BRIEF REPORT

Cloning of Syngeneic Hematopoietic Cells in the Spleens of Mice and Rats Pretreated with Cytotoxic Drugs

By GEORGE W. SANTOS AND MANSOUR HAGHSHENASS

The cloning of hematopoietic cells in the spleens of lethally X-irradiated mice has proven to be a useful technic for the experimental hematologist.1 Facilities for the X-irradiation of rodents however, are not always conveniently available to the investigator. The purpose of this report is to describe a method for the quantitative cloning of hematopoietic cells in the spleens of rats and mice pretreated with cytotoxic drugs.

MATERIALS AND METHODS

Animals*

Hybrid female rats, age 10-12 weeks, of the LBNF₁ (Lewis × BN)F₁ strain and female* mice, age 10-12 weeks, of the CD₂F₁ (BALB/c × DBA/2)F₁ strain were used as experimental animals. Rats were housed in plastic cages, four to a cage and mice in polycarbonate cages with filter tops, 10 mice to a cage. They were provided with Purina Chow and tap water ad lib.

Drugs*

Busulfan (BU) was prepared in 25 per cent methylcellulose and cyclophosphamide (CY) was prepared in saline. Both drugs were given as single i.p. injections.

Cell Preparations

Cell suspensions of spleen and bone marrow were prepared in cold Tyrode's solution as described previously.2 Total nucleated cell counts were performed with standard hematologic technics and viable cells estimated by trypan blue exclusion.3 Mouse and rat marrow suspensions were 2 to 5 per cent trypan blue positive and spleen suspensions were 10 to 15 per cent trypan blue positive. Appropriate concentrations of cell suspensions were injected i.v. in a 1 ml. volume in rats and in a 0.5 ml. volume in mice.

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*Rats were purchased from Microbiological Associates, Bethesda, Maryland. Mice were purchased from Cumberland View Farms, Clinton, Tennessee.

Hab Cages, Inc., Hackensack, N. J.

*CY was generously supplied by the Cancer Chemotherapy National Service Center. BU was kindly supplied by Dr. George Hitchings of Burroughs Wellcome and Co., Inc., Tuckahoe, New York.

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Table 1.—Lethality of Experimental Drugs*

<table>
<thead>
<tr>
<th>Animal†</th>
<th>Drug</th>
<th>LD50 ± 1 S.E. (mg./Kg.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>LBNF1 Rat</td>
<td>Busulfan</td>
<td>24.0 ± 2.1</td>
</tr>
<tr>
<td>LBNF1 Rat</td>
<td>Cyclophosphamide</td>
<td>291.5 ± 13.5</td>
</tr>
<tr>
<td>CD2F1 Mouse</td>
<td>Busulfan</td>
<td>89.3 ± 4.5</td>
</tr>
<tr>
<td>CD2F1 Mouse</td>
<td>Cyclophosphamide</td>
<td>456.2 ± 6.2</td>
</tr>
</tbody>
</table>

*Groups of 10-40 animals were injected intraperitoneally at various dose levels for each drug and observed for 42 days.
†Female animals 10-12 weeks of age.

Table 2.—Cloning of Syngeneic Hematopoietic Cells in the Spleens of Drug Treated Rodents*

<table>
<thead>
<tr>
<th>Host</th>
<th>Cells</th>
<th>Slope ± 1 S.E.</th>
<th>Ordinate Intercept ± 1 S.E.</th>
<th>Correlation Coefficient</th>
<th>Number of Animals</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mouse</td>
<td>Marrow</td>
<td>(2.65 ± 0.48)10⁻⁴</td>
<td>2.32 ± 2.66</td>
<td>0.59</td>
<td>58</td>
</tr>
<tr>
<td>Mouse</td>
<td>Spleen</td>
<td>(5.48 ± 0.60)10⁻⁴</td>
<td>0.33 ± 1.32</td>
<td>0.60</td>
<td>145</td>
</tr>
<tr>
<td>Rat</td>
<td>Marrow</td>
<td>(3.56 ± 0.63)10⁻³</td>
<td>2.34 ± 3.09</td>
<td>0.63</td>
<td>58</td>
</tr>
</tbody>
</table>

*Number of spleen colonies = slope X no. of cells injected + intercept.

**Assay**

Animals were sacrificed and spleens fixed in Carnoy’s solution prior to macroscopic colony counting. Sections, 4 microns thick, were cut at 20 micron intervals. Staining for differential counting was performed with hematoxylin and eosin or a modified Giemsa technique.

**Statistical Analysis**

All computations were made using methods described previously. In brief, regression lines were fitted by the method of least squares and then the slopes and ordinate intercepts were determined. The LD50 ± 1 S.E. was calculated by the method of Berkson.

**RESULTS**

**Lethality of Experimental Drugs**

The LD50 of CY and BU was determined in rats and mice given single injections of the drug. Groups of 10 to 40 animals were used at each of the several dose levels for each drug. Observations were made for 42 days. The LD50 ± 1 S.E. of CY and BU for rats and mice is entered in Table 1.

**Endogenous Colonies**

Four groups of 10 mice each were given either 300 mg./kg. of CY, 400 mg./Kg. of CY, 50 mg./Kg. of BU or 75 mg./Kg. of BU. Animals were sacrificed 7 days later and spleens were examined for macroscopic surface colonies which were seen in every case (endogenous colonies). Higher doses of these drugs resulted in prohibitive mortality prior to the seventh day after their administration. Four groups of 10 mice each were given 350 mg./Kg. of CY together with 50 mg./Kg. of BU. Animals were sacrificed 6, 7, 8, and 9 days.
Fig. 1.—Cloning of syngeneic marrow cells in CD2F₁ mice. ● Mean number of macroscopic surface colonies in the mouse spleen 7 days after cell transfer to CY and BU pretreated mice. Control spleens in 10 mice showed an average of 0.1 colony per spleen.

Fig. 2.—Cloning of syngeneic marrow cells in LBNF₁ rats. ● Mean number of macroscopic surface colonies in the rat spleen 7 days after cell transfer to CY and BU pretreated rats. Control spleens in 10 rats showed no visible colonies.
after drug. No endogenous colonies were seen on days 6 and 7. One spleen out of 10 and 1 spleen out of 8 examined 8 and 9 days after drug respectively showed one surface colony. Further experiments indicated that both the mortality and number of endogenous colonies per spleen increased after 9 days post drug treatment.

The situation was entirely analogous when rats were used. Neither drug proved useful when used alone. A combination of 200 mg./Kg. of CY and 30 mg./Kg. of BU proved satisfactory in that no endogenous colonies were seen up to 8 days after drug. A few colonies were seen on day 9 (average 0.1

<table>
<thead>
<tr>
<th>Species</th>
<th>Day</th>
<th>Erythrocyte</th>
<th>Erythrocyte Variant</th>
<th>Granulocyte</th>
<th>Undifferentiated</th>
<th>Mixed</th>
<th>Megakaryocyte</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mouse</td>
<td>7</td>
<td>25.8</td>
<td>22.0</td>
<td>1.5</td>
<td>39.7</td>
<td>1.1</td>
<td>9.6</td>
</tr>
<tr>
<td>Mouse</td>
<td>8</td>
<td>25.0</td>
<td>26.0</td>
<td>7.8</td>
<td>18.8</td>
<td>2.8</td>
<td>18.0</td>
</tr>
<tr>
<td>Rat</td>
<td>7</td>
<td>21.1</td>
<td>55.0</td>
<td>8.8</td>
<td>6.4</td>
<td>7.8</td>
<td>0.6</td>
</tr>
</tbody>
</table>

*Day after cell injection.
†From 150 to 200 colonies were examined in spleens containing 2 to 8 macroscopic colonies.
Cloning of Hematopoietic Cells

Mice were given 350 mg./Kg. of CY together with 50 mg./Kg. of BU and rats were given 200 mg./Kg. of CY together with 30 mg./Kg. of BU. Twenty-four hours later, various groups (10–20 animals per group) of mice or rats were given graded doses of nucleated syngeneic marrow or nucleated syngeneic spleen cells I.V. Animals were sacrificed 7 days after the injection of cells (8 days after drug) and spleens prepared for analysis. Regression lines were constructed to describe the relationship between the number of cells injected and the number of surface colonies seen. The slopes, ordinate intercepts and correlation coefficients of the regression lines are recorded in Table 2. The data representing the cloning of syngeneic marrow is displayed in Figure 1 for the mouse and in Figure 2 for the rat. Figure 3 depicts the gross appearance of hematopoietic colonies in the fixed spleens of drug pre-treated mice 8 days after the transfer of syngeneic marrow. Rat spleens were similar except that the colonies appeared to be slightly smaller.

Differential counts of the various colonies were performed on histologic sections (Table 3). No obvious difference in morphology was noted between rat or mouse derived colonies. Six types of colonies were seen: megakaryocyte, granulocyte, erythrocyte, erythrocyte variant, mixed and undifferentiated. Their spatial distribution in the spleen was similar to that described by Curry et al.7

Megakaryocyte colonies were too small to represent visible surface colonies but were included in the differential. A colony was arbitrarily defined as any grouping of 3 or more of these cells. Control mouse spleens contained
Fig. 5.—An erythrocyte spleen colony composed of cells of the red cell series at different levels of differentiation. Colony examined 7 days after cell injection in the drug pretreated mouse. Hematoxylin and eosin, × 2,000.

small but variable numbers of scattered megakaryocytes but rarely true colonies (< 0.5 per spleen). Rat control spleens were devoid of such cells.

Figure 4 shows a granulocyte colony in the mouse. Typical ring forms of the more mature granulocyte can be seen.

A typical erythrocyte colony in the mouse is noted in Figure 5. It is composed of more mature erythrocytes as well as some less differentiated members.

A mouse erythrocyte variant colony is depicted in Figure 6. In addition to large undifferentiated cells (Fig. 7) recognizable red cell precursors are seen. Mixed colonies containing two or more differentiated cell types were seen occasionally.

Figure 7 depicts an undifferentiated colony in the mouse. The cells are larger than those seen in Figure 5 and do not portray their ultimate differentiation.

DISCUSSION

The technic of cloning hematopoietic cells in the spleens of lethally irradiated mice has been used to investigate stem cell kinetics, erythropoiesis, radiation sensitivity, drug sensitivity, hematopoietic repopulation and the effect of micro-environment on stem cell differentiation. Recently this technic has been adapted to the irradiated rat. In the present studies, quantitative cloning of hematopoietic cells in the spleens of drug treated mice and rats is described. This modification frees the interested investigator from the requirements for x-ray facilities.

The cloning efficiency of mouse marrow in the present studies (e.g., number of surface spleen colonies per $10^4$ cells) is similar to the approximately one colony-forming cell per $10^4$ cells reported with x-ray. The results of the present studies would suggest that the mouse spleen contains 2–3
per cent as many hematopoietic colony-forming units (C.F.U.) for a given number of nucleated cells as does mouse marrow. Rat marrow contains about as many C.F.U. per number of nucleated cells as mouse marrow if one takes into account the differences in spleen volume in the two species. Indeed, this has been borne out in subsequent studies where rat marrow was cloned in the spleens of drug treated mice.\textsuperscript{15} The results reported herein are in extremely close agreement to the recent findings of Comas and Byrd for the cloning efficiency of rat marrow in the irradiated rat.\textsuperscript{14} These authors performed colony assay 12 days after irradiation and cell transfer. Considerable numbers of endogenous colonies were seen at this time and to attain an estimate of exogenous colonies they enumerated only those colonies larger than 1 mm. in diameter.

The differential counts noted in the present study for mouse marrow derived colonies examined 8 days after cell transfer are, within experimental limits, similar to that reported by others who employed X-irradiated mice as recipients.\textsuperscript{10} The data for the rat, however, suggest an earlier differentiation of cells as well as a decrease in the percentage of megakaryocyte colonies when compared to the mouse.

The present technic does not allow spleen colony assay beyond 9 days after drug treatment because of the appearance of endogenous colonies and/or prohibitive mortality in recipient mice and rats. This leads to a definite limitation of the method because it does not permit an easy identification and removal of small sized donor cell colonies from the unfixed spleens. Various approaches designed to overcome this limitation are currently being investigated.

Recently, Sensenbrenner et al.\textsuperscript{15} reported the cloning of rat, hamster and
Fig. 7.—An undifferentiated spleen colony 7 days after cell injection in the drug pretreated mouse. Hematoxylin and eosin, $\times 2,000$. The cells are larger than those seen in Figure 5 and show no evidence of differentiation. Hematoxylin and eosin, $\times 2,000$.

rabbit hematopoietic cells in the spleens of drug treated mice. In the same report, it was noted that allogeneic marrow cloned with the same efficiency as did syngeneic cells. This latter finding stands in apparent contrast to the report of McCulloch and Till$^{17}$ who noted that allogeneic cells cloned less efficiently than did syngeneic cells in X-irradiated mice. Reasons for the different observations are not entirely clear. However, it is quite probable that the doses of CY used are more immunosuppressive than the doses of x-ray used in cloning experiments.$^{18}$

**Summary**

A method is described for the quantitative cloning of syngeneic hematopoietic cells in the spleens of mice or rats pretreated with a combination of cyclophosphamide and busulfan. A linear relationship was demonstrated between the number of nucleated cells injected and the number of macroscopically visible surface spleen colonies counted 7 days later.

Six types of spleen colonies were encountered: megakaryocyte, granulocyte, erythrocyte, erythrocyte variant, mixed and undifferentiated. When compared to the mouse the rat showed an earlier differentiation of cells as well as a decrease in the percentage of megakaryocyte colonies.

This technic produces results, as far as cloning efficiency and morphology of spleen colonies are concerned, similar to those described in the mouse and rat following X-irradiation. The major advantage of the method is its independence from the requirements of x-ray facilities.

**SUMMARIO IN INTERLINGUA**

Es describite un methodo pro le clonage quantitativa de syngenic cellulas hematopoietic in le splenes de ratti et moses pretrattate con un combination de cyclophosphamida e busulfano. Un relation linear esseva demonstrate inter le numero de nucleate cellulas
SYNGENEIC HEMATOPOIETIC CELLS 637

injicite e le numero de macroscopicamente visible superficial colonias splenic contate septe dies plus tarde.

Sex typos de colonias splenic eseva incontrate: megakaryocytes, granulocytes, erythrocytes, variantes erythrocytic, cellulas mixte, e cellulas nondifferentiate. Un comparation del duo species animal usate monstrava pro le ratto un plus precoce differentiation cellular e etiam un plus basse procentaje de colonias megakaryocytes.

Le resultatos del technica, quanto al efficacia de clonage e al morphologia del colonias splenic, es simile a illos describite in muses e rattos post roentgeno-irradiation. Le major avantage del methodo es su independentia ab le requirimento de un apparatura de radios X.

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REFERENCES


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