Cellular Differentiation in a Murine Myelomonocytic Leukemia

By M. J. Cline and D. Metcalf

Mouse peritoneal macrophages and macrophages derived from normal bone marrow progenitors in vitro possess surface receptors whose predominant specificity is directed toward the IgG2a subclass of IgG molecules. Myelomonocytic leukemic cells also possess such IgG receptors but in an abnormally low frequency and without demonstrable subclass specificity. Within the leukemic population, monoblasts are distinguished from myeloblasts by their greater adhesiveness to glass, higher frequency of cells with surface receptors for IgG, and by erythrophagocytosis. Leukemic monoblasts, myeloblasts, and their morphologically mature progeny demonstrate impaired phagocytic ability. A concept of the development of surface receptors for IgG in the monocyte-macrophage cell line is presented.

A TRANSPLANTABLE MYELOMONOCYTIC LEUKEMIA of BALB/c mice is capable of giving rise to mixed colonies of partially differentiated mononuclear and granulocytic elements in agar cultures.1,2 In vivo, replication of tumor stem cells is influenced by organ-specific microenvironments.3

The present study was undertaken with the objective of identifying markers of differentiated function in the leukemic cells grown both in the intact animal and in vitro culture. The markers chosen were phagocytosis and the presence of a surface receptor for immunoglobulin G (IgG).4

MATERIALS AND METHODS

Cell Preparations

The B subline of the WEHI-3 myelomonocytic leukemia previously described 1·3 was maintained in inbred BALB/c mice.1·3 Single-cell suspensions of the WEHI-3 tumor were prepared by pressing minced spleen through a fine steel mesh followed by pipetting in either modified Eagle's medium3 or medium 199 containing 30% fetal calf serum (F-199). Bone marrow cells were isolated from normal C57BL or BALB/c mice by the gentle injection of Eagle's medium through femoral shafts. Peritoneal macrophages were obtained from C57BL or BALB/c mice by the method of Cohn and Benson11 and were suspended in F-199.

Cell Culture

Soft Agar: WEHI-3 cells and normal bone marrow cells were cultured in soft agar as previously described.3 Normal colonies were grown from BALB/c bone marrow using serum from endotoxin-injected C57BL mice to stimulate colony formation.12

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**Glass Adherence:** WEHI-3 cells or peritoneal macrophages in F-199 at a concentration of $2 \times 10^8$/ml were allowed to adhere to sterile cover slips at $37^\circ$C for 3 hr. Nonadherent cells were washed off with warm medium, and incubation of adherent cells was resumed for 2 or 48 hr. Agar colonies derived from normal bone marrow after 8–10 days in culture were dispersed in F-199, and the cells were allowed to form monolayers on glass cover slips under similar conditions.

**IgG-Receptor Analysis**

A 5-day primary and a 7-day secondary mouse antiserum to sheep erythrocytes (SRBC) was separated into IgM- and IgG-rich fractions, respectively, on Sephadex G-100/200. SRBC were sensitized by antiserum or globulin fractions at $37^\circ$C from 60 min.

Unsensitized or antibody-coated SRBC at a concentration of $1 \times 10^7$/ml were added to monolayers of glass-adherent WEHI-3 cells, bone marrow macrophages, or peritoneal macrophages and incubated at $37^\circ$C for 30 min. SRBC not bound or ingested by white cells were then washed from the cover slip with warm medium. The cover slips were fixed in methanol, stained with Giemsa, and scored for the number of leukocytes with adherent or phagocytized SRBC.

In an alternate technique for measuring IgG-coated SRBC adherence, sensitized erythrocytes ($5 \times 10^6$/ml) were incubated in suspension with leukocytes (approximately $5 \times 10^4$/ml) for 30 min at $37^\circ$C in microtiter plates; a drop of suspension was removed and placed under a cover slip, and the percentage of leukocytes with adherent SRBC was determined visually at a magnification of 400X.

The effects of specific immunoglobulins on the binding of IgG-coated SRBC to leukocytes were determined by adding purified globulin fractions to the leukocytes immediately before the SRBC. The globulin fractions were obtained from the sera or ascites fluid of mice bearing plasma cell tumors of defined type: IgA (HPC-1, 38), IgG1 (HPC-77, -9, -78, -3), IgG2a (GPC-7, -8 HPC-3), IgG2b (MPC-86, HPC-71, -84), IgM (MOPC-104, HPC-76), and light chains (MPC-76, HPC-4).

**Phagocytosis**

*Candida albicans* grown overnight in Sabouraud's medium were washed in saline and suspended in M-199 containing 10% normal mouse serum. *Candida* at a concentration of $5 \times 10^8$/ml were added to cell monolayers for 30 min at $37^\circ$C. Monolayers were washed, fixed, and stained.

**Morphologic Preparations**

Cell suspensions, deposited on glass slides by means of a cytocentrifuge (Shandon Instrument) or as monolayers, were fixed in methanol and stained with Giemsa.

In studies of $^3$H-thymidine incorporation, leukemic mice were injected intravenously with 100 μCi of $^3$H-thymidine 1 hr before sacrifice. Fixed cell preparations were coated with NTB emulsion (Kodak) and exposed for 7–10 days before development. Peroxidase staining was by the method of Rytomaa.

**RESULTS**

**Morphology**

Ten days after the injection of WEHI-3 cells, the spleens of leukemic mice contained a heterogeneous population of cells comprised of the following: myeloblasts and promyelocytes, 65–78%; monoblasts, 9–21%; mature macrophages, 9–13%; other cells including lymphocytes and mature granulocytes, 0–4% (Table 1). Of this population 48±9% were peroxidase positive.

In Giemsa-stained preparations, myeloblasts appeared as round cells 10–18 μ
diameter, with a high nuclear to cytoplasmic ratio. Nuclear chromatin was uniformly dispersed, and two to four prominent nucleoli were evident. In the thin rim of blue cytoplasm, granules were rarely evident in Giemsa-stained preparations, but with peroxidase staining, positive granular material was present in the perinuclear region.

Monoblasts were identified by their larger size of 18–30 μ and irregularly lobulated nucleus with a lacy chromatin structure containing one or two rather inconspicuous nucleoli. The cells had abundant pale blue cytoplasm that was often irregular in outline and contained occasional granules both in stained preparations and by phase microscopy. Monoblasts often contained small amounts of peroxidase-positive material.

**Labeling by ³H-Thymidine and Glass Adherence**

One hour after administration of ³H-thymidine to a leukemic animal, 33% of the myeloblasts and 39% of the monoblasts were labeled. No labeling was visible in either mature macrophages or differentiated granulocytes. When these cells labeled in vivo were incubated as a monolayer on a glass cover slip, the pattern of distribution of label among the glass-adherent cells was quite different from the pattern in the total population (Table 1). Whereas 34% of the total population contained radioactive label, only 10% of the glass-adherent cells were labeled. These data indicate that cells in S phase at the time of isolation are less likely to exhibit glass adherence than the general population. It was also noted that the glass-adherent population was enriched in monoblasts relative to myeloblasts (Table 1).

**Surface Receptors for IgG**

When cells isolated from a leukemic spleen were incubated in suspension with sheep erythrocytes coated with mouse IgG, 7±2% of the cells became surrounded with a rosette of red cells (Fig. 1). Rosette formation was not observed with uncoated SRBC or SRBC sensitized by IgM antibody at a concentration that produced a degree of hemagglutination equivalent to that produced by IgG antibody.

<table>
<thead>
<tr>
<th>Population</th>
<th>Total Population</th>
<th>Myeloblasts</th>
<th>Monoblasts</th>
<th>Macrophages</th>
<th>Others</th>
</tr>
</thead>
<tbody>
<tr>
<td>All cells</td>
<td>—</td>
<td>77</td>
<td>10</td>
<td>13</td>
<td>&lt;1</td>
</tr>
<tr>
<td>Glass-adherent cells</td>
<td>—</td>
<td>46</td>
<td>45</td>
<td>9</td>
<td>&lt;1</td>
</tr>
<tr>
<td>Cells labeled with ³H-thymidine</td>
<td>34</td>
<td>33</td>
<td>39</td>
<td>&lt;0.1</td>
<td>&lt;0.1</td>
</tr>
<tr>
<td>Glass-adherent cells labeled with ³H-thymidine</td>
<td>10.3</td>
<td>9.6</td>
<td>13.1</td>
<td>&lt;0.1</td>
<td>&lt;0.1</td>
</tr>
<tr>
<td>Glass-adherent cells with IgG receptors</td>
<td>30</td>
<td>9.6</td>
<td>47</td>
<td>55</td>
<td>&lt;0.1</td>
</tr>
</tbody>
</table>

*Values given are percentage distribution of morphologically identifiable components of various populations of WEHI-3 cells and are the means of at least four experiments. Range of values can be found in text.*
In contrast to the low levels of IgG rosette formation demonstrable with leukemic cells, 60±4% of mature macrophages derived from agar colonies of normal mouse bone marrow and 70±6% of newly isolated peritoneal macrophages formed rosettes in suspension. IgM-coated and unsensitized SRBC again did not form rosettes with normal bone marrow or peritoneal macrophages.10

Rosette formation and erythrophagocytosis by normal bone marrow and peritoneal macrophages are more efficient when these cells are adherent to a surface than free in suspension.7,14 When adherent, 88±12% of normal bone marrow colony macrophages and 83±15% of normal peritoneal macrophages demonstrate erythrophagocytosis and/or rosette formation. In contrast, 47±19% of adherent monoblasts and only 10±2% of adherent myeloblasts bound or phagocytized sensitized SRBC. Whereas erythrophagocytosis by monoblasts was occasionally observed, myeloblasts were never seen to be phagocytic. The numbers of SRBC adhering to monoblasts were always much greater than the numbers adhering to myeloblasts. Rarely did more than one or two erythrocytes adhere to myeloblasts.

The IgG receptors of normal mammalian macrophages are specific for certain subclasses of IgG molecule.15-17 To ascertain the IgG subclass specificity of macrophage receptors in the mouse, purified immunoglobulins at 300 µg/ml were added to monolayers of WEHI-3 cells or normal macrophages immediately before the addition of IgG-coated SRBC. Inhibition of rosette formation was taken as evidence of competition for a surface immunoglobulin receptor. As seen in Table 2, only proteins of subclass IgG2a produced marked inhibition of rosette formation by normal macrophages. Some IgG1 proteins produced modest inhibition. In contrast, comparable and even twofold higher concentrations of the same proteins failed to inhibit rosette formation by WEHI-3 cells.

**Phagocytosis**

Glass-adherent macrophages, derived from 10-day-old agar colonies of normal bone marrow or from the peritoneal cavity of normal mice, were
incubated with heat-killed *C. albicans* in vitro. Phagocytosis of multiple organisms was demonstrable in 97% of the normal bone marrow colony macrophages and 99% of the normal peritoneal cells. In contrast, only 7% of glass-adherent WEHI-3 cells were phagocytic, whether obtained directly from the animal or cultured in vitro. Phagocytosis was observed in mature macrophages and in rare monoblasts but not in myeloblasts.

*Cultivation of Cells In Vitro*

When normal bone marrow was cultured in vitro in soft agar, typical colonies developed. These were composed principally of granulocytic elements up to the fifth day of culture. Thereafter, macrophages became increasingly prominent until the colonies became moribund between day 12 and 15 of culture. Concomitant with a progressive increase in identifiable macrophages, an increase in the proportion of cells bearing IgG receptors occurred.

In contrast, WEHI-3 myeloblasts and monoblasts persisted in agar cultures for at least 10 days. The differential count of cells obtained from normal bone marrow and WEHI-3 colonies at day 8 of culture is shown in Table 3. The undifferentiated blast cells and morphologically mature WEHI-3 macrophages failed to show a normal complement of IgG receptor-positive cells.

<table>
<thead>
<tr>
<th>Competing Protein</th>
<th>Per Cent of Control SR BC Binding in Absence of Competing Protein</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Bone Marrow Colony</td>
</tr>
<tr>
<td>IgA</td>
<td></td>
</tr>
<tr>
<td>HPC-1</td>
<td>98</td>
</tr>
<tr>
<td>HPC-38</td>
<td>88</td>
</tr>
<tr>
<td>IgG1</td>
<td></td>
</tr>
<tr>
<td>HPC-77</td>
<td>86</td>
</tr>
<tr>
<td>HPC-9</td>
<td>51*</td>
</tr>
<tr>
<td>HPC-32</td>
<td>67</td>
</tr>
<tr>
<td>HPC-78</td>
<td>86</td>
</tr>
<tr>
<td>IgG2a</td>
<td></td>
</tr>
<tr>
<td>HPC-3</td>
<td>52*</td>
</tr>
<tr>
<td>GPC-7</td>
<td>60*</td>
</tr>
<tr>
<td>GPC-8</td>
<td>41*</td>
</tr>
<tr>
<td>IgG2b</td>
<td></td>
</tr>
<tr>
<td>HPC-84</td>
<td>92</td>
</tr>
<tr>
<td>HPC-71</td>
<td>92</td>
</tr>
<tr>
<td>MPC-86</td>
<td>84</td>
</tr>
<tr>
<td>IgM</td>
<td></td>
</tr>
<tr>
<td>MOPC-104</td>
<td>—</td>
</tr>
<tr>
<td>HPC-76</td>
<td>97</td>
</tr>
<tr>
<td>Light chains</td>
<td></td>
</tr>
<tr>
<td>MPC-76</td>
<td>96</td>
</tr>
<tr>
<td>HPC-4</td>
<td>97</td>
</tr>
</tbody>
</table>

*Denotes values significantly different from controls containing no competing protein; p <0.01.*
Table 3. Differential Counts of 8-Day Agar Colonies of Normal BALB/c Bone Marrow and WEHI-3

<table>
<thead>
<tr>
<th>Source</th>
<th>Myeloblasts</th>
<th>Monoblasts</th>
<th>Undiff. Blasts</th>
<th>Macrophages</th>
<th>Myelocytes</th>
<th>PMN</th>
<th>Unidentified</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal marrow</td>
<td>--</td>
<td>2</td>
<td>65</td>
<td>29</td>
<td>4</td>
<td></td>
<td>4</td>
</tr>
<tr>
<td>WEHI-3</td>
<td>72</td>
<td>18</td>
<td>3</td>
<td>3</td>
<td>4</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*One hundred colonies from six plates were pooled for counts.

DISCUSSION

Among the phagocytic leukocytes, the presence of strong surface receptors for IgG globulin appears to be a unique feature of the monocyte-macrophage cell line. Specificity is directed toward the Fc portion of the molecule and only toward certain subclasses of IgG molecules. The macrophage IgG receptor is similar, if not identical, to that which binds cytophilic antibody. The observations reported here indicate that such receptors are also demonstrable on primitive cells of a murine myelomonocytic leukemia. Both myeloblasts and monoblasts of this tumor possess such receptors, although the latter are more often positive. Two abnormalities in the development of this marker were demonstrable in leukemic cells. First, the fraction of leukemic cells developing this marker either in the animal or in culture was always small; the majority of leukemic cells lacked this differentiated characteristic. Second, no specificity for a subclass of IgG molecules could be demonstrated for the malignant cells. Whether this is a qualitative or quantitative abnormality of the receptor of leukemic cells is unknown.

Monoblasts and myeloblasts were equally labeled by $^3$H-thymidine in vivo. Consequently, no progenitor-progeny relationship could be detected between these morphologically distinguishable cell types. Monoblasts were characterized by their greater glass-adhesiveness, higher frequency of IgG receptor-positive cells, and erythrophagocytic ability. Monoblasts probably had a higher surface density of IgG receptors than myeloblasts, as evidenced by greater numbers of adherent red cells and red cells adherent when low concentrations of antibody were used for sensitization. Phagocytosis of a large microorganism (C. albicans) was subnormal in leukemic macrophages, was rarely observed in monoblasts, and was never observed in immature leukemic granulocytes.

We have recently observed that the IgG-receptor marker is detectable on the normal bone marrow "promonocyte" and on rare cells in the 12–14-day

![Diagram](image)

**Fig. 2.** Postulated sequence of development of IgG receptors in normal and malignant cells of monocyte-macrophage series.
fetal liver and yolk sac of the mouse. It is not detectable on hematopoietic stem cells of the early yolk sac, although such stem cells are capable of giving rise to typical IgG-positive macrophages in culture.\textsuperscript{20,21} Our present concept of the development of the macrophage IgG receptor is illustrated in Fig. 2. Once normal hematopoietic stem cells are committed to the development of the monocyte-macrophage pathway, IgG receptors are detectable at the cell surface and become increasingly prominent with maturation toward the macrophage. Leukemic monoblasts and myeloblasts also possess such receptors, but their development is quantitatively and perhaps qualitatively abnormal. The presence of IgG receptors on some myeloblasts is a further argument that these cells share a common progenitor with monoblasts.

REFERENCES

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