and criticisms during this work. We would like also to acknowledge our technical assistants Miss. Chantal Frey and Mrs. Leslie Goodwin.

Received for publication 3 October 1977.

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