Identification and Characterization of a Differentiation-Specific Antigen on Normal and Malignant Murine Erythroid Cells

By E. Rena Bacon and Arthur J. Sytkowski

Rauscher murine erythroleukemia cells grow continuously in vitro and undergo terminal differentiation in response to the physiological inducer erythropoietin. In the course of this developmental process they express many erythroid-specific markers. In order to investigate the expression of cell surface determinants during Rauscher cell differentiation we generated monoclonal antibodies to uninduced cells. Using an anti-Rauscher cell monoclonal antibody, we have identified a cell surface determinant, designated ERY-1, that is present on normal murine erythroid cells.

MURINE and human erythroleukemia cell lines have been studied extensively as model systems of erythroid commitment and differentiation. The Rauscher murine erythroleukemia cell line is particularly suitable for such investigation because of the ability of these cells to differentiate in response to the physiologic inducer erythropoietin as well as to nonphysiologic inducers, eg, dimethyl sulfoxide (DMSO).1,2 The cell line was derived originally from Rauscher virus–infected DBA mice and was cloned to establish more homogeneous populations.2 Rauscher cells resemble immature erythroblasts morphologically, and they differentiate into hemoglobin-containing erythroid cells after induction. They synthesize all three adult murine globins—alpha, beta major, and beta minor.3 Erythropoietin induction also results in increased levels of beta-adrenergic receptors4 and membrane spectrin (Bacon ER, unpublished observations). While the erythroid nature of Rauscher cells seems certain, the stage of differentiation of the uninduced cells relative to normal erythropoietic progenitors has not yet been determined clearly. Some experimental evidence suggests that noninduced Rauscher cells may be at a differentiation stage similar to that of the normal erythroid progenitor BFU-E. Rauscher cells growing in semisolid medium can exhibit a rather diffuse colony morphology reminiscent of the BFU-E–derived colony. Moreover, their erythropoietin dose-response relationship is remarkably similar to that of the normal mouse BFU-E.2,3

In order to examine the process of Rauscher cell differentiation further, we chose to investigate changes in the expression of cell surface determinants that occur during maturation and to compare these changes to those that occur during normal erythropoiesis. Therefore, we generated monoclonal antibodies against noninduced (and thus immature) Rauscher cells. We anticipated that the antibodies would also recognize bone marrow erythroid cells, including progenitors. We describe here the identification and characterization of an erythroid-specific surface antigen, designated ERY-1, which is expressed during certain stages of maturation of normal erythroid progenitors and of Rauscher cells.

MATERIALS AND METHODS

Production of Monoclonal Antibodies

Male Lewis rats were immunized intraperitoneally with 3 × 107 Rauscher cells (clone 404) at monthly intervals. Sera from immunized rats were assayed for anti-Rauscher cell antibodies by enzyme-linked immunosorbent assay (ELISA; see below), and an animal with a high anti-Rauscher cell antibody titer was identified. Four days prior to the fusion, the animal selected was boosted intravenously with 5 × 107 cells. At the time of the fusion the animal was killed by CO2 asphyxiation, and the spleen was removed. The fusion protocol was modified from the method of Kennett. Briefly, 7.5 × 106 spleen cells and 1.5 × 106 P3X63Ag8653 myeloma cells (Institute for Medical Research, Camden, NJ) were fused in the presence of 0.3 mL of 30% polyethylene glycol 1500 (PEG; BDH, Carle Place, NY). The cells were incubated at 37 °C for two minutes and then centrifuged at 260 × g at 25 °C for six minutes. The supernatant was aspirated, and the cell pellet was diluted slowly with 30 mL of HY-HT medium (70% Dulbecco's MEM, 20% fetal calf serum, 10% NCTC-109 [GIBCO, Grand Island, NY], supplemented with 1.6 × 10−3 mol/L glucose, 2 mmol/L glutamine, 1 mmol/L oxaloacetic acid, 0.5 mmol/L pyruvic acid, 0.2 U/mL insulin, 1 × 10−4 mol/L hyposorcine, 1.6 × 10−3 mol/L thymidine, and 2.5 × 10−3 mol/L 2-mercaptoethanol [Sigma, St Louis, Mo]). The cells were incubated overnight in a 150 mm plastic petri dish in 10% CO2 at 37 °C. The next day, the cells were centrifuged and resuspended in 30 mL HY-HT medium containing 4 × 10−5 mol/L aminopterin (HAT medium). The cells were plated into six 96 well Costar (Cambridge, MA) microculture dishes on a feeder layer of human fibroblasts and incubated in a humidified incubator in 10% CO2 at 37 °C for two minutes. The supernatant was aspirated, and the cell pellet was diluted slowly with 30 mL of 30% polyethylene glycol 1500 (PEG; BDH, Carle Place, NY). The cells were incubated at 37 °C for two minutes and then centrifuged at 260 × g at 25 °C for six minutes. The supernatant was aspirated, and the cell pellet was diluted slowly with 30 mL of HY-HT medium (70% Dulbecco's MEM, 20% fetal calf serum, 10% NCTC-109 [GIBCO, Grand Island, NY], supplemented with 1.6 × 10−3 mol/L glucose, 2 mmol/L glutamine, 1 mmol/L oxaloacetic acid, 0.5 mmol/L pyruvic acid, 0.2 U/mL insulin, 1 × 10−4 mol/L hyposorcine, 1.6 × 10−3 mol/L thymidine, and 2.5 × 10−3 mol/L 2-mercaptoethanol [Sigma, St Louis, Mo]). The cells were incubated overnight in a 150 mm plastic petri dish in 10% CO2 at 37 °C. The next day, the cells were centrifuged and resuspended in 30 mL HY-HT medium containing 4 × 10−5 mol/L aminopterin (HAT medium). The cells were plated into six 96 well Costar (Cambridge, MA) microculture dishes on a feeder layer of human fibroblasts and incubated in a humidified incubator in 10% CO2 at 37 °C. The cells were fed weekly with 50 uL of HAT medium (70% Dulbecco's MEM, 20% fetal calf serum, 10% NCTC-109 [GIBCO, Grand Island, NY], supplemented with 1.6 × 10−3 mol/L glucose, 2 mmol/L glutamine, 1 mmol/L oxaloacetic acid, 0.5 mmol/L pyruvic acid, 0.2 U/mL insulin, 1 × 10−4 mol/L hyposorcine, 1.6 × 10−3 mol/L thymidine, and 2.5 × 10−3 mol/L 2-mercaptoethanol [Sigma, St Louis, Mo]). The cells were incubated overnight in a 150 mm plastic petri dish in 10% CO2 at 37 °C. The next day, the cells were centrifuged and resuspended in 30 mL HY-HT medium containing 4 × 10−5 mol/L aminopterin (HAT medium). The cells were plated into six 96 well Costar (Cambridge, MA) microculture dishes on a feeder layer of human fibroblasts and incubated in a humidified incubator in 10% CO2 at 37 °C. The cells were fed weekly with 50 uL of HAT medium per well. The supernatants from the wells containing macroscopic hybridoma colonies were screened by ELISA for antibody to Rauscher cells, to syngeneic adult murine liver cells, and to thymocytes. Colonies whose supernatants contained antibodies to Rauscher (R) cells but not to adult liver cells (L) or thymocytes (T) (R+L−T− antibodies) were selected for further investigation and cloned. One such antibody was found to recognize a determinant found on erythroid precursors (see Results).

Assays for Reactivity of Monoclonal Antibodies

Enzyme-linked immunosorbent assay (ELISA). Rauscher cells, adult murine liver cells, or thymocytes were centrifuged for five

From the Division of Hematology and Oncology, Children's Hospital, the Dana-Farber Cancer Institute, and the Department of Pediatrics, Harvard Medical School, Boston.

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Address reprint requests to Arthur J. Sytkowski, MD, Division of Hematology/Oncology, New England Deaconess Hospital, 185 Pilgrim Rd, Boston, MA 02215.

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chromophore was measured at 490 nm with an EIA reader (Bio-Tek, 
diamine/0.01% H2O2, (Sigma) substrate reagent was added to each 
well. The reaction was stopped by adding 2N HCl, and the resulting 
saline (PBS), 1% bovine serum albumin (BSA; Miles Scientific, 
concentration of 106 cells/mL (0.1 mL/well). The wells were 
washed twice and blocked with Dulbecco’s phosphate-buffered 
saline (PBS), 1% bovine serum albumin (BBA; Sigma) substrate reagent was added to each 
well. The reaction was stopped by adding 2N HCl, and the resulting 
was measured at 490 nm with an EIA reader (Bio-Tek, 

**Immunofluorescence.** Antibody binding to Rauscher cells or to 
DBA/23 murine bone marrow cells, splenocytes, thymocytes, neut-
rophils, peritoneal macrophages, and platelets was assessed by 
fluorescence flow cytometry (FFC) with an Ortho Cytofluorogra-
System 50 interfaced with 2150 Ortho computer. Cells (5 × 106) 
were incubated with 100 μL of R LA T- hybridoma supernatant for 
30 minutes at 4 °C. They were washed three times with PBS and 
then incubated with 100 μL of 1:25 fluorescein isothiocyanate-
conjugated rabbit antirat IgG (Cooper Biomedical). The cells 
were washed and resuspended in 20 mL of 0.1 M NaOH, 1% (w/v) 
bovine serum albumin (BSA) in PBS. The slides were incubated with 
fluorescein isothiocyanate-conjugated rabbit antirat IgG (Cooper 
Biomedical). The slides were washed and resuspended in 25 mL of 
HEPES-buffered MEM, 2% fetal calf serum, pH 7.3, for fluorescein analysis and sorting. 
Sorted cells were collected into 35 mm petri dishes containing 1 mL of 
heat-inactivated fetal calf serum. For subsequent morphologic 
examination, the cells were washed and resuspended in fetal calf 
saline, cytocentrifuged onto glass slides (Shandon-Southern Cyto-
spin SCA-0030, Sewickley, PA) and stained with Wright’s Giemsa 
(Harleco). For differential counts, at least 500 cells per slide were 
counted. The slides were scored for both erythroid 

**RESULTS**

**Binding of Monoclonal Antibody ERY-1 to Rauscher Cells**

Numerous supernatants from the fusion contained anti-
odies that bound to Rauscher cells but not to liver cells or 
lymphocytes in the screening ELISA (R LA T- antibodies; 
see Materials and Methods). The binding of these antibodies 
to Rauscher cells and to normal marrow cells was examined 
by FFC. The Rauscher cell-binding profile of one of these 
antibodies, designated ERY-1, is shown in Fig 1. Greater 
than 80% of Rauscher cells bound MAb ERY-1 compared to 
the control rat IgG. In contrast, only 26% of Friend murine 
erthythroleukemia cells bound MAb ERY-1 (not shown), and 
this was at far lower fluorescence intensities than the 
Rauscher cells. Human K-562 cells did not bind MAb 
ERY-1.

The initial flow cytometric analysis of MAb ERY-1 
binding showed that a population of cells within normal
murine bone marrow bound the antibody (Fig 2A). Those cells that exhibited MAb ERY-1 binding were a continuous rather than discrete population whose fluorescence distribution was a broad shoulder of gradually increasing fluorescence intensity when compared to the control cell population that was incubated with irrelevant rat IgG (Fig 2A). This fluorescence distribution was different from that obtained when an antimyeloid monoclonal antibody, MAb MYELO-2, was used (Fig 2B). In this case a discrete population of cells was well separated from the control (and nonreactive) population.

Using MAb ERY-1, we sorted normal bone marrow cells into “positive” (ERY-1+) and “negative” (ERY-1−) populations (see Fig 2A for sorting gates) and examined them morphologically after spinning onto glass slides, fixing, and staining (Table 1). The ERY-1+ population consisted of 44% erythroid cells, 17% lymphoid cells, and 39% myeloid cells. This was in contrast to the ERY-1− population, which contained 0% erythroid cells, 14% lymphoid cells, and 86% myeloid cells. These data suggested to us that MAb ERY-1 recognized a determinant that was at least predominantly erythroid and prompted further investigation using highly enriched populations of hematopoietic cells.

**Binding of Monoclonal Antibody ERY-1 to Enriched Populations of Hematopoietic Cells**

Further confirmation of the erythroid nature of the ERY-1 antigen was sought by examining the binding of MAb ERY-1 to fetal and adult liver cells. Liver erythropoiesis is quite brisk in the 12- to 13-day-old murine fetus. However, it begins to decline markedly at day 14 and has essentially ceased at the time of birth. Using fluorescence flow cytometric analyses, we found that the 13-day murine fetal liver contained 70% ERY-1+ cells (Table 2). This value decreased to 35% ERY-1+ at day 14 and, finally, to a total absence of ERY-1+ cells in the adult. Thus, the pattern of MAb ERY-1 binding to murine fetal liver cells paralleled that of hepatic erythropoiesis, supporting the identification of the ERY-1 determinant as an erythroid antigen.

In contrast, MAb ERY-1 did not bind to enriched populations of neutrophils, splenic lymphocytes (predominantly B cells), thymocytes, peritoneal macrophages, or platelets (Table 2). These results provided strong evidence that the ERY-1 determinant is restricted to the erythroid lineage and that the presence of nonerythroid cells in the ERY-1+ population obtained after sorting normal bone marrow (Table 1) was due to the gate settings of the Cytofluorograf.

### Table 1. Binding of ERY-1 Monoclonal Antibody to Normal Murine Bone Marrow Cells Assessed by Fluorescence Flow Cytometry

<table>
<thead>
<tr>
<th>Population</th>
<th>Erythroid</th>
<th>Lymphoid</th>
<th>Myeloid</th>
</tr>
</thead>
<tbody>
<tr>
<td>ERY-1+</td>
<td>44</td>
<td>17</td>
<td>39</td>
</tr>
<tr>
<td>ERY-1−</td>
<td>0</td>
<td>14</td>
<td>86</td>
</tr>
<tr>
<td>Unsorted</td>
<td>16</td>
<td>22</td>
<td>62</td>
</tr>
</tbody>
</table>

### Table 2. Binding of ERY-1 Monoclonal Antibody to Murine Hematopoietic Cells

<table>
<thead>
<tr>
<th>Cell Type</th>
<th>ERY-1+ Cells (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fetal liver</td>
<td></td>
</tr>
<tr>
<td>13 day</td>
<td>70</td>
</tr>
<tr>
<td>14 day</td>
<td>35</td>
</tr>
<tr>
<td>Adult liver</td>
<td>0</td>
</tr>
<tr>
<td>Neutrophils</td>
<td>&lt;1</td>
</tr>
<tr>
<td>Lymphocytes, spleen</td>
<td>&lt;1</td>
</tr>
<tr>
<td>Thymocytes</td>
<td>&lt;1</td>
</tr>
<tr>
<td>Macrophages, peritoneum</td>
<td>&lt;1</td>
</tr>
<tr>
<td>Platelets</td>
<td>&lt;1</td>
</tr>
</tbody>
</table>
Identification of ERY-1- Cells in the Bone Marrow, Spleen, and Peripheral Blood During Erythropoietic Stress

Our initial flow cytometric and morphological examination of MAB ERY-1 binding to normal bone marrow indicated that the monoclonal antibody bound to erythroid precursors. Therefore, conditions of erythropoietic stress that result in higher frequencies of erythroid precursors should cause an increase in the relative number of ERY-1+ cells. We compared the percentages of ERY-1+ cells present in the bone marrow, spleen, and peripheral blood of normal animals to those in animals with phenylhydrazine-induced hemolytic anemia (Table 3). The number of ERY-1+ cells in the normal bone marrow of three mice tested ranged from 12% to 19%. This value increased to 38% during phenylhydrazine anemia. Similarly, the number of ERY-1+ cells in the spleen rose from less than 1% in the normal spleen to 32% in the erythropoietic spleen of phenylhydrazine treated animals. Lastly, the peripheral blood of normal animals contained 0% ERY-1+ cells. This value rose markedly to 40% during phenylhydrazine anemia.

Binding of Monoclonal Antibody ERY-1 to Progenitor Cells

The data presented thus far indicated that the ERY-1 antigen is present on developing erythroid precursors but that it disappears sometime before erythroid maturation is complete. Since this pattern implied that the expression of ERY-1 is specific for certain states of erythroid differentiation, we considered it important to determine the distribution of this determinant among various progenitor cells. The analysis was performed as follows. Normal bone marrow cells were reacted with ERY-1 monoclonal antibody and separated into ERY-1- and ERY-1+ populations by FFC. Approximately 15% to 20% of the normal cells were designated ERY-1+. Sorted populations of cells that were either ERY-1- or moderately to strongly ERY-1+ were collected and assayed in vitro for BFU-E, CFU-E, and CFC-E, and for granulocyte progenitors (CFU-C) (Table 4).

The most primitive of the erythroid progenitors, BFU-E, was completely absent from the ERY-1- population and was present in the ERY-1+ population. This was in marked contrast to the more mature progenitor CFU-E which was found almost exclusively (98%) in the ERY-1+ population. This appearance of the ERY-1 antigen on the CFU-E was maintained into the most mature progenitor population, the CFC-E, all of which were ERY-1+. As expected, the granulocyte progenitors, CFU-C, were found highly concentrated in the ERY-1+ population. This value increased to 137% of the starting density after two days and a dramatic decrease to less than 7% of maximum after six days of induction with erythropoietin. This pattern of ERY-1 expression, which was identified in three replicate experiments, strongly resembles the pattern found during normal erythropoiesis, further confirming the similarity of Rauscher cell differentiation to normal bone marrow erythroid development. In similar experiments using dimethyl sulfoxide induction, no initial increase in ERY-1 was detected. As DMSO induction was continued, however, ERY-1 expression diminished correspondingly and was less than 10% after six days.

Western Blot Analysis of ERY-1

The pattern of ERY-1 antigen expression occurring during normal erythropoietic development was reflected during the differentiation of Rauscher cells. As shown in Fig 4, the density of ERY-1 antigen was slightly higher after six hours and doubled within the first day of erythropoietin induction, analogous to the increase found as BFU-E develop into CFU-E. This was followed by a reduction to 137% of the starting density after two days and a dramatic decrease to less than 7% of maximum after six days of induction with erythropoietin. The pattern of ERY-1 expression, which was identified in three replicate experiments, strongly resembles the pattern found during normal erythropoiesis, further confirming the similarity of Rauscher cell differentiation to normal bone marrow erythroid development. In similar experiments using dimethyl sulfoxide induction, no initial increase in ERY-1 was detected. As DMSO induction was continued, however, ERY-1 expression diminished correspondingly and was less than 10% after six days.

ERY-1 Expression During Rauscher Cell Differentiation

ERY-1 Expression During Rauscher Cell Differentiation

erythropoietic spleen of phenylhydrazine treated animals.

Approximately 1

Table 3. Identification of ERY-1- Cells in Bone Marrow, Spleen, and Peripheral Blood of Normal and Phenylhydrazine-Treated Animals

<table>
<thead>
<tr>
<th>Cells</th>
<th>ERY-1- Cells (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bone Marrow</td>
<td></td>
</tr>
<tr>
<td>Normal</td>
<td>12, 19, 17*</td>
</tr>
<tr>
<td>Phenylhydrazine</td>
<td>38</td>
</tr>
<tr>
<td>Spleen</td>
<td></td>
</tr>
<tr>
<td>Normal</td>
<td>&lt;1</td>
</tr>
<tr>
<td>Phenylhydrazine</td>
<td>32</td>
</tr>
<tr>
<td>Peripheral Blood</td>
<td></td>
</tr>
<tr>
<td>Normal</td>
<td>0</td>
</tr>
<tr>
<td>Phenylhydrazine</td>
<td>40</td>
</tr>
</tbody>
</table>

*Three separate determinations.

Table 4. Binding of ERY-1 Monoclonal Antibody to Progenitor Cells

<table>
<thead>
<tr>
<th>Progenitor</th>
<th>Sorted Population*</th>
</tr>
</thead>
<tbody>
<tr>
<td>BFU-E</td>
<td>ERY-1+ 12</td>
</tr>
<tr>
<td>CFU-E</td>
<td>ERY-1+ 6</td>
</tr>
<tr>
<td>CFC-E</td>
<td>ERY-1+ 0</td>
</tr>
<tr>
<td>CFU-C</td>
<td>ERY-1+ 555</td>
</tr>
</tbody>
</table>

*Progenitors per 10^6 cells.
ERYTHROID DIFFERENTIATION ANTIGEN

Fig 4. Effect of erythropoietin induction on Rauscher cell ERY-1 membrane antigen density. Units of antigen density are linear fluorescence units as volts converted from the log scale.13,14 The fluorescence obtained using equivalent concentrations of irrelevant rat IgG was less than 0.05 units at all time points and has been subtracted from the ERY-1 values. The points represent the mean ± SEM of three replicate experiments.

DISCUSSION

In the present study we have employed the continuous cell line Rauscher murine erythroleukemia as immunogen and have generated a monoclonal antibody that recognizes an antigenic determinant, designated ERY-1, present on normal erythroid progenitors and precursors. The data indicate that the ERY-1 antigen is restricted to the erythroid lineage and is expressed only during certain stages of erythropoiesis.

Initial FFC analyses (Fig 2A) showed that the population of bone marrow cells expressing ERY-1 was continuous with the negative population rather than separated discretely (Fig 2B). Hence, the fluorescence intensities employed to discriminate “positive” from “negative” cells were necessarily somewhat arbitrary. The consequences of this partial overlap of populations is seen in Table 1. Thus, although no erythroid precursors were found in the ERY-1- population (ie, all erythroid precursors were ERY-1+), the ERY-1+ population in this analysis was not exclusively erythroid. Although an alternate explanation for this observation is that a portion of both myeloid and lymphoid cells actually expresses the ERY-1 determinant, this seems quite unlikely, since the ERY-1 determinant was not found on highly enriched populations of these and other nonerythroid hematopoietic cells (Table 2).

ERY-1 antigen was not found on the early erythroid progenitor, BFU-E. In this regard it is important to note that the lower limit of detection of cell surface determinants using conventional FFC, such as that employed in this study, is approximately 10,000 molecules per cell. Thus, the designation of BFU-E as ERY-1- must be considered as relative rather than absolute. Nonetheless, ERY-1 expression is clearly increased markedly in the CFU-E and CFC-E and decreases thereafter during precursor maturation. Importantly, this pattern of ERY-1 expression was exhibited with remarkable fidelity during erythropoietin-induced Rauscher cell differentiation, supporting the use of these cells as a model system for the study of erythropoiesis.

Monoclonal antibodies that recognize surface determinants restricted to immature mammalian erythroid cells have been reported previously. Yokochi et al described two antibodies that bind to immature human erythroid cells at different stages of differentiation.16 A third MAb, reported recently from this same laboratory, binds to CFU-E, erythroblasts, and mature erythrocytes.17 Tonevitsky et al have developed a rat MAb that recognizes a 69 kD antigen found on murine nucleated red cells and reticulocytes.16 Although many other MAbs that bind to erythroid cells have been described, strict lineage specificity is quite unusual.

At present, the identity and function of the ERY-1 antigen is unknown. It does not appear to be associated with the transferrin receptor since it exists at a very low density on Friend cells which are known to express greater than 107 copies of the transferrin receptor per cell.17 Moreover, the absence or presence of excess transferrin has no effect on the binding of MAb ERY-1 to Rauscher cells (data not shown). Since the pattern of ERY-1 expression during differentiation of erythroid progenitors resembles the pattern of erythropoietin sensitivity, it is possible that the ERY-1 antibody recognizes the erythropoietin receptor or a macromolecule associated with it. The addition of ERY-1 antibody to Rauscher cell cultures results in striking cell clustering but does not interfere with the ability of the cells to grow or to respond to erythropoietin (data not shown). Nevertheless, the antibody could recognize a portion of the receptor remote from the hormone-binding domain. Another possibility to be considered is a relationship between the ERY-1 determinant and the fibronectin binding sites found on erythroleukemia cells and on immature, normal erythroid cells.20,21 ERY-1 expression during normal and Rauscher cell erythropoiesis is remarkably similar to that reported for these sites. Finally, the similarity of ERY-1 to EP-1, a determinant found on human erythroid cells,16 must be noted. Establishing the identity and function of ERY-1 and other related differentiation-specific antigens may be pivotal in elucidating the processes of erythropoietic commitment and differentiation.

REFERENCES


Identification and characterization of a differentiation-specific antigen on normal and malignant murine erythroid cells

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