Effects of Storage on CFU of Mouse Bone Marrow Cells*

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ABSTRACT

Mouse bone marrow cells were stored for 0.5, 16 and 24 hours under one of the following four conditions; saline G at room temperature, saline G at ice temperature, CMRL 1066 at room temperature and CMRL 1066 at ice temperature. Their viability was then examined by counting CFU in the spleen of irradiated recipient mice.

1) The CFU decreased rapidly with storage time under all four conditions. Among four storage conditions, the storage in saline G at ice temperature seemed to give slightly more favorable results than others.

2) Types of micro-colonies were not significantly different under the present storage conditions.

3) Since the size of endogenous colonies were relatively small compared to exogenous colonies, the observed colonies are likely to be resulted from injected bone marrow elements and some endogenous cells.

4) It is concluded that the storage conditions in the present paper are not suitable for the storage of bone marrow cells. This emphasizes the need of exploration of a better storage method.

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INTRODUCTION

The spleen colony technique which was first described by Till and McCulloch\textsuperscript{1}, has found wide use as a tool in studying cellular radiation sensitivity\textsuperscript{1}, stem cell kinetics\textsuperscript{2} immune reactions\textsuperscript{3}, anemic mutants\textsuperscript{4,7}, erythropoiesis\textsuperscript{8,17}, etc. In addition numerous studies describing morphology\textsuperscript{15,18-20} and types of spleen colonies have been reported using different treatments, techniques and criteria of colony evaluation at various times after injection of marrow or spleen cells.

In spite of the wide use of these techniques, little attention has been focused on the effects of temperature, storage and suspending media on the viability of colony forming units (CFUs) and morphology of resultant colonies (An exception being the few experiments on frozen marrow\textsuperscript{27-29}).

The importance of maintaining high levels of bone marrow cell viability during marrow transplants for therapeutic or investigative reasons is self-evident. We have reported significant differences in viability based on macroscopic and microscopic study of colonies seen after varying experimental storage conditions\textsuperscript{30,31}. The preliminary results of the microscopic examinations and histological scoring of colony type led us to further investigate the influence of storage time, media and temperature on colony type.

MATERIALS AND METHODS

Animals and Irradiation

Eight week old white male mice of the BNL strain were used as both donors and recipients. In this experiment there were 5 recipient mice in each group. Recipient mice received a whole body exposure of 750 rads 1 hour prior to the donor bone marrow injection. While no donor cells were injected to the irradiated mice for endogenous colony studies. All irradiations were performed using a 250 kvp x-ray machine operating at 250 kvp, 30 mA, and at a dose rate of approximately 120 rads per minute.

Bone Marrow Cell Preparation

In order to obtain bone marrow cells in the shortest time the Stoner and Bond method\textsuperscript{32} was used. The entire femur and tibia from donors were ground with mortar and pestle in less than 1 ml of each of the two suspending media. (This method not only shortens the preparation time but also leads to a higher yield of CFU's than the more common aspiration technique.) After the bone marrow cell suspensions were obtained, they were divided into equal amounts with one portion of each being maintained at room temperature while the other was placed in an ice bath.

The cellular content of the solutions was determined electronic counting with a Coulter Model A counter using a 100µ aperture. Standard injection solutions were prepared at concentrations of 600,000 and 2,500,000 cells per ml. These solu-
tions were stored for 30 minutes, 16 hours and 24 hours at room and ice temperature in each suspending media. At selected time intervals 1/10 ml (60,000 cells for 30 min. storage, 250,000 cells for 16 and 24 hours storage) were injected via tail vein into each irradiated recipient.

**Suspending Media**

The suspending media used were Puck's saline G, a buffered nutrient saline mixture routinely used for marrow suspension in this laboratory, and CMRL 1066, the tissue culture media indicated by Till and McCulloch.

**Histological Preparations**

Seven days after donor cell injections recipient mice were sacrificed, spleens removed, fixed in Bouin's solution or 24 hours, and the macroscopic colonies counted, using a 3× magnifier. The colony number corrections were made in the groups which had been injected with 250,000 donor cells, to make them comparable with the group receiving 60,000 cells. From the groups of spleens used to make the macro-colony counts, two spleens were randomly selected for sectioning from each group. The spleens from the group of no donor cell injection were also selected for macro, and microscopic colony counts. From sections through the longitudinal axis the entire spleen was evaluated. Every sixth section (5μ thickness) was mounted and stained with hematoxylin and eosin. These were photographed and a 25× enlargement made of each section. Then each colony was examined on the slide and classified according to histological type. Photographs of the sections facilitate counting the total number of colonies and aids in the identification of the microscopic colonies not visible on the surface.

**Determination of Colony Volume**

From mounted sections the area of each colony was determined by eyepiece micrometer (Whipple-Hauser). Thirty-six erythrocytic and 27 granulocytic colonies, randomly selected from 2 spleens, were evaluated individually by Reincke and ourselves, and the volumes calculated. The some data on the volume of endogenous colonies were referenced from Reincke34). In order to compare the actual measured volume and the theoretical volume, the following formula was used assuming the colony as a sphere.

\[
\overline{V} = \frac{4}{3} \pi r^3
\]

\[V=\text{volume of colony in } \mu^3 \quad \text{where } r = \frac{T_s + T_i}{2} \times N\]

\[T_s=\text{thickness of section in } \mu\]

\[T_i=\text{thickness of interval to next section in } \mu\]

\[N=\text{number of section}\]
RESULTS

*Macro-colony*

As previously reported, for periods up to 8 hours, the macro-colony counts have suggested that all suspensions survived longer at ice temperature. In addition, the saline G media was found superior to the CMRL 1066. In the present study, the temperature effect was still evident at 16 hours, however the media effect was not apparent. By 24 hours, no temperature effect was evident in the CMRL 1066 media. (Fig. 1)

*Histological nature of spleen-colony type.*

Colonies were classified as erythrocytic, granulocytic, and undifferentiated,
megakaryocytic, or mixed. Lymphatic colony have never been identified in this studies.

In all experimental conditions, the percent of erythrocytic colonies ranged from 36.5 to 58.2%, and granulocytic from 31.6 to 54.3% of the total colonies. The mean percentage of each colony type as affected by media and temperature are shown in Table 1. When the ratios of erythrocytic to granulocytic colony formation were calculated, the ratio ranged from 0.98 to 1.31, with no evidence of significant difference attributable to suspending media, temperature and/or storage time. In contrast, the proportion of megakaryocytic colony formation was twice as high in CMRL 1066 as in saline G suspension, however, no temperature effect on megakaryocytic colony formation was evident. At 30 minutes storage the highest erythrocytic, granulocytic and total counts were noted in saline G ice temperature solutions, whereas the lowest number were in CMRL 1066 ice temperature (Fig. 2). After 16 hours storage, the total microscopic colony count was reduced to approximately 20, and the erythrocytic and granulocytic to approximately 10. In all ex-

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<th>Experimental Conditions</th>
<th>Colony type in spleen (mean per cent of total)</th>
<th>Colony ratio</th>
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<tr>
<td></td>
<td>Colony type</td>
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<tr>
<td>Suspending media</td>
<td>Erythroid</td>
<td>Granuloid</td>
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<tr>
<td>Saline-G</td>
<td>50.0</td>
<td>38.2</td>
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<tr>
<td>CMRL 1066</td>
<td>43.0</td>
<td>44.3</td>
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<tr>
<td>Temperature</td>
<td>Room temp.</td>
<td>47.3</td>
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<tr>
<td></td>
<td>Ice temp.</td>
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Fig. 2. Effects of temperature, suspending media and storage time on viability changes in the microscopic colonies.
There is a relationship between colony volume and number of section which is a function of colony diameter. The straight line shows theoretical colony volume assuming the colony as a sphere.
cept the saline G room temperature solution, these values were maintained throughout the 24 hour test period.

Colony size distribution.

The erythrocytic and granulocytic colony sizes were determined microscopically from serial sections. The largest erythrocytic colony observed was identified in 55 consecutive sections (Fig. 3). Assuming a spherical shape, the number of sections in which a colony appears is a function of the diameter of the colony. Accordingly, there is a relationship between colony size and number of sections (diameter). This relationship is shown in Fig. 3. In our determinations, volume measurements by optical sizing are slightly lower than the corresponding mathematical volume calculation.

With few exceptions, endogenous colonies were usually small, mainly granuloid and appeared in 3 or fewer sections (Figs. 3 and 4).

In all experimental conditions, the greatest number of erythrocytic colonies ranged in volume between $50 \times 10^6 \mu^3$ to $200 \times 10^6 \mu^3$ while granulocytic colonies ranged from $2.0 \times 10^6 \mu^3$ to $10 \times 10^6 \mu^3$ (Figs. 3 and 5).

DISCUSSION

Using the CFU technique, many investigators have studied the hematopoietic colonies which are visible on the surface of the spleen and to a lesser extent

![Size Distribution of Endogenous Colony](image)

**Fig. 4.** Endogenous colony size distribution.
Fig. 5. Colony size distribution of erythroid and granuloid colonies under various conditions.
colonies found in microscopic sections. In this study, experiments were designed to study both the surface macro-colonies and the microscopic colonies in histological subserial sections of 24 spleens following various handling of the marrow transplant. Approximately 100 sections were evaluated in each spleen.

The criteria used for defining a microscopic colony were as follows:

a) over 8 cells in at least 1 section, or
b) at least 4 cells in 2 sections or more.

In practice, most of the microscopic colonies observed were over 50 cells in their largest cross-section. This was particularly true in megakaryocytic colonies. As Curry & Trentin\(^2\) have described, the large predominant, early appearing colonies are generally erythrocytic. Our observations confirm this. In the development of the colonies, Reincke et al\(^3\) have demonstrated that erythrocytic microcolonies can be seen at day 3 in the spleen while granulocytic can be seen by careful histological examination at day 5. By day 7, undifferentiated primitive elements are frequently observed alone, or surrounding the periphery of the matured erythrocytic colonies.

At early times after marrow injections, granulocytic colonies are usually small, growing along the trabeculae or in subcapsular spaces of the spleen and do not appear as surface colonies. Less frequently, they are found in the Malpighian body.

As shown in Fig. 6 granulocytic elements are observed in the erythrocytic colony with well defined borderlines and without granulocytic and erythrocytic cell mixing. These granulocytic elements may be found in any portion of the colony, but always are seen in several consecutive sections. In our investigations, these colonies are not classified as mixed unless 2 or more kinds of cells are truly intermixed. Granulocytic elements in erythrocytic colonies resemble granulocytic colonies, suggesting that they really are colonies surrounded by the larger erythrocytic colony.

According to the data of Curry and Trentin\(^2\) and other authors\(^10,11,19-21\) the number of mixed colonies increases whereas the granulocytic colonies decrease with increased harvest time. We believe that these mixed colonies were originally separated and differentiated individual cell lines. Accordingly in our data, the granulocytic colony distributions are observed to be higher than those of the other authors\(^15,22-35\) because of our different criteria e.g. colony ratios are calculated as 1.0, as described in Table 1. Another reason for our observing high numbers of granulocytic colonies is our use of thinner sections. We have examined sections at 30\(\mu\) intervals whereas others used 50, 100, or 200\(\mu\) intervals.

The colony volume as measured by the eyepiece was smaller than the mathematical volume, as shown in Fig. 3. This is most likely due to the shape of the colony. Theoretically colonies assumed a spheroid shape but in reality they were often irregularly shaped.

Further evidence pertaining to the shape-volume calculation discrepancy has
been reported by Reincke\textsuperscript{36}, in which it was shown that in order to gain a close correlation between the measured colony size and the calculated volume, it was necessary to assume an ellipsoidal shape for the colonies.

In our early findings\textsuperscript{37}, for periods up to 8 hours, viability of erythroid colonies is depressed by storage time and temperature, however in the case of CMRL 1066, it compared to total viability is not strongly affected by temperature. In spite of this findings, no viability change is noted in this study between 16 and 24 hours storage time in saline G ice, and CMRL 1066 at room and ice temperature. What dose this indicate? Could they maintain the same level of inability to their further storage? Or did almost all donor’s bone marrow cells become depressed by storage?

This indication of stable viability of the stored cells between 16 and 24 hours may be explained in either of two ways. First, a small percentage of the stored elements may be extremely resistant and maintain their viability. Second, the colonies noted at the later times are endogenous.

To explain this phenomenon on the basis of endogenous colonies is some what tenuous, since there is an apparent effect on the number of granulocytic colonies when storage was in saline G at room temperature (Fig. 2). Conversely it is equally difficult to explain the total effect on the basis of surviving stored marrow cells.

\textbf{Fig. 6.} Granuloid colony which is observed within erythroid colony. 
H and E staining (250×). Granuloid colony is seen growing along the trabeculae with well defined borderline in erythroid colony.
because of the following: From Figs. 3 and 4 we note that endogenous colonies are generally small in size and mostly granuloid. If we then compare Fig. 4 to the colony distribution graphs for 16 and 24 hours storage in Fig. 5, we note that a number of colonies present are not small and granuloid and therefore cannot logically be explained as endogenous. These are most likely due to surviving stored marrow cells. Our findings therefore must be taken to indicate a combination of these two possibilities, i.e. that there are indeed some surviving stored elements, but as one would suppose, there are also present a small number of endogenous colonies at the later time periods.

REFERENCES


