Embryonic Origin of the Mouse Macrophage

By M. J. Cline and M. A. S. Moore

Progenitor cells capable of giving rise to functional macrophages in in vitro culture were first detectable in the fetal yolk sac of the mouse between day 7 and 8 of gestation. Macrophage progenitors were not detectable outside the yolk sac until day 11. Although the early yolk sac contained macrophage precursors, no cells with the morphologic or functional characteristics of mouse promonocytes or more mature macrophages were observed. Promonocytes and macrophages were first identified in the 10-day yolk sac and 11-day fetal liver. These cells were characterized by surface receptors for IgG immunoglobulin, peroxidase activity (promonocytes), glass adherence, and phagocytosis of a large yeast particle (macrophages). From these observations, we conclude that the early fetal yolk sac is the embryonic site of origin of the macrophage precursor and that this precursor is "proximal" to the promonocyte on the pathway of sequential macrophage maturation.

The blood islands of the yolk sac are the first sites of hematopoiesis in both birds and mammals.1-3 Hematopoietic stem cells, originating in these blood islands, migrate from the yolk sac into the circulation and subsequently colonize and proliferate within the hematopoietic organs of the developing embryo.1-4 The hematopoietic stem cell content of yolk sac is demonstrable in experiments in which chick or mouse embryo yolk sac cells are used to repopulate both the myeloid and lymphoid tissues of irradiated recipients.2-5 Furthermore, multipotential stem cells detected by the in vivo spleen colony assay and progenitor cells of granulocytes and macrophages detected by the in vitro agar colony-forming assay have been demonstrated within the mouse yolk sac and subsequently within developing fetal liver.6

Recently, a bone marrow precursor of the monocyte-macrophage cell line has been identified in adult mice.6 This promonocyte has been characterized by its morphology, glass adheriveness, limited phagocytic ability, DNA-synthetic capacity, and kinetic analysis of its relationship to more mature cells of this line.6,7 We have also recently observed that very primitive cells of the monocyte-macrophage line also possess the surface IgG-receptor that is characteristic of the monocyte and mature macrophage.8-13 On the basis of this series of observations, we undertook a study with the dual objectives of identifying the embryonic site of origin of the macrophage precursor and of determining whether this precursor was similar to the adult promonocyte.

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MATERIALS AND METHODS

Cell Preparations

The uterus was removed from 7 to 18-day pregnant C57BL mice 7–18 days after detecting a vaginal plug subsequent to mating with CBAT6T6 mice. The embryos, together with the yolk sacs, were dissected in Eisen’s balanced salt solution (EBSS) to remove trophoblast, Richter’s membrane, and the ectoplacental cone. Cell suspensions were prepared from the yolk sacs of embryos of 7–9 days’ gestation and from the isolated livers of 10–18-day embryos as previously described, and cells at $1 \times 10^6$/ml were suspended in EBSS or in medium 199 containing 30% fetal calf serum (F-199).

Peritoneal macrophages were isolated according to the method of Cohn and Benson and were suspended in F-199 at a concentration of $2 \times 10^6$/ml.

Bone marrow cells were isolated by the forcible injection of F-199 through the isolated femurs of 3–4-mo-old C57BL mice and suspended in modified Eagle’s medium or F-199.

Agar colonies grown from either bone marrow or fetal tissues for 8–10 days in vitro were dispersed in medium 199 or F-199 by repeated pipetting through a small orifice.

Cell Culture

Glass Adherence: Cells from the four sources cited above were allowed to adhere to glass covers slips for 2–3 hr at 37°C. Nonadherent cells were then removed by washing with warm medium, and culture was resumed for 4 or 18 hr before functional tests were performed on the adherent cells.

Agar Culture. Bone marrow or fetal cells were grown in soft agar according to the method of Bradley and Metcalf, using semipurified colony-stimulating factor from human urine or serum from endotoxin-treated mice.

Analysis of IgG Receptor Activity

Serum from CBA mice given serial i.p. injections of washed sheep red cells (SRBC) was used as the source of sensitizing antibody. A 5-day primary and a 7-day secondary serum were separated into IgM- and IgG-rich fractions, respectively, on Sephadex G-100/200.

SRBC were sensitized with various concentrations of heat-inactivated antibody at 37°C for 60 min and washed thrice by centrifugation and suspended in EBSS.

Unsensitized or antibody-coated SRBC (1 $\times 10^7$/ml) were added to cell monolayers for 30 min at 37°C. SRBC not bound or ingested were removed by washing with warm EBSS before the monolayers were fixed, stained, and scored for the number of cells with adherent or ingested SRBC.

In an alternate technique, control or sensitized SRBC (5 $\times 10^6$/ml) were incubated in suspension with cells (approximately 5 $\times 10^4$/ml) in EBSS for 30 min at 37°C. The percent of cells with rosettes of SRBC was determined visually at 400× magnification.

The subclass specificity of cell IgG receptors was determined by competition studies between IgG-coated SRBC and purified immunoglobulins obtained from serum or ascites fluid of mice bearing plasma cell tumors of defined type: IgA (HPC-1), IgG1 (HPC-39), IgG2a (GPC-7, -8), IgG2b (HPC-71), IgM (MOPC-104E), and light chains (MPC-76). Purified immunoglobulins were added to cell monolayers immediately before sensitized SRBC.

Dr. N. L. Warner generously provided these purified proteins.

Phagocytosis

Candida albicans were prepared as previously described. S. adelaide was obtained from Commonwealth Serum Laboratories, Victoria. Microorganisms (approximately 5 $\times 10^8$/ml in 20% mouse serum) were added to cell monolayers for 30 min at 37°C. Non-phagocytized organisms were removed by washing.
Morphology and Radioautography

Cell suspensions deposited on glass slides by a cytocentrifuge (Shandon Instruments) or as adherent monolayers were fixed in methanol and stained with Giemsa. Promonocytes were identified by the criteria described by Van Furth et al.2,7 Peroxidase staining was by the method of Rytomaa.19

In some studies, mice were injected i.v. with 100 μCi 3H-thymidine (23 Ci/mM) 1 hr before preparation of bone marrow cells. Fixed cell preparations were coated with NTB emulsion (Kodak) and exposed for 7-10 days.

RESULTS

Identification of Macrophages in Fetal Tissues

Morphology: In suspensions of cells isolated from fetal yolk sac of 7-9 days' gestational age, there were no cells identifiable as promonocytes, monocytes, or macrophages in Giemsa-stained preparations. By the tenth day, cells with the morphologic characteristics of mature macrophages14 were detectable in the yolk sac population and by the 10th-11th day comprised 0.5 ± 0.3% of the total population. The identity of these cells was confirmed by functional criteria.

Cells with the appearance of monocytes and macrophages at various stages of development were apparent in the 12-day fetal liver and by day 18 comprised approximately 6% of the population (Table 1).

Advantage was taken of the glass-adherent properties of promonocytes6,7 and more mature monocytes and macrophages14 to provide an enriched population of these cells from the total fetal population. The time allowed for adherence was either 6 or 24 hr. Under these conditions, rare glass-adherent cells were observed in the 7-9-day yolk sac and in the 7-9-day whole fetus. In Giemsa-stained preparations and by phase macroscopy such cells did not

| Table 1. Differential Counts of Cells Prepared From Fetal Tissues: Total Cells and Glass-adherent Cells |
|---|---|---|---|---|---|---|
| Cell Source | Promonocyte | Monocyte | Macrophage | Granulocytes | Normoblasts | Undifferentiated Blast Cells |
| All cells in suspension | N.D. | N.D. | N.D. | N.D. | 48 | 52 |
| 7-9 day yolk sac | 0.5 | N.D. | <1 | 96 | 1.5 | 2 |
| 10-day yolk sac | 1. | 1. | N.D. | 87 | 9 | 2 |
| 12-day liver | 1. | 0.5 | N.D. | 91 | 6 | 1.5 |
| 14-day liver | 2. | 4. | 41 | 42 | 4 | 7 |
| Glass-adherent cells | N.D. | N.D. | N.D. | N.D. | N.D. | 100 |
| 7-9 day yolk sac | 31 | 40 | — | — | — | 29 |
| 10-day yolk sac | 12 | 5 | — | — | — | 1 |
| 12-day liver | 4 | 58 | — | — | — | 38 |
| 18-day liver | 8 | 41 | 36 | — | 2 | 13 |

*N.D. = not detectable.
resemble macrophages. Furthermore, they lacked the functional characteristics of monocytes and macrophages.

In contrast, in the 10- and 11-day yolk sac and in fetal livers of age 12 days or older, glass-adherent cells with the typical appearance and functional characteristics of macrophages were observed. The proportion of glass-adherent cells with this appearance is given in Table 1. Such cells constituted an increasing proportion of glass-adherent cells in the liver between days 12 and 14; thereafter, the proportion of these cells declined as glass-adherent granulocytes made their appearance in this organ.

Receptors for IgG: Because morphologic appearance alone is an insufficient criterion for identifying cells of the macrophage series, the presence of the IgG surface receptor and phagocytosis were used as markers of identification.9,13,20

Two techniques for demonstrating IgG receptors were used. In the first, IgG-coated SRBC were incubated with fetal cells in suspension; in the second, sensitized SRBC were added to glass-adherent cell monolayers. The results are shown in Table 2.

In cells isolated from 7–9-day yolk sacs or whole embryos, none with the capacity to form rosettes with IgG-coated SRBC was observed either in suspension or adherent to glass. In contrast, rosette-forming cells were seen in the 11-day yolk sac and with increasing frequency in fetal liver from 11 days onward (Table 2). By the 12th day, rare erythrophagocytosis was noted.

Phagocytosis: Phagocytosis of C. albicans and S. adelaide was also used as a marker of differentiated cells at or beyond the promonocyte stage.6,7 Very few of the fetal granulocytes were glass adherent until day 18, and the few that were adherent were readily distinguishable by morphology. Consequently, phagocytosis could be used as a monocyte-macrophage marker. Phagocytosis by 7–9-day yolk sac cells in suspension or glass-adherent was not seen (Table 2). Glass-adherent phagocytic cells made their appearance in the 11-day yolk sac. Between 0.1% and 0.33% of all fetal liver cells between 11 and 14 days' gestational age phagocytized Candida when in suspension (Table 2); a higher proportion of glass-adherent liver macrophages were phagocytic. Glass-

<table>
<thead>
<tr>
<th>Cell Source</th>
<th>% Cells Forming Rosettes With IgG SRBC</th>
<th>% Cells Phagocytizing C. albicans</th>
<th>In Vitro Colony-forming Cells (CFC)* (CFC/10⁶ Cells)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>In Suspension</td>
<td>Glass-adherent</td>
<td>In Suspension</td>
</tr>
<tr>
<td>7–9 day yolk sac</td>
<td>&lt;0.1</td>
<td>&lt;0.1</td>
<td>&lt;0.1</td>
</tr>
<tr>
<td>7–10 day liver</td>
<td>&lt;0.1</td>
<td>&lt;0.1</td>
<td>&lt;0.1</td>
</tr>
<tr>
<td>11-day yolk sac</td>
<td>2.1</td>
<td>27.0</td>
<td>&lt;0.1</td>
</tr>
<tr>
<td>11-day liver</td>
<td>&lt;1.5</td>
<td>—</td>
<td>&lt;0.1</td>
</tr>
<tr>
<td>12-day liver</td>
<td>0.5±0.2</td>
<td>3.7</td>
<td>&lt;0.1</td>
</tr>
<tr>
<td>14-day liver</td>
<td>1.4±0.4</td>
<td>40.0</td>
<td>0.33±0.1</td>
</tr>
<tr>
<td>18-day liver</td>
<td>3.7±0.3</td>
<td>21.0</td>
<td>2.0</td>
</tr>
</tbody>
</table>

*Mean colony incidence in two experiments performed on pooled material from all embryos in a litter.
Stimulated by 0.1 ml of 1/6 endotoxin serum.
adherent macrophages from fetal liver at or beyond the 12th day were also seen to be phagocytic for S. adelaidae.

*Bone Marrow Promonocytes:* Bone marrow cells from untreated or ^3^H-thymidine-injected animals were allowed to adhere to glass cover slips under conditions used to identify promonocytes. Under these conditions, labeled peroxidase-positive cells with the appearance of promonoblasts could be identified. Approximately one in six of these cells formed rosettes with IgG-coated red cells. Erythrophagocytosis or phagocytosis of *Candida* by these cells was only rarely observed, whereas bone marrow monocytes and macrophages demonstrated active phagocytosis of both these particles.

*Production of Macrophages by Fetal Tissues*

Cells from 7–10-day yolk sac and 11–18-day fetal liver were grown in soft agar in the presence of colony-stimulating activity. As previously described, these cells gave rise to colonies of granulocytes and mononuclear cells (Table 2). Incidence of in vitro colony-forming cells in yolk sac and early fetal liver are considerably higher than reported previously, due to the use in this study of a considerably more active source of colony-stimulating factor with consequent increase in "plating efficiency."

The mononuclear cells arising in these colonies had the characteristic of mature macrophages by the criteria of morphology, lack of peroxidase activity, phagocytosis of microorganisms, and IgG receptors. From studies in which free immunoglobulins competed with IgG-coated SRBC for binding, the subclass specificity of the IgG receptors was deduced. As seen in Table 3, fetal yolk sac and liver colonies contained macrophages of identical specificity to those of peritoneal and bone marrow macrophages.

Proteins of class IgG_{2a} were inhibitory, whereas those of IgA, IgM, IgG_{2b}, and light chains were not. The IgG_{1} protein tested was slightly inhibitory. From these observations we conclude that the early fetal yolk sac contains

<table>
<thead>
<tr>
<th>RBC-sensitizing Agent</th>
<th>Competing Protein (100-150 μg/ml)</th>
<th>% Phagocytes With Bound or Ingested SRBC</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Peritoneal Macrophage</td>
</tr>
<tr>
<td>IgM antibody</td>
<td></td>
<td>0</td>
</tr>
<tr>
<td>IgG antibody</td>
<td></td>
<td>1± 1</td>
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<tr>
<td>IgG antibody</td>
<td>IgA (HPC-1)</td>
<td>83±15</td>
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<tr>
<td>IgG antibody</td>
<td>IgG_{1} (HPC-39)</td>
<td>64± 8</td>
</tr>
<tr>
<td>IgG antibody</td>
<td>IgG_{2a} (GPC-7)</td>
<td>19±15*</td>
</tr>
<tr>
<td>IgG antibody</td>
<td>IgG_{2a} (GPC-8)</td>
<td>49±10*</td>
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<tr>
<td>IgG antibody</td>
<td>IgG_{2a} (HPC-71)</td>
<td>64± 8</td>
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<tr>
<td>IgG antibody</td>
<td>IgM (MOPC-104E)</td>
<td>81± 4</td>
</tr>
<tr>
<td>IgG antibody</td>
<td>Light chain (MPC-76)</td>
<td>67± 5</td>
</tr>
</tbody>
</table>

*Statistically different from control values in absence of competing protein at p <0.01.
cells capable of giving rise to macrophages, although no cells at or beyond the promonocyte stage are detectable in either the 7–9-day yolk sac or embryo.

**DISCUSSION**

The identification of hemic cells purely on the basis of morphologic criteria is fraught with hazard; consequently, several identifying markers were used to follow the appearance of cells of the monocyte-macrophage line in fetal tissues and to establish the nature of the progeny cells in culture. The markers used were glass adherence, peroxidase staining, phagocytosis of a large particle (*C. albicans*), and the presence of a surface receptor for a subclass of IgG. Peroxidase is found in cells of the granulocytic series from the level of the progranulocyte and in promonocytes and monocytes but not in more mature macrophages. Phagocytosis is characteristic of cells of the granulocytic line from approximately the myelocyte stage and of cells of the monocyte-macrophage series. The presence of strong surface receptors for IgG molecules is probably unique to monocytes and macrophages among the phagocytic leukocytes and, in our own studies in the mouse, has not been identifiable in neutrophils. Rare cells with the fine structural features of lymphocytes appear to have similar receptors, but these cells are distinguishable by their lack of phagocytic ability.

On the seventh day of gestation, the mouse yolk sac appears as the central part of the egg cylinder wall and then grows rapidly forming an extensive membrane enveloping the amnion and exocoelomic cavity. During this period blood islands appear in the mesodermal layer of the mouse yolk sac. In these islands the hemangioblasts develop either as undifferentiated blast cells or as progressively more mature erythroid precursors. In the yolk sac of 7–9-days' gestational age, differentiated cells of the granulocytic series were never seen either in this or in previous studies. Furthermore, no cells could be identified in the early yolk sac that were similar to the immature bone marrow promonocyte of the adult animal, a cell characterized by glass adherence, limited phagocytic ability, and (as shown in these studies) by some surface IgG-receptor function. Despite the absence of identifiable monocyte-macrophage precursors, early yolk sac cells were capable of giving rise in culture to typical mature macrophages characterized by abundant phase-dense granules, absence of peroxidase, avid phagocytosis, and surface IgG-receptor activity. The IgG subclass specificity of macrophages derived from the early yolk sac was identical to that of mature peritoneal macrophages from the same strain of mice.

In the late yolk sac, monocytes, macrophages, and cells with the characteristics of promonocytes were readily identifiable. At this time the yolk sac is open to the fetal circulation, consequently it is not known whether these macrophages originate in situ or outside the yolk sac.

No progenitor cells capable of giving rise to macrophages were demonstrable in the fetal liver or the remaining embryo other than the yolk sac until the tenth day of gestation. At a time when such progenitor cells were readily demonstrable (day 11), the fetal liver contained well-differentiated cells of the monocyte-macrophage line characterized by glass adherence, phagocytic abil-
ity, and surface receptors for IgG globulin. From the 11th day, these cells comprised a progressively larger fraction of the total population. Macrophages were prominent in the fetal liver before cells of the granulocytic series could be identified.

From these observations, we conclude that the fetal yolk is the site of origin of the macrophage precursor in the mouse embryo. The yolk sac appears to serve a similar function in the production of immunoglobulin-producing cells. The macrophage progenitor in the yolk sac is "proximal" to the bone marrow promonocyte on the pathway of sequential development that culminates in the monocyte and mature macrophage. The early yolk sac does not itself support differentiation of the progenitor to the promonocyte stage or beyond until after the ninth or tenth day of gestation. Thereafter, the opening of the yolk sac to the fetal circulation obscures the origin of the macrophages observed in this organ. Thus, the situation described for the macrophage progenitor appears to be similar to that of the progenitor of granulopoiesis. Precursors of the latter cell line exist in the early yolk sac, but granulopoiesis does not occur in this site.

REFERENCES

17. Metcalf, D.: Transformation of granulocytes to macrophages in bone marrow


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