Relationship of Colony-stimulating Activity to Apparent Kill of Human Colony-forming Cells by Irradiation and Hydroxyurea

By Hal E. Broxmeyer, Peter R. Galbraith, and Fraser L. Baker

Suspensions of human bone marrow cells were subjected to $^{137}$Cs irradiation in vitro and then cultured in semisolid agar medium. Cultures of irradiated cells were stimulated with colony-stimulating activity (CSA) of different potencies, and it was found that the amount of stimulation applied to cultures influenced the apparent kill of colony-forming cells (CFC). It was also found that the effects of irradiation on colony formation were not confined to CFC kill since medium conditioned by cells during irradiation exhibited stimulatory and inhibitory properties after treatment by 600 and 1000 rads, respectively. Studies in which irradiated cells were pretreated with hydroxyurea indicated that CFC in the DNA synthetic phase of the cell cycle were particularly sensitive to low doses of irradiation. The proliferative capacity of CFC surviving 1000 rads was undiminished as judged by their ability to form large colonies. Estimates of CFC kill by hydroxyurea were also affected by the level of CSA.

During the course of studies on the effects of irradiation on the survival of human marrow colony-forming cells (CFC), it became evident that the potency of colony-stimulating activity (CSA) used to stimulate cultures of irradiated cells influenced the $D_0$ value obtained (the dose of irradiation required to reduce survival to 37% of control); the higher the level of CSA the greater the $D_0$ value. Similarly, estimates of the percentage kill of CFC by hydroxyurea, an agent which kills cells in the DNA synthetic phase of the cell cycle, were influenced by the level of CSA. The studies reported in this paper document these phenomena.

MATERIALS AND METHODS

Colony-forming cells (CFC) were obtained from bone marrow aspirated from the manubrium of normal volunteers. The bone marrow aspirate was collected in sterile heparin tubes, centrifuged at 500 g for 10 min, and the buffy coat was drawn off. The nucleated cell-rich fractions were washed three times in tissue culture medium and treated as follows:

Irradiation

One-milliliter aliquots of washed cells, suspended in Falcon polystyrene tubes (17 x 100 mm), were chilled to 4°C to 10°C and mixed using a vortex mixer immediately prior to irradiation in an attempt to avoid hypoxia. The tubes were placed on their sides in a lucite container and irradiated using $^{137}$Cs at 112 rads/min (Gamma cell-20, Atomic Energy of Canada), washed twice, and then plated over various sources of CSA.

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Dosimetry was based on air ionization measurements made with a Victoreen condenser chamber calibrated by the National Research Council of Canada and also with a ferrous sulfate dosimeter. The dose rate measured by both methods agreed. Within each experiment the number of cells plated in culture was kept constant, but in different experiments plating doses of 2, 5, or $8 \times 10^5$ cells were used.

**Hydroxyurea**

Washed marrow cells were incubated with 100 μg hydroxyurea/ml (37°C for 50 min), washed twice, irradiated with doses from 0 to 1000 rads, washed and plated in cultures stimulated by different levels of CSA.

**Cell Culture**

The two-layer semisolid agar culture system was used throughout. Marrow cell suspensions containing control CFC and CFC treated by irradiation and/or hydroxyurea were plated over basal acellular agar layers to monitor for spontaneous (unstimulated) colony formation due to CSA production by endogenous colony-stimulating cells, and, in parallel, cultures were stimulated by one or more of the following sources of CSA as specified. Different sources of CSA provided different levels of stimulation of colony formation by control CFC.

**Sources of Colony-stimulating Activity (CSA)**

**Feeder layers.** Feeder layers were prepared using separated blood leukocytes, irradiated with 1000 rads ($^{137}$Cs), washed and plated at 0.5, 1.0, and $2.0 \times 10^6$ cells/ml in 0.5% agar. At a given concentration, normal blood contained approximately 1% the number of CFC present in marrow, and irradiation of blood cells with 1000 rads eliminated the background formation of cell aggregates in the feeder layer.

Feeder layers were also prepared from irradiated buoyant blood leukocytes obtained from a density separation similar to that of Moore et al., except that the density cut was made at 1.070 g/cu cm. Removal of dense cells has been shown to enhance CSA.

**Sera.** Normal human serum was dialyzed and added to the overlayer in a concentration of 20%, since this was found to provide the maximum stimulation by serum.

**Conditioned media (CM).** Unseparated and buoyant blood leukocytes were incubated in tissue culture media for 7 days in concentrations of 2 to $12 \times 10^6$ cells/ml. The medium conditioned by buoyant leukocytes was found to be the more potent and was added to the overlayer in a concentration of 20%.

**Colony Counts**

Colonies were scored (five plates per point unless otherwise stated) after 7 days and 14 days of incubation, since the time for optimal colony growth varied. In all cases, the colony counts given are those from 14 day cultures. Colonies (> 50 cells) were scored using an Olympus inverted microscope at ×40 and at ×100 when it was necessary to ascertain that a clone contained more than 50 cells. Samples of colonies were removed from the agar with a finely drawn pasteur pipette, streaked on a microscope slide, and stained for the peroxidase reaction.

**Statistics**

Radiation survival lines were obtained by least-squares linear regression analysis. Probability of differences between samples was determined using the Student's t test.

**RESULTS**

**Effect of CSA Level on "Radiation Survival" of CFC**

The relative potency of CSA derived from different sources is indicated in Table 1. Using these sources to stimulate cultures of irradiated cells we found that $D_0$ (the dose of irradiation required to reduce survival to 37% of control)
Fig. 1. Effect of CSA on radiation survival ($D_0$) of colony-forming cells. CSA was supplied by: (a) 20% conditioned media (c); (b) 20% dialyzed sera (e); (c) $1 \times 10^6$ unseparated blood leukocytes (a); (d) $2 \times 10^6$ unseparated blood leukocytes (e); (e) $0.5 \times 10^6$ buoyant blood leukocytes (a); (f) $1.0 \times 10^6$ buoyant blood leukocytes (c); exp, number of experiments composing survival line. The straight lines were obtained by least-squares linear regression analysis. The 95% confidence limits for $D_0$ of (a) to (f) were: (a) 35–40; (b) 75–85; (c) 71–95; (d) 75–103; (e) 121–139; (f) 146–174.

Table 1. Effect of Colony-stimulating Activity (CSA) on "Radiation Survival" of Colony-forming Cells (CFC)

<table>
<thead>
<tr>
<th>Source</th>
<th>Potency *</th>
<th>$D_0$ (rods)</th>
<th>Maximal Dose Range Over Which Colonies Were Detected (rods)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Conditioned media</td>
<td>14.0 ± 2.0</td>
<td>45</td>
<td>50</td>
</tr>
<tr>
<td>Dialyzed sera</td>
<td>18.0 ± 3.0</td>
<td>75</td>
<td>200</td>
</tr>
<tr>
<td>Unseparated blood</td>
<td>25.0 ± 4.0</td>
<td>95</td>
<td>400</td>
</tr>
<tr>
<td>Unseparated blood</td>
<td>22.5 ± 3.0</td>
<td>100</td>
<td>600</td>
</tr>
<tr>
<td>Buoyant blood</td>
<td>38.0 ± 2.0</td>
<td>135</td>
<td>800</td>
</tr>
<tr>
<td>Buoyant blood</td>
<td>51.0 ± 3.0</td>
<td>165</td>
<td>1000</td>
</tr>
<tr>
<td>Buoyant blood</td>
<td>52.5 ± 2.5</td>
<td>165</td>
<td>1000</td>
</tr>
</tbody>
</table>

* CSAs assayed using $2 \times 10^5$ unirradiated marrow cells as the source of target CFC. Colony counts ± 1 SEM and $D_0$ values are given for a single representative experiment. CSA was obtained from one normal donor, while bone marrow was from another normal donor.

† A summary of the 21 experiments is shown in Fig. 1.
was influenced by the level of CSA used to stimulate the cultures. Thus, the survival curves shown in Fig. 1 and the data summarized in Table 1 indicate that low $D_0$ values, and hence an obviously erroneous estimate of sensitivity to irradiation, are obtained when cultures are stimulated with CSA of low potency (and in unstimulated cultures).

$D_0$ values obtained with a given source of CSA were the same when marrow cells were seeded at 2, 5, or $8 \times 10^3$ cells/ml. This finding suggested that the higher plating dose of $8 \times 10^3$ marrow cells/ml did not exhaust CSA or affect the plating efficiency.

Feeder layers prepared from buoyant blood leukocytes (density $<1.070$ g/cu cm), which had been irradiated with 1000 rads, provided the greatest stimulation and allowed us to detect colonies with higher irradiation dosages. The $D_0$ value was 160 rads (95% confidence range of 146–174 rads) and the $n$ value was unity. ($n$, or hit number, is derived by extrapolating the straight line portion of the irradiation survival curve to zero dose). The $D_0$ and $n$ value compared favorably with those reported by Senn and McCulloch for man and mouse. Although colony number was reduced by irradiation, colony size was not. Thus, after treatment with 800 rads, some of the 14 day colonies contained more than 1000 cells, and colonies of more than 650 cells have been found after treatment with 1000 rads. These were as large as colonies grown in cultures of unirradiated cells, and all contained peroxidase-positive cells.

**Effect of Irradiation on Endogenous CSA of Irradiated Marrow Cells**

In an experiment to determine the effect of irradiation upon marrow CSA (endogenous CSA), the CSA of $4 \times 10^3$ washed marrow cells that had been treated with 0, 600, and 1000 rads, and that of media conditioned by these cells during irradiation was assayed with $4 \times 10^3$ unirradiated cells. The results of a typical experiment, repeated a total of three times, are shown in Fig. 2. In control cultures, the expected linear dose-related increase in colony formation oc-
curred when $4 \times 10^5$ unirradiated marrow cells were added to cultures containing $4 \times 10^5$ marrow cells. The addition of Millipore-filtered medium conditioned by marrow cells during irradiation with 600 rads, increased colony formation by 80% ($p < 0.001$). Addition of the cells alone treated with 600 rads also enhanced colony formation beyond that which would have been expected due to the addition of 600 rad survivors (i.e., $0.8 \pm 0.3$ colonies). In contrast, medium conditioned by cells during irradiation with 1000 rads, and cells treated with this dose, decreased colony formation ($p < 0.01$ and <0.001, respectively). Moreover, the net inhibitory effect of 1000 rads to marrow cells was not overcome with the additional stimulation provided by a potent leukocyte feeder layer. These effects were not due to irradiation effects on the culture medium, since additions of unconditioned medium irradiated with 600 or 1000 rads did not affect colony formation.

These studies show that irradiation of cell populations may affect colony formation independent of its effects upon CFC.

**Effect of CSA on Apparent CFC Kill by Hydroxyurea**

Strength of stimulation by CSA had an effect upon apparent CFC kill by pretreatment with hydroxyurea (Fig. 3). The highest estimated percentage "kill" was obtained when the most potent CSA was used. Qualitatively similar results have been obtained in each of eight additional experiments done using stimulation from both unseparated and buoyant blood leukocytes. The highest apparent CFC kill by hydroxyurea was 73%. To exclude the possibility that time of incubation in hydroxyurea influenced results obtained,$^9$ control studies were...
Fig. 4. Effect of irradiation dosage on colony number in cultures of hydroxyurea-pretreated and control marrow cell suspensions. Brackets indicate ± 1 SEM (eight plates per point). p values indicate the level of significance between mean colony numbers of hydroxyurea-pretreated and control cultures. - - - - e, without prior treatment with hydroxyurea; - - - - - O, with prior treatment with hydroxyurea.
done in which CFC were incubated with and without hydroxyurea for 60-180 min. In three separate experiments of the above design, time of incubation did not influence apparent CFC kill.

Use of Hydroxyurea to Investigate Cell Cycle Characteristics of CFC Surviving Irradiation

Representative results of four separate experiments are shown in Fig. 4. Approximately 40% of unirradiated CFC were killed by treatment with hydroxyurea. Cells surviving hydroxyurea were not killed by 50-100 rads, whereas in this dosage range, cells not exposed to hydroxyurea were killed in log-dose fashion. With irradiation doses between 100-1000 rads, the actual number of colonies formed was the same, whether or not the cells had been pretreated with hydroxyurea.

The apparent radiation survival of hydroxyurea pretreated cells (four experiments) is expressed as log-dose survival in Fig. 5. An initial shoulder is present, and the exponential portion of the curve gives a $D_0$ of 165 rads (95% confidence range of 157–183 rads), which is within the range found when cells were not exposed to hydroxyurea. The $n$ value was 1.5.

As in the case of irradiated cells, we could not distinguish hydroxyurea survivors from untreated CFC in the kind of colony type or size.

DISCUSSION

It has been shown that irradiation sensitivity is phase dependent in a number of experimental cell lines$^{11,12}$ and hemopoietic pluripotent stem cells,$^{12-15}$ al-
though the results for the latter are conflicting. Our results suggest that CFC at different phases of the cell cycle may have different radiation sensitivities. For example, approximately 40% of CFC are apparently killed by hydroxyurea, and presumably the same 40% are killed by low doses of irradiation in the range of 50–100 rads. At higher doses of irradiation, curves of hydroxyurea pretreated and control CFC survival are superimposable (Fig. 4). Thus it appears that smaller doses of irradiation have killed only those cells in S-phase, while higher doses are required to affect CFC in other stages of the cell cycle.

In unirradiated cultures, and within limits, the number of colonies formed is a function of the amount of CSA provided. Thus, if no other factors are involved, log-dose irradiation survival curves of CFC plated with different levels of stimulation should parallel one another, and $D_0$ values should be identical. That a family of nonparallel “survival curves” using different levels of stimulation was found suggests that other factors must be involved. Clearly, irradiation has effects other than those on CFC, and high levels of stimulation may be necessary to overcome the effects of inhibitory substances released from irradiated cells (Fig. 2). In addition, it is possible that CFC at different phases of the cell cycle have different thresholds for damage by irradiation and stimulation by CSA. For example, if CFC in S-phase are not only most sensitive to irradiation, but also have the lowest threshold to CSA, then the observed relationship between CSA and apparent $D_0$ would be expected. Our data could also be explained if irradiation-injured CFC require progressively higher levels of stimulation to proliferate.

It was also necessary to provide high levels of CSA to obtain high estimates of the proportion of CFC in S-phase using the hydroxyurea kill technique. It is important to realize that information concerning phase threshold response of CFC to CSA cannot be deduced from our data. “Kill” of CFC in S-phase occurred before the cells were cultured in the presence of colony-stimulating factor, and CFC, in cell cycle but not in S-phase, which survived exposure to hydroxyurea, might be expected to proceed into S on schedule. Our data, shown in Fig. 3, should not yet be interpreted to indicate that CFC in S-phase require higher levels of CSA to proliferate to form colonies.

Hydroxyurea, like tritiated thymidine, is lethal to cells in the DNA synthetic phase. The wide range of estimates of CFC kill reported in the literature may be due to differences in level of CSA used to stimulate cultures.

Finally, it is noteworthy that CFC surviving 1000 rads lost little, if any, capacity to proliferate, as judged by the fact that the colonies formed were as large as those in cultures of unirradiated cells. The actual proportion of CFC surviving 1000 rads was exactly as predicted from the results of earlier studies in which irradiation dosages had been carried up to 600 rads.

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