Effect of Cyclophosphamide on the Murine Hematopoietic Stem Cell Compartment as Measured by Different Assay Techniques

By Samuel Hellman and Helen E. Grate

Three different methods have been used to measure the survival of the hematopoietic stem cell pool following treatment with cyclophosphamide. Two of these systems measure the stem cell pool by its ability to proliferate and differentiate into mature progeny. In both these methods irradiated recipient mice receive syngeneic bone marrow from either normal or cyclophosphamide-treated animals. A period of time is allowed for the transplanted progenitor cells to divide and differentiate, and then the progeny produced are assayed. Ability to form red blood cells is assessed by the amount of radioactive iron incorporated into newly formed erythrocytes. Capacity for granulocyte formation is measured by peripheral white blood cell counts following endotoxin stimulation. The pool as measured by its ability to produce erythrocytic progeny appears to be more sensitive than as measured by its ability to produce granulocytic progeny. The spleen colony assay gives results similar to the assay of granulocytic progeny. These results, taken with previous data indicating decrease in erythroid precursors in spleen colonies derived from cells surviving cyclophosphamide, are interpreted as indicating a decrease in ability for erythroid differentiation in cells surviving cyclophosphamide.

The stem cell compartment of mouse bone marrow can be assayed by the ability of transplanted syngeneic bone marrow cells to form colonies in the spleens of irradiated mice. This technique, introduced by Till and McCulloch, measures a class of colony-forming cells that appears to be capable of both self-replication and differentiation to a variety of hematopoietic forms. "Stem cells" will be used synonymously here with "progenitor cell" to mean a cell capable of extensive self-replication, as well as progressive differentiation to one or more mature cell types.

Another approach to measuring the stem cell compartment is by the use of repopulating assays. In such techniques, irradiated recipient animals are injected with syngeneic bone marrow cells. These cells are allowed to proliferate for a period of time, and then the newly formed progeny of the injected cells are measured. If one waits a period of time sufficient for proliferation and differentiation of the injected bone marrow progenitor cells, this measurement of progeny produced can be used as an assay of the number of such progenitor cells in the injected bone marrow. The erythrocyte progenitor...
progeny are determined by measuring the incorporation of \(^{59}\text{Fe}\) into newly formed erythrocytes in the peripheral blood.\(^4\) This has been shown to be proportional to the number of bone marrow cells injected, and if one compares normal bone marrow with bone marrow from irradiated animals, a radiation survival curve can be determined.\(^6\) A similar assay for granulocytic progeny has also been described.\(^5\) The granulocytic progeny are measured by the white blood cell response to bacterial endotoxin. Survival fractions can be similarly determined. We have previously described these methods in detail and have indicated that increased demand for erythrocytes will decrease the ability of bone marrow to give rise to granulocytic progeny as measured by these assays.\(^7\)^\(^8\) thus, indicating some competition between the two assays presumably for a common stem cell pool. In a previous report, we have indicated that the response to radiation is different as measured by the three assays.\(^9\) This present report describes the response to cyclophosphamide as measured by these assays. A portion of this data has been briefly reported previously.\(^10\)

**Materials and Methods**

Animals used in all experiments were either male C3H/HEJ mice or, when specifically described in the text, female AKR/J mice all 8–12 wk old (Jackson Laboratories, Bar Harbor, Maine). X-irradiation factors were 250 kV, 15 mA, HVL, 1.5 mm of copper, focus to skin distance of 50 cm. The dose rate for whole animal irradiation was approximately 110 rads/min. Dose measurements were made in a mouse "phantom" using a Baldwin-Farmer dosimeter whose calibration was confirmed by the National Bureau of Standards. Before and after irradiation, animals were placed in cages of six to eight animals and supplied with food and water ad libitum.

Cyclophosphamide (cytoxan by Mead Johnson, Inc.) was prepared by dissolving 100 mg of the drug and 45 mg of sodium chloride in 5 cc of distilled water. The appropriate dose of drug was injected intravenously unless stated otherwise in the text. All injections were made with freshly prepared drug.

Bone marrow cell suspensions were prepared by flushing the medullary cavity of the tibia and femur with cold sterile Tyrode solution. Cell suspensions were kept on ice until used and were injected intravenously into the lateral tail vein not longer than 90 min following removal. A sufficient volume of solution was used so that the injected bolus varied between 0.05 and 0.4 cc. Cell counts were made using a hemocytometer. Donor marrow was taken either from donor mice immediately or at scheduled times following cyclophosphamide. All donor marrow was pooled from both hind limbs of at least two donors.

**Assay of Erythrocytic Repopulating Ability (ERA) of Transplanted Bone Marrow**

Recipient animals were exposed to 700 rads whole body irradiation and within 2 hr injected with graded doses of syngeneic normal bone marrow suspended in Tyrode solution. Similar litter-mate animals were injected with treated bone marrow. This marrow was taken either from donor mice immediately or at indicated times after cyclophosphamide. Seven days were allowed for the cells to proliferate. This period of time appears sufficient to assay the continued proliferative ability of progenitor cells.\(^9\) The animals then received 0.5 \(\mu\)Ci of \(^{59}\text{Fe}\) as ferrous citrate. Two days later, a cardiac puncture was made, and 0.5 ml of blood removed and counted in a well scintillation counter. The per cent of \(^{59}\text{Fe}\) incorporation was then calculated, assuming a blood volume of 0.66 cc/10 g of body weight. The per cent of \(^{59}\text{Fe}\) incorporation was then plotted against the number of bone marrow cells injected. This can be used to estimate the number of normal cells necessary to produce the same \(^{59}\text{Fe}\) uptake as that produced by a larger number of treated cells. From this, a survival fraction can be determined as described by Hodgson.\(^8\) Each determination used a test group of six
to eight mice injected with treated marrow and three groups of six mice, each group receiving a different dose of normal bone marrow. An additional group of six mice served as an uninjected control.

**Assay of Granulocytic Repopulating Ability (GRA) of Transplanted Bone Marrow**

This system has been described in detail previously.\(^5\,9\) Recipient animals were exposed to 700 rads whole body irradiation and within 2 hr injected intravenously with graded doses of syngeneic normal bone marrow. Similar litter-mate animals were injected with treated marrow. This marrow was taken from donor mice either immediately or at indicated times after cyclophosphamide. Nine days were allowed for the bone marrow cells to proliferate, and then the animals were injected intravenously with 10 μg of a preparation of lipopolysaccharide endotoxin isolated from *Salmonella abortus equi* by the Westphal Method (Difco Co.). Nine days appears sufficient to assay the continued proliferative ability of progenitor cells.\(^9\) The white blood cell count of each animal was measured immediately before and 2, 4, and 6 hr following the endotoxin injection using a Fisher Autocytometer. The maximum white blood cell responses in the animals were then grouped and plotted against the number of cells injected earlier. Compared to this was the white blood cell response in that group of animals injected with treated bone marrow. From this, one can estimate the number of normal cells necessary to produce the same white blood cell count that a larger number of treated cells produced, and a survival fraction can be determined. White blood cell counts were used, since the response to endotoxin in this method has been shown to be a granulocytic response,\(^9\) thus obviating the need for differential counts. Each determination of survival fraction used a test group of six to eight mice injected with treated marrow and three groups of six mice, each group receiving a different dose of normal bone marrow. An additional group of six mice served as an uninjected control.

**Spleen Colony Assay (SCA)**

Recipient animals received 600 rads, then 3–6 hr later received 450 rads, and within 2 hr following this second dose they received the injected bone marrow cells. Delivering the radiation in this divided fashion allowed more surviving animals with the background colony number still less than 0.1 per animal. Nine days later, the surviving animals were sacrificed, and the spleens removed and placed in Bouin's solution. Twenty-four hours later, macroscopically visible colonies were counted. The number of colony-forming units was calculated, and a survival fraction determined.\(^11\) Each determination was based on counting at least ten spleens in each normal and treated bone marrow group. An uninjected control group was scored with each experiment.

**RESULTS**

**Survival of Bone Marrow Progenitor Cells 24 Hr Following Cyclophosphamide**

Animals were treated with cyclophosphamide intraperitoneally. Twenty-four hours later bone marrow was removed from hind limbs, and the sensitivity to cyclophosphamide scored using the three assay techniques. Figure 1 shows the results using the spleen colony technique. A similar experiment was done using AKR mice, and these are indicated on the same figure. The stem cells in AKR mice appear more sensitive to cyclophosphamide. A linear relationship is assumed. However, the points are so dispersed that this relationship may be questioned. The limitation on values derived from marrow removed 24 hr after cyclophosphamide is discussed below. In Fig. 2 the three stem cell assay techniques are compared. There is a large and striking difference between the SCA and GRA as compared to survival scored by the ERA with the latter appearing far more sensitive.
HEMATOPOIETIC STEM CELL COMPARTMENT

Fig. 1.—Spleen colony assay survival fraction measured 24 hr after i.p. administered cyclophosphamide. Closed circles indicate C3H mice; open circles indicate AKR mice. Each point is indicated with its standard error. Line is fitted by regression analysis using methods of least squares for C3H points.

Survival of Bone Marrow Progenitor Cells Removed 3 Hr Following Cyclophosphamide

Table 1 shows that bone marrow cellularity is markedly reduced at 24 hr. Because of this cell depletion and possible compensatory homeostatic mechanisms, the experiments reported above were repeated using intravenously administered cyclophosphamide with bone marrow cells removed 3 hr thereafter. Figure 3 shows the results using the SCA. In contrast to Fig. 1, we see a nonlinear relationship and apparent “shoulder” of the survival curve. After initial “resistance,” there is a rapid decrease in survival with increasing doses of cyclophosphamide. Over the doses studied, the curve becomes less concave and may, at the terminal portion, show an exponential decrease in survival.

Fig. 2.—Survival fraction measured 24 hr after i.p. administered cyclophosphamide. Closed circles indicate GRA, open circles indicate ERA, and closed triangles indicate SCA. Each point is indicated with its standard error. Lines are drawn by eye: solid line through GRA points, and broken line through ERA points. All determinations done in C3H mice. Note differences in scale from Fig. 1. GRA, granulocytic repopulating ability; ERA, erythrocytic repopulating ability; SCA, spleen colony assay.
Table 1.—Comparison of Bone Marrow Cell Number at 3 hr and 24 hr After Cyclophosphamide

<table>
<thead>
<tr>
<th>Dose of Cyclophosphamide (mg)</th>
<th>Hind Limb Cellularity as Function of Normal Control</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>3 hr</td>
</tr>
<tr>
<td>2 mg</td>
<td>0.95</td>
</tr>
<tr>
<td></td>
<td>1.04</td>
</tr>
<tr>
<td>4 mg</td>
<td>0.95</td>
</tr>
<tr>
<td></td>
<td>0.96</td>
</tr>
<tr>
<td></td>
<td>0.77</td>
</tr>
<tr>
<td></td>
<td>0.87</td>
</tr>
<tr>
<td>6 mg</td>
<td>0.75</td>
</tr>
<tr>
<td></td>
<td>0.89</td>
</tr>
<tr>
<td></td>
<td>0.99</td>
</tr>
</tbody>
</table>

Numbers in parentheses indicate SE.

Again, AKR stem cells were tested and appear to be more sensitive than the C3H.

Figure 4 compares the survival fractions as determined by the three assays. Under these circumstances, the GRA appears most resistant, and the ERA appears extremely sensitive. The SCA is perhaps somewhat more sensitive than the GRA at the 6 mg dose but not nearly as sensitive as the ERA.

![Fig. 3.—Spleen colony assay survival fraction measured 3 hr after i.v. administered cyclophosphamide. Closed circles indicate C3H mice; open circles indicate AKR mice. Each point is indicated with its standard error. Curve is drawn by eye.](image-url)
Fig. 4.—Survival fraction measured 3 hr after i.v. administered cyclophosphamide. Closed circles indicate GRA, open circles indicate ERA, and closed triangles indicate SCA. Each point is indicated with its standard error. Lines are drawn by eye. All determinations done in C3H mice. Note difference in scale from Fig. 3. GRA, ERA, and SCA as in legend of Fig. 2.

Recovery From 4 mg Cyclophosphamide

Recipient mice were given 4 mg cyclophosphamide intravenously, and at varying times following this administration bone marrow cells were removed, and the surviving fractions were scored using the repopulation assays. The results are seen in Fig. 5. The initial point again demonstrates the marked sensitivity to cyclophosphamide as measured by the ERA as compared to the GRA. Both have an initial dip and subsequent recovery. Recovery appears to be complete in 6 days as measured by the GRA. With the ERA one sees an

Fig. 5.—Fraction of cells surviving i.v. administered cyclophosphamide (4 mg) as measured by the GRA or ERA at various times after drug administration. All points are indicated with their standard errors. All determinations done in C3H mice.
abortive rise with a subsequent trough at 8 days before beginning to return to normal. It thus appears that the bone marrow stem cells are both more sensitive initially and recover more slowly as measured by the ERA than by the GRA.

**Discussion**

The stem cell compartment appears clearly more sensitive by ERA than by GRA. We have previously shown this is true with X-ray, although the disparity is much less than seen with cyclophosphamide. Since we have previous evidence showing that increase in ERA is associated with concomitant decrease in GRA, we feel that these assays, at least in part, measure a common stem cell pool. Both these data and the X-ray data can be interpreted as indicating that either stem cell differentiation to erythropoiesis is preferentially impaired as compared to granulopoiesis, or that in addition to a common pool of stem cells the ERA measures a specific pool of unipotent erythroid progenitors that are quite sensitive to cytotoxic agents, and further, that such cells are not scored by either the GRA or the SCA. If this latter were true, then the spleen colonies produced from cells surviving cyclophosphamide treatment should have a similar distribution of differentiated forms within them as do those from untreated bone marrow cells. This is not the case. The colonies are quite different in that there is a marked reduction in erythroid differentiation in those produced by cells surviving alkylating agents. This problem is discussed in greater detail in a separate report. We conclude, however, that the present data are most consistent with the notion that cyclophosphamide affects erythrocytic differentiation of stem cells to a much greater extent than granulocytic differentiation.

Bruce et al. reported the survival of colony-forming cells 24 hr after intraperitoneal cyclophosphamide. Their data, using AKR/J mice, are quantitatively different from ours using C3H/HEJ mice. Because of this, we repeated some of our results using AKR/J shown in Figs. 1 and 3. These show a strain difference in the response to cyclophosphamide. Further, in the study of Bruce et al the SCA is performed 24 hr after intraperitoneal administration. The data here reported indicate some of the problems in interpreting such survival data. Since by 24 hr there is marked bone marrow cell depletion (Table 1), one wonders whether compensatory homeostatic mechanisms resulting in further stem cell depletion due to differentiation pressures may not be operative, resulting in the observation that 24-hr values are regularly two- to fourfold less than the 3-hr values with the GRA and ERA. Thus, we favor use of the 3-hr determination for evaluation of the direct effect of cyclophosphamide on the stem cell.

The presence of a “shoulder” on a cyclophosphamide survival curve has been interpreted in murine leukemia as indicating the ability of such cells to repair or accumulate sublethal damage. This is based on the similarity to radiation survival curves. While this interpretation may be correct, there are other alternatives when considering cyclophosphamide. For example, since the drug must be activated in vivo, this shoulder may represent the kinetics of such activation and/or possible inactivation. Evidence against there being repair
HEMATOPOIETIC STEM CELL COMPARTMENT

of sublethal damage to alkylating agents are studies with the alkylating agent nitrogen mustard that do not show a similar shoulder in tissue culture14 or in murine colony-forming cells.15

In order to study the recuperation of the ERA and GRA, bone marrow was removed from donor animals as a function of time after 4 mg of cyclophosphamide (Fig. 5). These data show that not only is the ERA affected more initially, but recovery is more protracted. This is unlike X ray where the stem cells appear more sensitive initially but show more rapid recuperation as measured by ERA than by GRA.9,10 With cyclophosphamide, there appears to be an abortive rise reaching a maximum at day 6. The explanation of the abortive nature of this rise is not evident from the data reported but suggests compensatory homeostatic mechanisms may be involved in this secondary reduction in erythropoiesis, perhaps in response to the reduced peripheral granulocyte level present at that time. The ERA remains significantly depressed even when the GRA has returned to normal. Such results appear consistent with the clinical observation of failure of erythropoiesis and consequent anemia in patients chronically receiving cyclophosphamide.

These data here reported indicate that the alkylating agent cyclophosphamide not only kills stem cells, but those stem cells surviving have a markedly impaired erythropoietic potential. This is substantiated by analysis of the 59Fe incorporation in spleen colonies produced by cells surviving cyclophosphamide.11 The mechanism by which this occurs is unknown.

REFERENCES

12. Bruce, W. R., Meeker, B. E., and Valeriote, F. A.: Comparison of the sensi-


Effect of Cyclophosphamide on the Murine Hematopoietic Stem Cell Compartment as Measured by Different Assay Techniques

SAMUEL HELLMAN and HELEN E. GRATE