STROMAL COLONIES FROM MOUSE MARROW:
CHARACTERIZATION OF CELL TYPES,
OPTIMIZATION OF PLATING EFFICIENCY AND ITS
EFFECT ON RADIOSENSITIVITY

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SUMMARY
Modifications to the colony assay in vitro for stromal progenitor cells in mouse femoral marrow
have been studied so as to optimize the efficiency of colony formation (CFE). The highest reproduc-
tible concentration achieved was about 30 colonies (containing fibroblasts, macrophages and endo-
theloid cells) per $10^6$ nucleated marrow cells (range 20-50) in mice 3-4 months old, and higher by
50% in mice 14-15 months old. Each of many slight technical modifications could reduce these
values by more than 30%.

The importance of optimization was demonstrated by a reduced radiosensitivity when the CFE
was reduced by a factor of 3 using α-medium stored at 4°C for 15 days. The $D_0$ value was $3.9 \pm 0.8\ \text{Gy}$
compared to $1.6 \pm 0.1\ \text{Gy}$ using freshly prepared medium, and this could be due to the
selection of a radioresistant subpopulation.

The modifications studied may partly explain the marked variations in CFE and in radiosensitiv-
ity reported in the literature.

INTRODUCTION
Several methods have been developed for growing colonies of fibroblasts in vitro
from mouse marrow. The cells producing the colonies are considered responsible in
part for maintaining the supportive haemopoietic 'stroma' (e.g., see Friedenstein et
al. 1974). The methods include the liquid culture developed by Friedenstein,
Chailakhyan & Lalaykina (1970) using guinea-pig marrow and, later, mouse marrow
(Friedenstein, Gorskaya & Kulagina, 1976), the agar culture described by Metcalf
(1972), the methyl-cellulose system described by Wilson, O'Grady, McNeill & Munn
(1974) and the collagen technique described by Lanotte, Schor & Dexter (1981). The
colony-forming efficiency (CFE) of nucleated marrow cells is generally about 20
fibroblastic colony-forming units (CFU-F) per $10^6$ cells, although there are examples
from 2-50 CFU-F per $10^6$ murine cells and up to 235 in man (Table 1). Also, the
range of values reported in experiments by a single investigator can vary by up to a
factor of 20 (Table 1). These variations are much larger than reported for assays in
vitro of murine haemopoietic progenitors; e.g., 50-200 per $10^5$ cells for GM-CFC

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Table 1. Reported concentrations of CFU-F in bone marrow

<table>
<thead>
<tr>
<th>Species</th>
<th>Media</th>
<th>Sera</th>
<th>CFU-F/10⁶ cells</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Guinea pig (adult)</td>
<td>Parker's 199</td>
<td>Bovine serum</td>
<td>4.0 ± 0.5</td>
<td>1.7-7.6</td>
</tr>
<tr>
<td>Guinea pig (adult)</td>
<td>Parker's 199</td>
<td>FCS</td>
<td>25</td>
<td>4-78</td>
</tr>
<tr>
<td>C57Bl mouse (10-12 weeks)</td>
<td>Eagle's MEM</td>
<td>HS</td>
<td>—</td>
<td>0-2</td>
</tr>
<tr>
<td>Balb/c mouse (8-12 weeks)</td>
<td>(199 + methyl-</td>
<td>FCS</td>
<td>—</td>
<td>Wilson (1974)</td>
</tr>
<tr>
<td>(CBAxCS7Bl)</td>
<td>Easge's cellulose</td>
<td></td>
<td>—</td>
<td>Friedenstein et al. (1976)</td>
</tr>
<tr>
<td>F1 mouse (adult)</td>
<td>Alpha</td>
<td>FCS</td>
<td>22</td>
<td>3-45</td>
</tr>
<tr>
<td>CF-1 mouse (8-10 weeks)</td>
<td>M-199</td>
<td>FCS</td>
<td>10.3 ± 0.7</td>
<td>Werts et al. (1980)</td>
</tr>
<tr>
<td>B6D2F1 mouse (11-13 weeks)</td>
<td>Alpha</td>
<td>FCS</td>
<td>32 ± 2.7</td>
<td>20-50</td>
</tr>
<tr>
<td>Human (adult)</td>
<td>Alpha</td>
<td>FCS</td>
<td>13.6 ± 2.0</td>
<td>Castro-Malaspina et al. (1980)</td>
</tr>
<tr>
<td>Human (adult)</td>
<td>RPM11640</td>
<td>FCS</td>
<td>235 ± 20*</td>
<td>Nagao et al. (1981)</td>
</tr>
</tbody>
</table>

HS horse serum
FCS foetal calf serum.
*colonies of 6 cells or more scored.

(Dexter & Testa, 1976), and 10-50 per 10⁵ cells for BFU-E (Heath, Axelrad, McLeod & Shreeve, 1976). Large variations in plating efficiency could lead to selection of subpopulations of cells with different properties and responses, as shown for example, by the growth of human GM-CFC in different media where the apparent cell radiosensitivity could be varied by a factor of 4 (Broxmeyer, Galbraith & Baker, 1976). Some possible reasons for the variation in CFE have now been investigated using the liquid culture technique for assaying CFU-F, and the effect of the variation on the apparent radiosensitivity of CFU-F is reported.

MATERIALS AND METHODS

Mice

Eleven to thirteen-week-old female B6D2F1 mice were used in the experiments unless otherwise stated. Femoral marrow cells in Fisher's medium were pooled from 3-5 mice and plated usually within 15 min.

Culture medium

Alpha MEM medium (α-medium) powder (GIBCO, N.Y.) was used for colony growth. For preparing the culture medium, three methods were used: (1) stored single-strength medium: a package of α-medium powder (10-2g) was dissolved in 947 ml sterile double-distilled water, to which 50 ml 4.4% sodium bicarbonate, 2.5 ml benzine penicillin (50 000 units) and 0.5 ml streptomycin (50 mg) were added. The medium was stored at 4°C; (2) stored double-strength medium:
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the same procedure as for single-strength medium was used, except that the total volume for a package was 500 ml; this stored medium was diluted to single strength immediately before use; and (3) the medium was freshly prepared.

Fischer's medium was used for preparing the bone marrow suspensions. Single-strength medium was prepared fortnightly from 10x concentrated medium and stored at 4°C (Testa & Lajtha, 1973).

**CFU-F culture**

Femoral marrow cells (0.6-1 × 10⁷ cells) were inoculated into Falcon T-25 plastic flasks (3013F) containing 5 ml of α-medium plus 5% foetal calf serum (Sera Lab, U.K.). The flasks were incubated at 37°C in humidified air plus 5% CO₂ for 7-12 days. The medium was changed on day 7 for the longer incubation times. The colonies were stained with May/Grunwald/Giemsa and those containing at least 50 cells were counted under a stereo microscope (×17.5). In some experiments, silver impregnation was used to investigate the presence of collagen.

**Phagocytosis**

Either 10⁷ latex particles (Uniscience Ltd, Cambridge), 1.1 μm diameter, suspended in 0.2 ml of medium, or 0.05 ml of an undiluted suspension of carbon particles (Pelikan C11/1431), were added to each flask. After incubation at 37°C for the times stated in Results, cells were stained as described above.

**Removal of macrophages from the culture**

Superfine silica (Dowson & Dobson, U.K.) was sterilized by 3×10⁴ Gy electrons and suspended at a concentration of 10 mg per ml in normal saline. These particles are ingested by, and kill, macrophages (O'Rourke, Halstead, Allison & Platts-Mills, 1978). The optimal concentration of silica for killing the macrophages was found to be 0.6 ml suspension per flask. The particles were added at the beginning of the culture and were removed on days 4 or 7.

**Irradiations**

When feeder cells were added, they were irradiated with ¹³⁷Cs γ-rays (4.5 Gy per min). 300 kVp X-rays were used at 1.54 Gy per min for measuring the radiosensitivity of the CFU-F, irradiated in vitro.

**Autoradiography**

Two glass microscope slides were placed on the bottom of each 100 mm Petri dish (Falcon 30003), to which was added 3 × 10⁶ femoral marrow cells in 15 ml medium, and also, at different times of incubation, 15 μCi [³H]thymidine (0.25 Ci/mmol). On day 7 the cultures were chased with 'cold' thymidine and then stained with Schiff's reagent. The slides were dipped in Ilford KS emulsion and exposed for 14 days.

**Electron microscopy**

The cultures were prepared for scanning (SEM) or transmission (TEM) electron microscopy as described in detail elsewhere (Allen & Dexter, 1982).

**RESULTS**

**Properties of fibroblastic colonies**

**Cell morphology.** The attached population was clearly divided into three cell types: fibroblasts, mononuclear phagocytes or macrophages, and endothelial cells. Using the light microscope the fibroblasts were seen to be typically bipolar, occasionally tripolar or dendritic, with an ovoid nucleus and two to five nucleoli. When latex or carbon particles were added to the culture and it was incubated for 1 h at 37°C, the
Fig. 1. SEM of a region of the culture showing the typical bi- or tri-polar appearance of the fibroblasts, which also display different degrees of spreading. In all cases the typical surface morphology of microvilli and ridges is retained. ×900.

Fig. 2. SEM of fibroblasts (f) and endothelial cells (e) showing the variation in surface morphology. The central fibroblast is underlapping an area of cell–cell attachment between the adjacent endothelial cells (arrows). ×1850.
fibroblasts were not phagocytic, although some phagocytosis was evident if the incubation period was extended to 3 days. Using the SEM, the fibroblast population was characterized by a well-marked microvillous or ridged surface morphology (Fig. 1). This surface topography was maintained even when the cells were well-spread, and was in sharp contrast to the almost completely smooth surface of the endothelial cells (Fig. 2). An actively migratory behaviour of the fibroblasts was also suggested by their relatively sparse distribution unless high cell concentrations were present (Fig. 1, and below), and their behaviour with respect to the regions of endothelial

Fig. 3. Section through fibroblast cut parallel to the substratum. The nuclear profile shows a thin rim of condensed chromatin and two nucleoli. The cytoplasm has both rough and smooth reticulum, numerous mitochondria and occasional lysosomes. ×9600.

Fig. 4. Detail from cell periphery showing fibrous extracellular matrix. ×13600.
growth, where they showed regular underlapping (Fig. 2). In sections from material embedded in situ and cut parallel to the substratum, the cell profiles demonstrated typical fibroblast morphology (Fig. 3) with a relatively smooth nuclear profile and a narrow peripheral rim of condensed chromatin. The cytoplasm contained numerous round or ovoid mitochondrial profiles, both smooth and rough endoplasmic reticulum and occasional lysosomes (Fig. 3). Fibrous extracellular matrix material confirmed the presence of the silver-stained collagen observed in the light microscope (Fig. 4).

Endothelial cells displayed a polygonal cell shape and round nuclear profile in the light microscope, and a smooth surface in the SEM (Fig. 2). Endothelial cell growth tended to occur in regions of confluence even with small numbers of cells, indicative of a reduced migratory behaviour in comparison with the fibroblasts. Sections cut parallel to the substratum showed an increased amount of rough endoplasmic reticulum and more vermiform mitochondrial profiles than the fibroblasts. The sub-membranous cytoplasm was also rich in microfilaments, often forming bundles in areas of membrane apposition between adjacent cells, possibly indicating junctional complexes (Fig. 5).

Monocytes or macrophages were characterized by their avid phagocytic behaviour, and were also clearly identified in the SEM by their rounded morphology, surface folding, and retraction fibres (Fig. 6). This was confirmed by sections, which also showed typical nuclear morphology and numerous phagocytic inclusions (Fig. 5).

**Colony development.** On the second day of culture, most adherent cells were macrophages, but a few fibroblasts existing as single cells or small cell clusters could be recognized among the macrophages. From day 2 to day 5, the number of fibroblasts per colony increased exponentially with a doubling time of about 23 h, reaching 30 fibroblasts per colony on average at day 5. The colony size continued to enlarge between day 5 and day 14 although at a slightly reduced rate. By days 12–14, large colonies were formed containing hundreds of fibroblasts, macrophages and endothelial cells. After day 14, the number of fibroblasts and macrophages did not increase much further but the number of endothelial cells continued to increase. Eventually, each colony was often surrounded by 10–50 endothelial cells in the third to fourth week.

The proliferative status of the cells during colony development was assessed by mitotic and labelling indices. Surprisingly, no typical mitotic cells were observed even in the colonies developing most rapidly, although about 2% mitotic cells would be
expected if mitosis takes 0-5 h and the cell doubling time is 23 h. It was possible that the fibroblasts in mitosis were less adherent and could be lost from the culture when they were fixed and stained. The labelling studies demonstrated that most fibroblasts could be labelled if [3H]thymidine was added to a CFU-F culture 3 days before it was harvested. After 4 h, or 1, 3, 5 and 7 days of labelling, the percentages of labelled fibroblasts were, respectively, 9, 44, 71, 83 and 87. Evidence supporting the concept that the CFU-F in mitosis might be less adherent was the fact that a few CFU-F (2-10%) were always in the supernatant of a culture during the first 2 weeks of incubation, as assessed by transfer between flasks.

The importance of macrophages in the colony growth was investigated by their removal at different times of culture using silica particles. The particles were added at the beginning of culture, left for 4 or 7 days incubation, and then removed and the cells surviving were allowed to grow in fresh medium for another 3-5 days. The fibroblasts did not ingest silica particles intensely and appeared to proliferate virtually normally to at least day 10. Surprisingly, the endothelial cells, which were not phagocytic, were not present after treatment with silica.

Relationship between cell inoculum and colony number. The relationship between the number of marrow cells inoculated and the number of colonies that developed is shown in Fig. 7. Feeder bone-marrow cells irradiated with 50 Gy were added to

![Graph](image-url)

Fig. 7. Relationship of colony number to number of bone marrow cells cultured. Curves I and II, results of two separate experiments with higher and lower CFE, respectively (mean ± s.o.). The broken curves are taken from the literature: A, (CBA × C57B1)F1 mice (Friedenstein et al. 1976); B, CF-1 mice (Werts, Gibson, Knapp & DeGowin, 1980); C, guinea pig (Friedenstein et al. 1970); D, human (Castro-Malaspina et al. 1980); and E, human (Nagao et al. 1981).
make the total number of cells $10^7$ per flask. Other published data are shown for comparison. When the CFE was low there was sometimes a curvature in the relationship between colony number and number of cells plated (curves A and II in Fig. 7). However, the relationship was linear if the CFE was high (curve I in Fig. 7).

**Culture technique**

*Storage and pH of α-medium.* In the initial experiments, using α-medium that had been stored at 4°C for up to 14 days, the CFE was low and variable at 0.5–6 colonies per 10^6 cells. In order to enhance CFE, several modifications were tried. Neither a greater serum concentration, nor an addition of mouse red cells (Metcalf, 1972), nor a supplement of 200 mM fresh l-glutamine was able to increase the CFE. However, it was found that there was a close relationship between the storage time of α-medium and the colony number: the CFE dropped from 28–35 CFU-F with fresh medium to 2–16 with medium stored for 9 days or more. Similar results were obtained with double-strength medium stored at 4°C for similar periods, or with single-strength medium stored at −20°C. Therefore, only freshly prepared medium was used in subsequent experiments. The pH of the stored medium was at least partially responsible for the poor CFE. When medium was stored in air plus 5% CO₂, the CFE was 70% of the control after 17 days storage. Also, the CFE was generally higher if the flasks were gassed with CO₂ before (as well as after) the marrow cells were plated, in order to minimize transient changes in pH.

To determine the optimal pH of the medium for preparing bone marrow cell suspensions, α-medium was buffered with Hepes (Sigma Chem. Co. U.S.A.) and different amounts of NaHCO₃ were added to adjust the pH. The cells were suspended in these media with different pH values, and kept on ice for 1 h before culture in fresh medium at pH 7.2–7.4. There was no significant influence of the initial pH on the CFE if it was within the range of 7.2–7.7, but, the survival of CFU-F decreased rapidly when the initial pH was above 8.

*The time of cells in suspension before seeding.* The CFE decreased progressively to 70% after 1 h and to 25% after 7 h when the cells were suspended in Fischer's medium in vials (gassed with 5% CO₂ to maintain a physiological pH) on ice before culture. The decline in CFE could be prevented if the cells in the contralateral femora were left in the shafts on ice and suspended immediately before plating. Thus the decline could be due either to the adhesion of CFU-F on the surface of the vials, or to the death of CFU-F in suspension. This was investigated by keeping the inoculated flasks at 4°C for varying times and transferring the contents to new flasks for incubation. In this case, the adherent cells were not transferred. Other inoculated flasks were kept at 4°C and then the temperature was raised to 37°C, so that the adherent cells remained in the flasks. The decline in CFE was similar in both cases indicating that adherence was not the cause of the decline in CFE.

*The osmolality of α-medium.* The osmolality of α-medium when prepared to manufacturers' specifications differs from one preparation to another by as much as 70 mosM. In three experiments, mean values of 36±13 and 33±9 CFU-F per 10^6 cells
were obtained at 280 and 312 mosM, respectively, with similar concentrations of CFU-F also at three intermediate osmolalities.

$O_2$ concentration in the gas phase. When the concentration of $O_2$ in the gas phase was decreased from 20 to 5%, the CFE increased from 21 to 53 CFU-F per $10^6$ cells.

The age of mice

The number of CFU-F per femur, and also to some extent the concentration of CFU-F, increased steadily with the age of the mice up to 15 months: in 3- to 4-month-old mice, there were 390 ± 30 CFU-F per femur, compared to 830 ± 80 at 17–18 months of age.

Radiosensitivity of the CFU-F grown in different media

Irradiation was performed in vitro within 1 h after the cells were plated. Various inoculum sizes were used to enable the colony number after irradiation to be mostly in the range 50–500. The survival curves plotted in Fig. 8 showed a $D_0$ of 1.6 ± 0.1 Gy and an extrapolation number ($n$) of 2.7 ± 0.2. When the α-medium was stored for 15 days was used, the $D_0$ increased to 3.9 ± 0.8 Gy with an extrapolation number of 1.2 ± 0.4.

![Fig. 8. Dose-response curves of CFU-F irradiated in vitro. (●—●) Cells cultured in fresh medium, mean of five experiments. (○---○) Cells cultured in stored α-medium, mean of three experiments.](image-url)
DISCUSSION

Colony growth

Several methods are available for measuring stromal colony-forming cells in vitro, but whether they are growing the same types of cells is not clear. In the original paper describing stromal colonies from mouse marrow, Friedenstein et al. (1976) described the colonies as being composed of large polygonal fibroblasts and stellate histiocytes (macrophages). Later, according to morphology, Werts, Gibson & DeGowin (1979) indicated that their colonies were principally composed of four cell types: i.e., large polygonal cells, round cells, spindle-shaped cells and small stellate cells. Most cells phagocyted opsonized yeast, except the large polygonal cells. In the present study, apart from the fibroblasts and macrophages, both of which are similar to those described above, we also found a number of endothelial cells, which were either not present, not described or considered to be fibroblasts by other authors (Wilson et al. 1981). However, endothelial cells are generally found in liquid and collagen gel cultures of marrow (Mori et al. 1979; Lanotte et al. 1981). The endothelial cells are clearly separable from the fibroblasts by behaviour, size, shape and surface morphology as seen in the SEM, and internal structure as seen in the TEM. Also, it appears that they are stimulated by fibroblasts since they were seen only in the developed colonies and always in close contact with fibroblasts. They may also be stimulated by macrophages, because when macrophages were removed using silica particles the endothelial cells also disappeared, although they were not phagocytic.

The population of fibroblasts labelled with $^{3}H$thymidine increased with the labelling time to a plateau of about 90%. This takes longer than was found by Friedenstein et al. (1974) and Nagao, Komatsuda, Tamauchi & Arimori (1981), who observed 90% of guinea-pig or human fibroblasts labelled after 60 h of cultivation with $^{3}H$thymidine. It is clear from these that colony development is dependent mainly on proliferation, rather than on aggregation of non-dividing cells. While the clonal nature of the fibroblasts in the mouse CFU-F assay has been reported (Piersma, Ploemacher & Brockbank, 1982), the origin of the two other cell types remains to be determined. It is reasonable to postulate active migration and/or proliferation of the macrophages in close contact with the fibroblastic cells. The presence of foci of endothelial cells may represent an attempt to establish the complex cell-associations between these different cell types that are necessary to maintain haemopoiesis in vitro (Dexter & Testa, 1976). Work directed to select the conditions that favour endothelial cell growth is in progress.

Growth conditions

Several media have been used to cultivate bone marrow fibroblastic colonies (see Table 1). Alpha medium was found to provide optimal growth of human CFU-F (Castro-Malaspina et al. 1980). In the present study, we found that the $\alpha$-medium also provided satisfactory conditions for mouse CFU-F. The $\alpha$-medium is not, however, suitable for storage, and whether this is also true for other media has not been reported.
Table 2. Variation in conditions for culturing CFU-F

<table>
<thead>
<tr>
<th>Condition</th>
<th>Good conditions*</th>
<th>Poorer conditions†</th>
</tr>
</thead>
<tbody>
<tr>
<td>Concentration of foetal calf serum (%)</td>
<td>5–15</td>
<td>&lt; 5</td>
</tr>
<tr>
<td>Storage time of α-medium (days)</td>
<td>&lt; 1</td>
<td>&gt; 9</td>
</tr>
<tr>
<td>Gassing with 5% CO₂</td>
<td>Before and after cell seeding</td>
<td>After cell seeding</td>
</tr>
<tr>
<td>pH of medium for cell suspension</td>
<td>7–2–7.7</td>
<td>&gt; 8.0</td>
</tr>
<tr>
<td>Time of cells in suspension at 4°C before seeding</td>
<td>&lt; 15 min</td>
<td>&gt; 1 h</td>
</tr>
<tr>
<td>Osmolarity of medium (mosm)</td>
<td>280–312</td>
<td>NT†</td>
</tr>
<tr>
<td>O₂ concentration in gas phase (%)</td>
<td>5</td>
<td>20</td>
</tr>
</tbody>
</table>

* CFU-F/10⁶ cells = about 40.
† Decrease of 30% or more in CFE.
NT†, not tested.

The best conditions obtained in the present study for culturing CFU-F, which provided a CFE of about 40 CFU-F per 10⁶ bone marrow cells, are shown in Table 2. The main factors responsible for a poor CFE were found to be storage of medium, high pH and delayed plating. Less important but detectable features, resulting in a CFE at the lower end of the normal range are the lack of gassing of the culture flasks with 5% CO₂ before seeding the cells, and the delay before cold-seeded cultures were warmed to 37°C. Thus some of the variations in CFE observed by other investigators (Table 1) could be due to these factors. It should be noted that the change in CFE for CFU-F using the best conditions in the present study was similar to the change seen in concurrent experiments with GM-CFC (100–200 per 10⁵ bone marrow cells). The use of 5% O₂ and 5% CO₂ in nitrogen, produced a reproducible increase in CFE, as in colony assays for haemopoietic precursor cells and also in cultures of mouse embryo fibroblasts (Bradley, Hodgson & Rosendaal, 1978).

Radiosensitivity

The importance of a reproducible and high CFE for experimental use is shown by the measurement of radiosensitivity. The use of stored medium results in a lower CFE and a lower sensitivity; i.e., in the apparent selection of a resistant subpopulation. The radiosensitivity of CFU-F using fresh medium ($D_0 = 1.64$ Gy) is similar to that recently reported of $D_0 = 1.5$ Gy (Xu & Hendry, 1981). Other values in the literature, reviewed by Hendry & Lord (1983), range from 1.5 Gy (above) to 3 Gy (Wilson et al. 1974), and hence this variation could be due partly to differences in CFE. These responses differ from those reported by Broxmeyer et al. (1976) for GM-CFC, where low CFE values resulted in an increase in apparent sensitivity by up to a factor of 4. Interestingly, the radiosensitivity of CFU-F derived from skin or lung (with $D_0$ values in the range 0.75–1.8 Gy) is generally greater than for CFU-F derived from marrow (see review by Hendry & Lord, 1983). Whether this is intrinsic or related to growth conditions is unknown.
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REFERENCES


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