Purification of Erythroblastic Nests

By Alberto J. L. Macario, Charles Dugan, Ines L. Perez-Lloret, and Everly Conway de Macario

We describe a method for preparing purified erythroblastic nests in large numbers (~10^7/run) in three steps: (1) induction of splenic erythropoiesis in mice, (2) preparative differential centrifugation for the removal of erythrocytes and single cells from spleen cell suspensions, and (3) sedimentation in an isokinetic gradient of Ficoll 400 in Joklik's modification of minimum essential medium. Viability of isolated EN is very high, as demonstrated by the trypan blue exclusion and in vitro erythrocyte formation methods.

An erythroblastic nest (EN) is a multicellular structure composed of a central, non-erythrocyte cell surrounded by one or more contiguous rows of erythroblasts. EN have been found in the bone marrow of several mammalian species but not in birds, and in the erythropoietic mouse spleen after induction of erythropoiesis by various means. EN are also called erythroblastic islands, but we reserve this name for clusters of erythroblasts without a non-erythrocyte central cell.

Although numerous morphological studies of EN have been performed in vivo and in culture, information on its biologic role and on the mechanism of its formation and participation in the erythropoietic process is scarce. It has been suggested, for example, that the central cell provides the surrounding erythroblasts with ferritin and perhaps other molecules, nutrients or otherwise, and a mechanism for the extrusion of the nucleus. However, there is no proof of these suggested functions.

Analysis of EN has been restricted by the difficulty of producing workable amounts of purified preparations. Here we describe a procedure for the production and purification of EN that yields large numbers of EN devoid of other cells or cell structures.

MATERIALS AND METHODS

Mice

Female C57BL/6J (The Jackson Laboratory, Bar Harbor, Me.) 8-wk-old mice were used throughout.

Erythropoiesis

Spleens stimulated to increased erythropoiesis were obtained by a method based on our earlier observation that irradiation increases erythropoiesis in the spleens of mice made anemic by bleeding. Each mouse was bled for three successive mornings; the hematocrit had dropped to 20% ± 2% by the afternoon of the third bleeding day. Six hours after the third bleeding, whole body irradiation (700 rad, 137Cs source, M-Gammator Model M38-1, Isomedix, Inc., Parsippany N.J.) was performed. Fourteen hours later, each mouse was transfused with 70 x 10^6 syngeneic spleen cells from normal, untreated donors. Spleens for preparation of erythroblast suspensions containing EN were excised 5 days after the cell transfer, the day on which erythropoiesis peaks.

Cell Suspensions

Suspensions of spleen cells were prepared as described previously. Spleen from normal untreated and from erythropoietic mice were teased through a stainless steel screen (Mark 80, Small Parts, Inc., Miami, Fla.) and the cells suspended in Joklik modified minimum essential medium (MEM) (Gibco, Grand Island, N.Y. and Division of Laboratories and Research, New York State Department of Health) containing penicillin (100 U/ml), streptomycin 100 µg/ml, and 10% fetal bovine serum (FBS). The spleen cells from normal untreated mice, which were used for transfusion into irradiated mice, were washed 3 times with medium without FBS before inoculation. The erythroblasts and EN for cultivation in vitro were suspended in alpha modified MEM (Flow Laboratories, McLean, Va. and Division of Laboratories and Research, New York State Department of Health) containing 30% FBS, antibiotics as above, and 10^-4 M β-mercaptoethanol.

Cell viability was determined by the trypan blue exclusion method. Differential cell counts were performed on smears obtained with a cytocentrifuge (Shandon Southern Instruments, Inc., Sewickley, Pa.) and stained with Wright's Giemsa (Hematek Slide Stainer, Ames Co., Elkhart, Ind.).

Preparative EN Enrichment

Cell suspensions obtained from erythropoietic spleens were suspended in Joklik medium with 10% FBS and antibiotics and centrifuged at 500 rpm (30.7 g at the mean distance of 11 cm) in a Sorval GLC-2B centrifuge (DuPont Instruments Biomedical Products Division, Newton, Conn.) for 5 min at 5°C. The supernatant was collected and the pellet resuspended and centrifuged again. The whole operation was repeated twice. Cell concentration, viability, and differential counts were determined in the supernatants and the final pellet. The results are expressed as arithmetic means (AM) and 95% confidence intervals (CI).

Ficoll Gradient

Final purification of EN using the enriched pellet obtained by the preparative procedure (see above) was carried out in an isokinetic gradient of Ficoll constructed in Joklik's modification of MEM. Gradients were formed in 100-ml centrifuge tubes (2806, International Equipment Co., Needham Heights, Mass.) over a 4.5-ml cushion of 45% Ficoll 400 (Pharmacia, Piscataway, N.J.)
N.J.) in Joklik's medium with 10% FBS and antibiotics. The linear gradient of Ficoll was formed on top of the cushion, from 2.70% to 4.87% at 13.7 - 23.7 cm from the center of rotation, respectively, with a total volume of 64 ml.

Seven milliliters of the starting cell suspension, containing $4 \times 10^6$ cells/ml (single nucleated cells plus EN) were layered on top of the gradient. Centrifugation followed at 400 rpm (24.5 g at the sample-gradient interface, 13.7 cm from the center of rotation) in a PR-2 centrifuge (International Equipment Co.) for 8 min at 4°C.

The centrifuge speed was monitored during each run by a phototachometer (Power Instruments, Inc., Skokie, Ill.).

To collect fractions from the gradient, a tapping cap (Halpro, Rockville, Md.) was inserted and 55% sucrose was pumped into the bottom of the tube. The first 7 ml were discarded. Sixteen 4-ml fractions were then collected on ice and each was centrifuged at 1000 rpm (153.6 g at 13.7 cm from the center of rotation) for 10 min at 4°C in the Sorvall GLC-2B centrifuge. The supernatants were discarded, and the pellets were resuspended in tissue culture.

![Fig. 1. EN in erythropoietic mouse spleens: The central cell appears as a macrophage (A), monocyte (B), or monoblast (C). Wright-Giemsa (x 4230).](image)
medium and recentrifuged. The final pellets were resuspended at the proper concentrations for cell countings (1–2 × 10⁶/ml), preparation of cytocentrifuge slides (1 and 0.5 × 10⁶/ml), and cultivation (10⁵ cells/ml in alpha medium).²⁸³⁰

**Culture of Cells and EN**

Triplicate or quadruplicate 1-ml samples from each cell suspension (10⁶ cells/ml) were cultured in wells of a Falcon tissue culture plate (3008, Becton-Dickinson, Oxnard, Calif.). The plates were then incubated at 37°C in a humid atmosphere with 5% CO₂ and 95% air. The contents of the 3 or 4 wells corresponding to each test sample were collected after 20 hr of cultivation, pooled, and examined. Cell counting, viability, and differential counts were determined as above.

**RESULTS**

**Induction of EN in the Mouse Spleen**

Although EN can be induced in various numbers in the mouse spleen by several methods,¹¹ our procedure, including bleedings, irradiation, and transfusion of syngeneic spleen cells, yields the highest proportions and absolute numbers of EN. The EN obtained can be grouped according to the morphology of the central cell: macrophage, monocyte, or monoblast-like (Fig. 1A–C). The frequency of occurrence of these EN types 5 days after transfusion is shown in Table 1.

**Preparative Procedure for EN Enrichment**

A tenfold enrichment of EN content in the spleen cell suspensions was attained by repeated differential centrifugation in tissue culture medium containing FBS²⁸ (Table 2). Recovery was nearly 100% with most erythrocytes and single cells being removed.

**Purification of EN**

The EN were further purified by sedimentation on an isokinetic gradient of Ficoll. The EN concentrated in fraction 15, which contained 25% of the EN input, a result confirmed in 4 experiments (AM 27%; CI 15.3%–40.3%). Fraction 15 contained 50% of all EN recovered and, together with fraction 16, made up 70%.

Fraction 15 contained the highest proportion (greatest degree of purification) and the largest number (greatest yield) of EN in all experiments. The product of these two parameters was taken as the fraction efficiency index, which was consistently higher for fraction 15 (n = 4) (Fig. 2). Preceding fractions (nearer to the gradient's top) contained smaller cell clusters and single cells. The latter cells were highly prevalent in the top fraction (Fig. 3A–D). Although single cells were also found in fraction 15 (AM 46.3%; CI 40.2%–52.5%; n = 4), the great majority appeared close to the EN, as if detached from its periphery (Fig. 3D), an artifact produced by fractionation with a gradient of Ficoll.

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**Table 1. Classification and Frequency of EN According to the Morphology of the Central Cell**

<table>
<thead>
<tr>
<th>EN (Percent) Central Cell</th>
<th>Macrophage</th>
<th>Monocyte</th>
<th>Monoblast-Like</th>
</tr>
</thead>
<tbody>
<tr>
<td>AM*</td>
<td>78.4</td>
<td>17.0</td>
<td>4.6</td>
</tr>
<tr>
<td>95% CI</td>
<td>(75.4–81.4)</td>
<td>(14.4–19.4)</td>
<td>(3.6–5.5)</td>
</tr>
</tbody>
</table>

*Arithmetic mean and 95% confidence interval. Seven experiments including 26 mice; 2099 EN counted.

**Table 2. EN Enrichment by Preparative Differential Centrifugation**

<table>
<thead>
<tr>
<th>Cell Suspensions</th>
<th>Initial</th>
<th>Final</th>
<th>Percent Recovery*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Input</td>
<td>Output</td>
<td></td>
</tr>
<tr>
<td>Nucleated single</td>
<td>254.1†</td>
<td>45.1</td>
<td>18.6</td>
</tr>
<tr>
<td>(x 10⁶)</td>
<td>(103.6–366.4)</td>
<td>(19.0–65.7)</td>
<td>(15.0–21.9)</td>
</tr>
<tr>
<td>Erythrocytes</td>
<td>284.1</td>
<td>4.1</td>
<td>1.6</td>
</tr>
<tr>
<td>(x 10⁶)</td>
<td>(107.0–392.7)</td>
<td>(1.3–6.3)</td>
<td>(0.9–2.1)</td>
</tr>
<tr>
<td>EN</td>
<td>3.2</td>
<td>3.2</td>
<td>96.4</td>
</tr>
<tr>
<td>(x 10⁶)</td>
<td>(1.5–4.7)</td>
<td>(1.5–4.6)</td>
<td>(92.8–99.9)</td>
</tr>
<tr>
<td>Percent</td>
<td>0.66</td>
<td>6.28</td>
<td>10.65†</td>
</tr>
<tr>
<td></td>
<td>(0.43–0.83)</td>
<td>(4.85–8.53)</td>
<td>(7.70–13.26)</td>
</tr>
</tbody>
</table>

*Output/input x 100 – percent recovery.  
†Arithmetic mean (95% confidence interval); n = 7.  
‡Factor of enrichment = output/input.
most likely due to the centrifugation procedure involved in the cytocentrifuge preparations.

Viability of Purified EN

Cell viability of the purified EN (fraction 15) was demonstrated by two methods: (A) trypan blue exclusion: AM 94%; CI 89.7%-99.8%; n = 4; and (B) formation of erythrocytes in vitro.\textsuperscript{11} Cultures containing EN produced significantly higher numbers of erythrocytes than did cultures devoid of EN (Table 3).

DISCUSSION

The method we describe for the preparation of purified EN is based on our earlier findings that irradiation strikingly increases the amount of erythropoiesis in the spleen of mice made anemic by bleeding\textsuperscript{22} and that the erythropoietic mouse spleen induced by various means contains a relatively high number of EN.\textsuperscript{11}

The method includes first, the induction of massive erythropoiesis in the mouse spleen by prebleedings, irradiation, and cell transfer; second, differential centrifugations for EN enrichment; and third, final purification by sedimentation on an isokinetic gradient of Ficoll.

The EN concentrate in fraction 15. Single cells are also found in cytocentrifuge slides prepared from fraction 15, but most likely these cells are not contaminants from the single cell pool in the input suspension. More likely, they have been detached from the EN during the preparation of the cytocentrifuge slides. Most single cells in these slides from fraction 15 are close to EN, sometimes still joined with them by barely visible connections. Moreover, single cells are rarely found in suspensions of fraction 15 when count-

Table 3. Erythrocyte Production in Cultures of Purified EN

<table>
<thead>
<tr>
<th>EN at Time 0 (× 10⁶)</th>
<th>Erythrocyte Production Index*</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>1.75</td>
</tr>
<tr>
<td>1.50</td>
<td>(0.99-2.59)†</td>
</tr>
<tr>
<td>(0.67-1.93)</td>
<td>(5.93-12.45)</td>
</tr>
</tbody>
</table>

*Total number of erythrocytes at 20 hr of cultivation/total number of erythrocytes at time 0 (beginning of cultures). All cultures were initiated with 10⁶/ml purified erythroblasts with or without EN as indicated.
†Arithmetic mean (95% confidence interval). Data from 12 experiments.
ing in a hemocytometer. Thus, the method described yields virtually pure EN in large numbers.

The availability of purified EN will allow progress in studies of: (A) the nature of the bonds and the mechanism of surface interaction between the central and the surrounding cells; (B) the question of passage of substances to, from, or to and from the central cell; and (C) the in vitro behavior of EN, individually in microcultures or in groups, with or without association with other cells or structures (e.g., T cells or stromal cells, which are known to influence erythropoiesis). Such investigations, using purified EN, will help elucidate the role of these structures in erythropoiesis and their functional and morphological alterations in hemopoietic disorders. The EN may also serve as a model system of morphogenesis, in which tridimensional cellular structures consisting of different cell types, become closely associated to exercise their ultimate physiologic role.

**ACKNOWLEDGMENT**

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**REFERENCES**

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