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THE DIFFUSION CHAMBER TECHNIC AS PROGENITOR (STEM) CELL ASSAY*

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ABSTRACT

The hemopoietic response in mice to X-irradiation and treatment with hydroxyurea or cyclophosphamide was studied by comparing the diffusion chamber (DC) technic and the spleen colony technic. The hydroxyurea experiments showed that DCPCs (diffusion chamber progenitor cells) had a higher proliferative rate than spleen colony forming units (CFUs).

The radiation response indicated that 7 day DC culturing measures two different types of progenitor cells, one relatively radioresistant with D_o of 135 rads and one with radiosensitivity comparable to that of CFUs (50 rads).

Two hours after cyclophosphamide treatment, erythroid CFUs were almost eradicated indicating that cyclophosphamide selectively blocked the ability of multipotent stem cells to erythroid differentiation. The number of granuloid CFUs and DCPCs were less affected.

It is concluded that DC culturing probably measures a mixture of multipotent and unipotent stem cells.

INTRODUCTION

The environment of the diffusion chambers (DC) implanted intraperitoneally provides good growth conditions for stem cells, granulocytes and macrophages. After an initial loss of spleen colony forming units (CFUs) amounting to approximately 50 per cent, the CFUs will multiply in the DC with a population doubling time of 20 hours. Their number on day 3 is equal to[1] or greater than[2] the input value. The spleen colonies generated by CFUs harvested from 1-day or 4-day DC cultures showed a type distribution similar to that observed with normal marrow cells[2]. In irradiated animals, the DC-CFUs proliferate faster than in normal hosts[1].

The granulocytes and macrophages are growing rapidly from day 2-3 of culturing and after 7 days there may be a 10-fold increase in cellularity, when normal steady-state marrow cells have been inoculated[3]. Erythropoiesis has been observed when autologous cells are cultured in rabbits[4]. In mouse cell cultures there is no erythropoiesis unless the host animals are injected with erythropoietin or subjected to intermittent hypoxia[2]. Even then the erythropoiesis is too scant to be useful for most practical purposes.

The present paper describes attempts to characterize the progenitor cells measured by the DC technic. Hemopoiesis in the mouse was studied by comparing the DC-technic and the spleen colony assay (SCA).

MATERIALS AND METHODS

Details of the DC technic[5,6] and the SCA[2] have been described previously.

An estimate of the number of stem cells (diffusion chamber progenitor cells, DCPC) that are the ancestors of differentiated cells harvested after a given culture period may be obtained in two different ways:

An absolute estimate of DCPC may be obtained by limiting dilution analysis[6,7]. In short, the principles of this technic are as follows: Chambers containing small numbers of bone marrow cells are implanted. If myeloblasts and promyelocytes are found in a chamber smear after a culture period of 7 days, it is assumed that the chamber contained one or more "effective" DCPC initially. Chambers which contain no granulocytes or only mature granulocytes and/or macrophages are scored as empty. The probability, P(0) of having no DCPC in a chamber is given by the equation applying to a Poisson distribution.

1) $P(0) = e^{-\lambda}$ where $P(0) = \frac{\text{Number of empty chambers}}{\text{Total number of chambers}}$

and λ = the average number of DCPC per chamber which gives

2) $\lambda = -\ln P(0)$

Thus, the average number of DCPC per DC can be estimated by determining the fraction of empty chambers. By this technic we have found that in normal mouse marrow there is 1 DCPC per 1500 - 2000 bone marrow cells. This certainly is an under-

estimate, but it is a reproducible procedure. Further, it is not necessary to count the cells, and the interpretation of the smears is quite rapidly performed. The disadvantage is that a large number of chambers is required to get a reliable estimate.

A relative estimate of the DCPC may be obtained from the mean number of cells harvested per chamber. This estimate may be expressed as,

$$\text{Cytopoietic capacity (per femur)} = \frac{N_{out}}{N_{in}} \quad (N_{fem})$$

where N_{out} is the number of cells harvested, N_{in} the number of cells inoculated, and N_{fem} the number of cells per femur. If the number of cells per femur in the experimental group deviates from the control, a relevant comparison can only be made by calculating the cytopoietic capacity per femur, which expresses the number of cells that would have been harvested if all cells in one femur had been cultured.

RESULTS AND DISCUSSION

The relationship between the number of cells inoculated and the number of cells harvested is demonstrated in Fig. 1. In 7-day cultures grown in normal mice, the cytopoietic capacity decreased significantly with increasing inocula, in particular in the range from $2x10^4$ to $1x10^5$ cells per chamber. A similar but less pronounced effect was seen in 4-day cultures. Thus previous findings[8] indicating no feedback inhibition in 4-day cultures were not confirmed. The findings were almost similar

when the cells were cultured in irradiated hosts (500 rads). The problem of this cell-dose effect can largely be avoided by using inocula in the range $1\text{-}2\times10^5$; it should be kept in mind that the growth is sub-maximal with these greater inocula.

Table 1 shows the proliferative rate of progenitor cells, as determined with hydroxyurea injections. DCPCs had a higher proliferative rate than CFUs, so obviously the two assay systems do not assay identical populations. The CFUs in normal marrow are predominantly multipotent stem cells (MSC)[9,10], and it seems likely that the DC technic to a large extent measures unipotent (committed) stem cells (USC)[11].

Another comparative study is demonstrated in Fig. 2, which shows the radiosensitivity of progenitor cells. Appropriate corrections were made for the cell dose effect (Fig. 1) from experiments in which DC containing varying numbers of normal bone marrow cells were cultured in irradiated hosts (500 rads)[12]. The CFUs were highly radiosensitive, and the survival curve was exponential with a D_o of 50 rads. The radiation response of DCPCs as measured with 7-day cultures was not very different from that of CFUs for small doses, but above 60 rads the curve was exponential with a D_o of 135 rads. With 4-day cultures, the surviving fraction for small doses was somewhat larger than for 7-day cultures. For doses between 80 and 200 rads the curve exhibited a shoulder and for even larger doses the curve was exponential with a D_o of 132 rads.

These experiments indicate a significant different radiation sensitivity for CFUs and DCPCs, and they show that different progenitor populations are assayed with 4 and 7-day cultures. The survival curve for 7-day cultures may be a composite of

two exponential dose response curves with different D_o, corresponding to a very sensitive and a less sensitive cell type. The survival curve of the most resistant cell has a D_o equal to the D_o of our experimental curve for high doses, i.e. 135 rads. It follows from geometrical considerations that the extrapolation number for this part of the curve, which is 0.59, expresses the fraction of resistant cells. Thus, in 7-day cultures, we may be measuring a mixture of 40 percent very sensitive and 60 percent less sensitive progenitor cells. Using these values it is possible to show that a combination of two exponential curves, one with a D_o of 135 rads and the other with a D_o of 50 rads will give a curve that is almost idential to the experimental one for 7-day DC culturing. This may indicate that the most radiosensitive cell giving rise to differentiating cells in DC are CFUs.

The survival curve obtained with 4-day cultures reflects an even more complex radiation response than 7-day cultures. The broad shoulder may indicate the presence of an additional precursor cell type, which to some extent is able to accumulate sublethal radiation damage.

The use of cytotoxic agents may also be helpful in attempts to characterize progenitor cells by comparing the effect in different assay systems. We have examined the effect of one or two injections of cyclophosphamide[13] on CFUs and DCPCs (Fig. 3).

Two hours after the injection, the number of erythroid CFUs was reduced to 2.5 percent of the control. Thereafter, erythroid CFUs increased exponentially with a doubling time of 20 hours. A second injection 4 days later had a similar effect, showing that it was independent of their proliferative rate. The findings indicate that cyclophosphamide preferentially impairs the ability of MSC to differentiate towards the erythron. Granuloid CFUs and DCPCs were less affected. These two types of progenitor cells showed a similar pattern of depletion and regeneration, but the curves were not identical, again suggesting that the DCPC and CFUs are not identical.

In conclusion, the diffusion chamber technic apparently assays a mixture of MSC and USC. We are planning to extend the irradiation experiments in order to examine whether the CFU fraction of the DCPC population will increase when the culture period is prolonged.

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Table 1

Per cent of progenitor cells (per femur) surviving hydroxyurea treatment

CFU	Absolute number	Relative number
82 ± 11	53 ± 12	38 ± 8
(46)	(138)	(34)

Male (C_3H x DBA)F_1 hybrid mice were injected with hydroxyurea (900 mg/kg) or saline. Bone marrow was sampled two hours later to be tested with the spleen colony and the diffusion chamber techniques. Diffusion chamber progenitor cells (= DCPC) were determined in absolute number by limiting dilution analysis and in relative number from culture cellularity. The values are given as mean ± SE in per cent of saline-injected controls. The number of observations (spleens or chambers) are shown in parenthesis and the controls comprised a similar number.

LEGENDS TO FIGURES

Figure 1. The figure shows the cytopoietic capacity of various size inocula relative to the cytopoietic capacity of control chambers inoculated with 10^5 bone marrow cells. Each experiment comprised 7 chambers per cell dose and the number of experiments is indicated at each point. Means and their SE are shown.

Figure 2. The radiosensitivity of CFUs and DCPCs (diffusion chamber progenitor cells) of (C_3HxDBA) F_1 hybrid mice. The host animals were irradiated before and after chamber implantation in such a way that all mice received a total dose of 500 rads, whereas the implanted cells were irradiated with varying doses.

Each point represents 4-5 experiments each comprising 7 chambers. In the spleen colony experiments the animals were similarly irradiated before and after the injection of cells and received a total dose of approximately 900 rads (12-13 spleens per point).

Figure 3. Hale-Stoner-Brookhaven mice were injected with cyclophosphamide (200 mg/kg) and the bone marrow cells were harvested from two hours to 4 days after the injection and tested with spleen colony assay and by DC culturing (limiting dilution method). Groups of mice received a second injection of cyclophosphamide 4 days after the first injection. The spleens were examined histologically and each point represents 3-4 spleens.

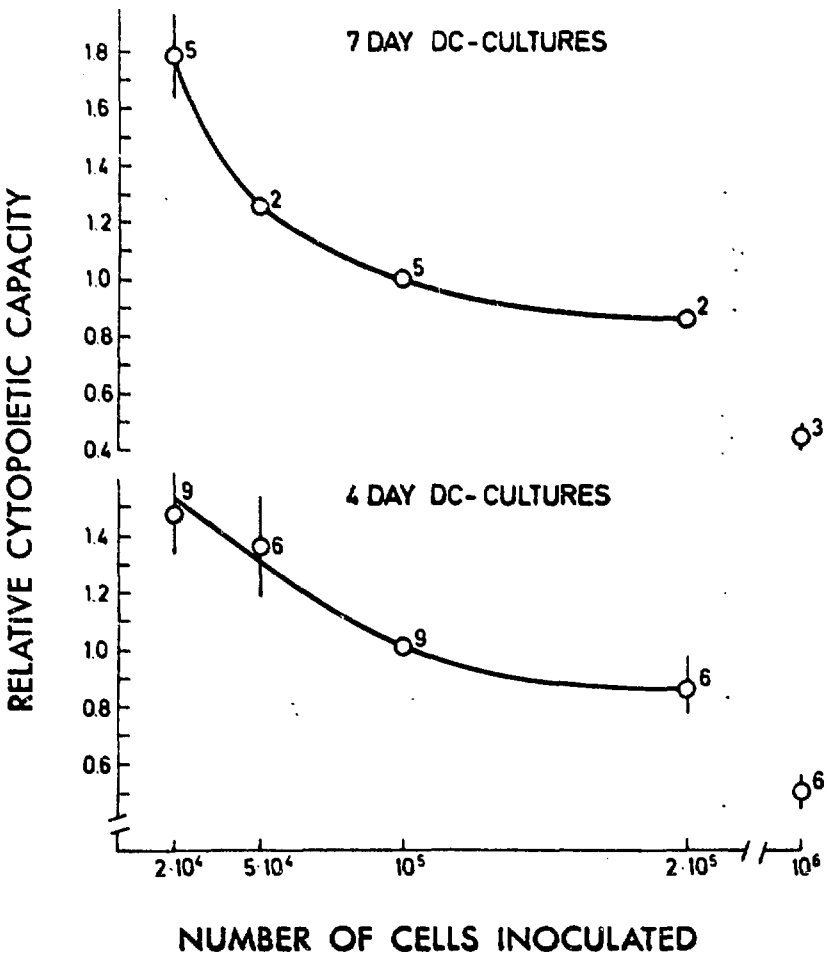

7 DAY DC-CULTURES
4 DAY DC-CULTURES
RELATIVE CYTOPOIETIC CAPACITY
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
5
2
5
2
3
9
6
9
6
6
2·10⁴
5·10⁴
10⁵
2·10⁵
10⁶
NUMBER OF CELLS INOCULATED

Figure 2

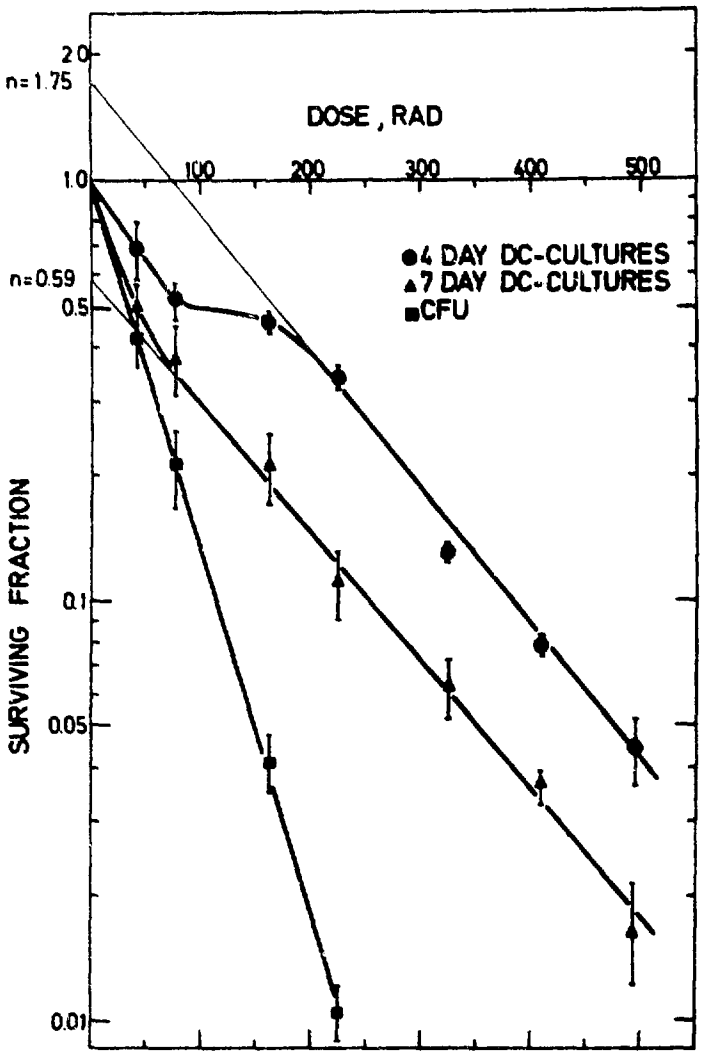
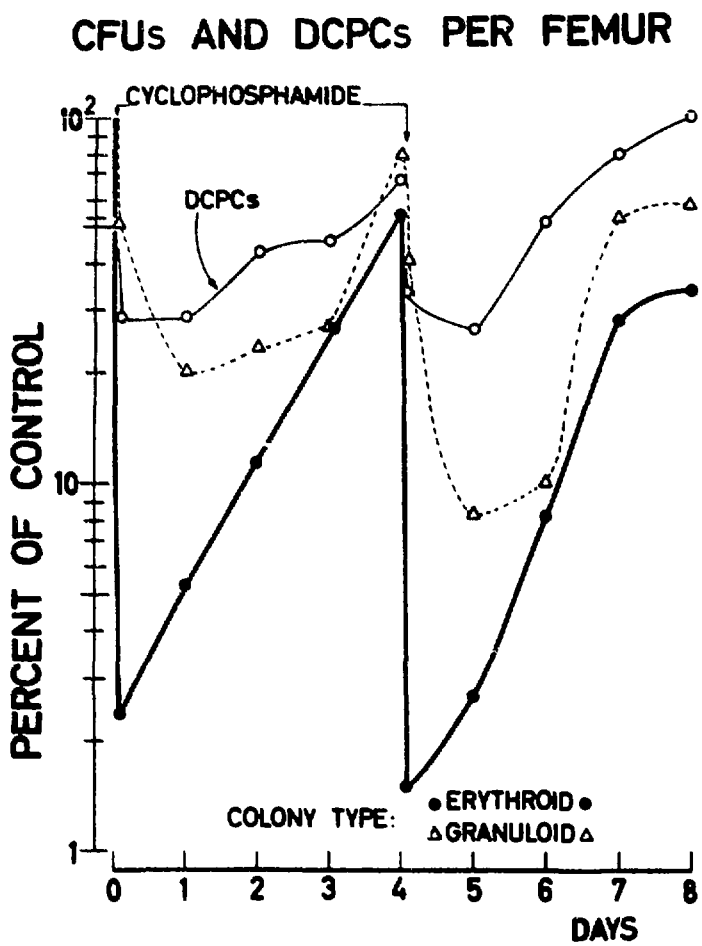

2.0
n=1.75
DOSE , RAD
100
200
300
400
500
1.0
n=0.59
0.5
●4 DAY DC-CULTURES
▲7 DAY DC-CULTURES
■CFU
SURVIVING FRACTION
0.1
0.05
0.01

Figure 3


CFUs AND DCPCs PER FEMUR
CYCLOPHOSPHAMIDE
DCPCs
PERCENT OF CONTROL
10^2
10
1
COLONY TYPE:
●ERYTHROID●
△GRANULOID△
0
1
2
3
4
5
6
7
8
DAYS