

Reculturing of Cells from Primary CFU-C Colonies

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This study was aimed at investigating whether cells of CFU-C derived colonies could form secondary colonies. Bone marrow cultures of volumes of agar medium between 25 μ l and 75 μ l contained in glass capillaries were stimulated with mouse lung-conditioned medium (MLCM) containing granulocyte/macrophage colony-stimulating factor (GM-CSF). Agar gels with colonies of up to ≥ 20 were blown out into identical culture medium, completely dispersed on a whirl-mix to single cell suspensions, and used for establishing secondary agar cultures. In these secondary cultures considerable numbers of secondary granulocytic, mixed granulocytic/macrophage and macrophage colonies as well as numerous clusters arose. In contrast, when single colonies were recultured, only few secondary cell aggregates were formed. When primary cultures containing up to ≥ 20 cell aggregates were used for serial reculture at intermittent intervals of 3 and 4 days, a 2–7-fold increase of colony-forming cells was found in tertiary cultures as was monitored by 7 day colony counts. And by use of different kinds of CSF-containing media, an over 4-fold increase of secondary over primary colonies was obtained with bovine lung-conditioned medium (BLCM) in primary and L-cell-conditioned medium (LCCM) in secondary 7 day cultures. Primary capillary cultures were found to be devoid of CFU-S. Also, setting up bone marrow cultures in petri dishes and stimulating with MLCM, growth of primary as well as secondary colonies was obtained. The results indicate some self-renewal potential of CFU-C *in vitro*.

Introduction

Hematopoietic stem cells are characterized by their potential for both differentiation and self-renewal [1–2]. Colony-forming cells with restricted differentiation capability such as those which form granulocytic and/or macrophage colonies in semi-solid culture (CFU-C) are regarded as not being capable of self-renewal [3–4].

The technique of suspending and reculturing cells from colonies in primary cultures allows to monitor for the presence of cells with same growth properties. By use of this technique, growth of secondary macrophage colonies in agar culture has been reported by others [5]. Here, we report on studies of murine CFU-C derived colonies in agar culture contained in glass capillaries [6] as well as petri dishes; colonies were recultured in identical medium to yield macrophage, granulocytic and mixed granulocytic/macrophage colonies. A numerical increase of colony-forming cells *in vitro* was observed following serial reculturing and by use of different kinds of CSF.

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Material and Methods

10–12 week old male C57Bl/6J mice were used. Bone marrow was flushed from femurs in ice-cold MEM Dulbecco medium (Seromed). Samples of 300 μ l culture mixture were prepared of: medium plus saline (33.3%; v/v), pre-tested horse serum (20%; v/v; Flow), agar (0.18%; w/v; Difco), and CSF (6.67% = maximum stimulatory concentration; v/v); for primary cultures the mixture usually contained 10^5 nucleated bone marrow cells per ml. Glass capillary cultures [6] of 75 μ l volume (score of up to ≥ 20 colonies) were incubated at 37 °C and 7.5% CO₂ in fully humidified air. For growth of single primary colonies the cell titer was 10^4 and the culture volume 25 μ l (refers to Table II); in experiments measuring CFU-C survival, no CSF was added and 10^6 cells/ml were inoculated in 75 μ l culture volume (refers to Fig. 3). The usual source of CSF was mouse lung-conditioned medium (MLCM) which was prepared as described elsewhere [7]. In one experimental series two other conditioned media were used additionally: L-cell-conditioned medium (LCCM), which was a gift from the Institute of Microbiology/Free University of Berlin, and bovine lung-conditioned medium (BLCM) concentrated by ammonium sulfate fractionation, chromatography using phenyl-

sepharose and ultrafiltration as described elsewhere [8–9].

Following incubation of cultures, each gel with primary colonies was blown out into 300 μ l fresh culture medium devoid of bone marrow cells and was then completely dispersed within 60–90 seconds on a whirl-mix. Homogeneous single cell suspensions were obtained as was monitored by microscopic examination. Five secondary cultures of $\leq 75 \mu$ l were set up from each resuspended primary culture and incubated at identical conditions as the primary cultures. The incubation period was 1 hour to 7 days for primary cultures and always 7 days for secondary cultures. Day 7 cultures were scored for colonies (>50 cells) and clusters (17–50 cells) at $15\times$ magnification.

For classification of cell aggregates, 7 day culture gels were blown out onto slides, fixed with methanol, stained with May-Grünwald/Giemsa and examined at $400\times$ magnification.

The experimental scheme for intermittent 3 day/4 day serial reculture was as follows: 12 capillary cultures were set up, 6 of which were recultured (passage 1) after 3 days in each 5 new capillaries whereas the other 6 capillaries were used for colony counts on day 7. Of the passage 1 capillaries, half was recultured again (passage 2) after 4 days and half was used for 7 day colony counts representing the number of CFU-C at the time of the passage, etc. For serial reculture at intervals of 7 days, 6 capillaries were initially set up; prior to reculture the colonies were counted.

In order to test for the presence of CFU-S (colony-forming units spleen = pluripotent stem cells; [10]) in primary agar cultures, single 7 day culture gels (initial cell inoculum of 10^5 cells/ml) and single 4 day culture gels (inoculum 10^6 cells/ml) were blown out into 300 μ l MEM Dulbecco medium, completely dispersed as described and each sample was injected intravenously into a mouse which had received 780 rad X-rays 3 hours previously from a Stabilipan machine (Siemens) operating at 180 kVp and 20 mA (target distance 60 cm; dose rate 45 rad/min). The same volume of culture medium, but without cells, was injected into control mice. After 10 days the mice were killed, the spleens removed and fixed in Bouin's solution for 24 hours and the spleen colonies counted at $15\times$ magnification.

For petri dish (35 mm diameter) culturing of CFU-C colonies, 5×10^4 nucleated bone marrow cells

were inoculated in 1 ml McCoy's agar [11] with 100 μ l MLCM. After 7 days of culture the cell aggregates were scored and whole gels were dispersed on a whirl-mix in 5–10 ml culture medium. Cells were pelleted and used for setting up secondary cultures in identical medium for another 7 days of incubation. Single primary and secondary colonies were picked out of the gel using a Pasteur pipette, then placed on a microscope slide and stained with 0.6% orcein in 60% acetic acid.

Results

During 7 days of growth of primary CFU-C derived cell aggregates the presence of cells capable of colony and cluster formation was followed by daily reculturing. Fig. 1 demonstrates the result of such experiments indicating existence during the first

Reculturing of Murine GM-CFC Aggregates
(Primary Cultures 0–7 Days, Secondary Cultures 7 Days)

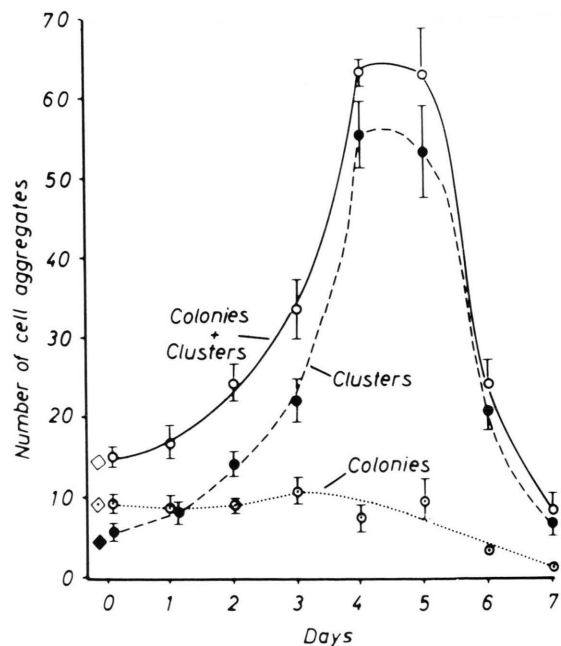


Fig. 1. Formation of secondary 7 day cell aggregates in dependence upon the time of reculture from dispersed gels with primary colonies (0–7 days; 0 day means 1 hour). Each point represents the mean and SE from reculture of 10 primary cultures. For reference, the number of 7 day primary cell aggregates is indicated by square symbols ($\diamond$ = colonies, $\blacklozenge$ = clusters, $\diamond$ = colonies plus clusters).

5 days of primary culture of about an equal number of cells capable of secondary colony formation as is the number of colonies originating in the primary cultures themselves; by day 7 of primary culture the number of colony-forming cells present for secondary colony formation decreases to 10–20% of those that form the primary colonies. The number of cells capable of secondary cluster formation shows a distinct peak on days 4–5 of primary culture. Secondary colonies appeared to be composed of less cells than primary colonies.

Of 79 secondary colonies classified following May-Grünwald/Giemsa staining, 19 (24.0%) were pure granulocytic (>98% of the cells/colony), 27 (34.2%) were of macrophage or mononuclear cell type, and 33 (41.8%) were of mixed granulocytic/macrophage nature. No clusters with granulocytic cells but only macrophage/mononuclear cells were seen.

In order to test for the possible presence of pluripotent stem cells (CFU-S), primary CFU-C cultures were suspended and injected into 780 rad irradiated mice. Table I gives the results of spleen colony assays with background levels of such colonies in all groups. No CFU-S were thus detected in 7 day CFU-C cultures as well as in 4 day cultures with 10-fold cell inocula.

Table I. Number of CFU-S derived spleen colonies 10 days after injection of 4 and 7 day old CFU-C cultures and matched control cultures devoid of cells.

Agar gels injected	No. of spleens	No. of spleen colonies	
		Mean	Range
CFU-C 7d	8	0.15	0–1
Control	8	0.25	0–2
CFU-C 4d	11	0.55	0–2
Control	9	0.22	0–2

Glass capillary agar cultures and spleen colony assays were done as described in 'Material and Methods'. CFU-C 7d cultures were seeded with the usual 10^5 cells/ml, CFU-C 4d cultures were seeded with 10-fold inoculum (10^6 cells/ml).

Table II gives the results obtained when single colonies or clusters were grown for 7 days and recultured for another 7 days. Day 7 of the primary culture was chosen since at this time small aggregates can hardly be missed microscopically as compared to

Table II. Reculturing of single cell aggregates: 7 day growth of both primary and secondary cultures.

	Colonies	Clusters	Gels Ø aggreg.
No. of 1°	111	82	120
No. of 2°	4	98	295

	2° aggregates from 1°		
	Colonies	Clusters	Gels Ø aggreg.
Mean	0.88	0.40	0.01
Variance	12.12	8.40	0.01
Coeff. variat.	3.95	6.30	4.95

Primary and secondary CFU-C cultures were set up as described in 'Materials and Methods'. The wide distribution of reculturable cells in primary aggregates is indicated by variance and coefficient of variation and further specified in 'Results'.

earlier days. It was noticed that the single primary colonies were smaller (about 2000–3000 cells) than colonies grown together with other colonies (about 3000–5000 cells). These data show that less secondary colonies were obtained than in experiments with several aggregates grown and recultured together after 7 days (Fig. 1). From most of the single primary colonies as well as clusters no secondary cell aggregates were obtained. 14 out of 111 primary colonies generated between 1 and 20 secondary clusters and at most 1 secondary colony with no more than about 1000 cells; only 3 of 82 primary clusters generated 1, 16 and 21 secondary clusters but no colonies. Matched primary agar gels which by random were devoid of aggregates were also 'recultured' to test for 'dormant' CFU-C. Out of 120 aggregate-free gels only 1 brought forth 1 secondary cluster.

The discrepancies in number of secondary colonies obtained between gels with up to ≥ 20 primary colonies recultured versus single primary colonies recultured (Table II, Fig. 1) and corresponding differences in sizes of primary colonies noticed may be partly explainable by the results of Table III. In this experiment, the optimum cell density for glass capillary cultures of 10^5 nucleated cells/ml was increased or reduced by a factor of 10. Primary cultures as well as secondary cultures established after 1 hour of primary culture did not show 10-fold different colony counts but only 5 times more versus over 20 times less colonies. Thus, many cells inoculated was inhibitory while few cells inoculated was sub-optimal for stimulation of colony growth.

Cells/ml	1° cultures			2° cultures		
	10 ⁶	10 ⁵	10 ⁴	10 ⁶	10 ⁵	10 ⁴
Colonies	con- fluent	17.2 ± 0.4	0	88.1 ± 2.7	16.6 ± 1.6	0.3 ± 0.2
Clusters		5.3 ± 0.2	0	25.8 ± 0.4	10.2 ± 0.9	0.3 ± 0.2

Table III. Comparison of 7 day CFU-C aggregates grown in primary culture and grown in secondary culture upon reculturing primary cell seeds after 1 hour.

Primary and secondary cultures were set up as described in the text. The mean and SE of 9 cultures each are given.

The results of serial reculture are shown in Fig. 2 for both intermittent 3 day/4 day passages (left) and 7 day passages (right) with 2 experiments, for which separate batches of MLCM were used. When aggregates were recultured every third or fourth day, 3 passages were possible with a distinct peak for colonies at passage 2 indicating numerical increase of the initial CFU-C number by a factor of 2–7; for clusters a pronounced peak was found at passage 3 (increase by a factor of 10–20). With reculturing at 7 day intervals, only 2 passages were possible (with a small peak for colonies at passage 1 in 1 experiment),

whereas pronounced peaks were found for clusters at passage 2 in both experiments (25–30-fold increase). From passage 2 on all colonies were of mononuclear cell type.

Table IV gives the colony counts of a series of experiments with combinations of CSF from 3 sources (MLCM, LCCM, BLCM). The yield of primary colonies was the same for MLCM- and LCCM-stimulated cultures (18–19 colonies) but much lower for those incubated with BLCM (5 colonies). When BLCM-stimulated primary cultures were recultured after 7 days with LCCM, up to 61 secondary colonies

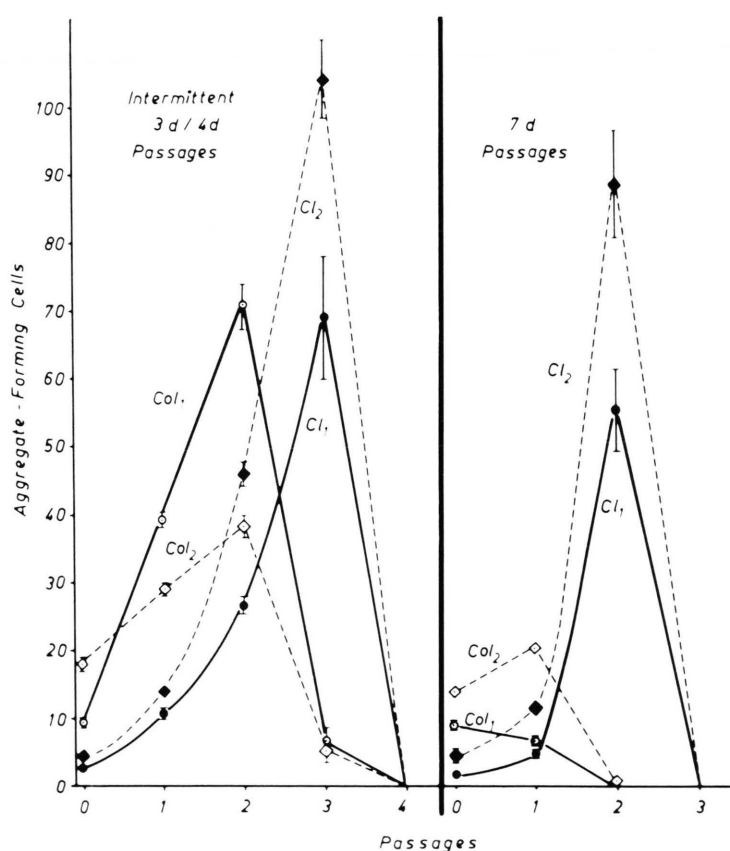


Fig. 2. Number of colony- and cluster-forming cells in primary cultures (passage 0) and upon serial reculture (passages 1–4) at both intermittent 3 day/4 day intervals (left) and 7 day intervals (right), as measured by 7 day counts of cell aggregates. Two experiments each with always 6 primary cultures; each point represents the mean and SE; Col_{1,2} = colony-forming cells exp. 1 and 2, resp.; Cl_{1,2} = cluster-forming cells exp. 1 and 2, resp.

Table IV. Number of 7 day CFU-C colonies in both primary and secondary cultures upon use of CSF from different sources (MLCM, BLCM, LCCM) and reculture at different times.

CSF in 2° culture	day of result.	CSF in 1° culture		
		MLCM	BLCM	LCCM
—	—	18.4 ± 0.6	4.9 ± 0.5	18.7 ± 0.7
MLCM	3	12.8 ± 1.8	27.5 ± 5.9	16.0 ± 1.2
	5	33.0 ± 4.6	27.2 ± 2.1	26.8 ± 1.3
	7	24.3 ± 1.6	24.3 ± 0.3	23.5 ± 0.9
BLCM	3	8.3 ± 2.0	0	9.8 ± 1.7
	5	21.7 ± 1.4	0	22.7 ± 1.1
	7	8.7 ± 1.5	0	18.2 ± 1.7
LCCM	3	23.5 ± 2.5	27.7 ± 8.7	29.3 ± 2.3
	5	35.2 ± 2.7	41.3 ± 3.9	58.7 ± 2.6
	7	25.2 ± 4.7	61.0 ± 4.6	44.5 ± 3.0

Primary and secondary cultures were set up as described in the text. The mean and SE of each 6 cultures is given.

were obtained (>4 times the number in primary cultures); no secondary colonies were generated with BLCM in secondary cultures. High colony counts were also found for other secondary LCCM-stimulated cultures, whereas low counts were obtained with secondary BLCM-stimulated cultures, and MLCM stimulation in secondary cultures was intermediate. The results for clusters were very similar to those for the colonies (data not shown). Granulocytic or granulocytic/macrophage colonies were only

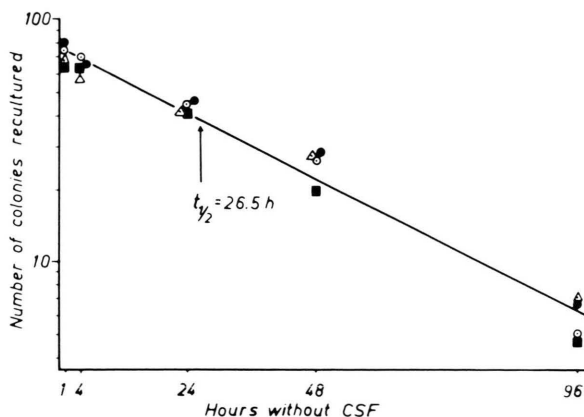


Fig. 3. Survival of CFU-C in primary cultures devoid of CSF as measured by reculturing with MLCM at various times after primary inoculation; secondary cell aggregates were counted after 7 days of secondary culture. Four experiments with each 6 primary cultures per point; the half-time was calculated following exponential regression analysis.

observed in MLCM-stimulated primary plus secondary cultures, whereas BLCM and LCCM stimulation yielded mononuclear cells.

Fig. 3 shows the dependence of the reculture success on the presence of CSF-containing medium; here, CSF-deprived primary cultures were recultured at different times with MLCM. The *in vitro* survival of CFU-C shows a half-time of 26.5 hours.

The reculture potential of cells from colonies in primary glass capillary cultures was confirmed using primary and secondary petri dish cultures (Table V). Besides macrophage/mononuclear cells also granulocytic cells were seen in secondary colonies grown in petri dishes.

Table V. Primary and secondary CFU-C aggregates grown in petri dish cultures and stimulated with MLCM.

	1° aggregates	2° aggregates
Colonies	28.7 ± 2.4	22.2 ± 4.1
Clusters	5.8 ± 0.8	7.1 ± 2.2

Primary and secondary petri dish cultures were set up as described in the text. The mean and SE of counts from 6 cultures is given.

Discussion

The results pertain to *in vitro* self-renewal of CFU-C. Not only that single cells from completely dispersed primary CFU-C derived colonies could be stimulated to form secondary colonies, but also a numerical increase *in vitro* of colony-forming cells was obtained. And it was shown that a) no CFU-S were present in CFU-C cultures (Table I) rejecting the possibility of CFU-S derived secondary CFU-C colonies, and b) no secondary colonies were formed when agar gels by random devoid of primary cell aggregates were recultured (Table II) thus indicating that probably no single cells have survived between the primary colonies to become stimulated in secondary culture.

The little yield with reculture of single cell aggregates (Table II) confirms results reported by others [12] that raised the impression of non-self-renewal *in vitro* [3]. As the results of Table III indicate, absence of potentiating effects of crowding (conditioning effects through other cells) on colony formation may be the cause of this limited reculture yield; this effect is discussed in detail by Metcalf [13]. The wide distribution of cluster-forming cells among CFU-C col-

onies (Table II) can be compared with the wide distribution of CFU-S among spleen colonies [14], although the maximum number of recultured cluster-forming cells is much smaller than the maximum secondary CFU-S number [14]. A wide distribution has also been reported for multipotent colony-forming cells among multipotent colonies *in vitro* [2]. Wide distribution indicates heterogeneity of the precursor cells and is likely to be caused by events on the single cell level.

The numerical increase of CFU-C in agar culture of up to a factor of 7 was measured by use of two methods, serial reculture and reculture with combinations of CSF-enriched conditioned media. Favoring conditions for this increase seem to be a) short culture periods so that the cells may be frequently interrupted in their differentiation process and have to adjust to the new environment, as observed with the 3 day/4 day serial reculture (Fig. 2), or b) some inhibitory effects from the conditioned medium, as obviously present in the BLCM preparation resulting in a low colony yield in primary but high yield in secondary LCCM-stimulated cultures (Table III). Granulocytic colonies were seen in primary and secondary MLCM-stimulated cultures while from tertiary cultures on only mononuclear cell aggregates were observed. This may point to differences in the regulation of self-renewal versus differentiation in the cells.

Since in the serial reculture experiments (Fig. 2) not more than 3 passages with an overall time of 2–3 weeks of culture were successful, a limited self-renewal capacity of CFU-C could be suggested being comparable with limitations of passaging for CFU-S [15] and multipotent stem cells [2]. But as more than 20 passages of macrophage colonies have been reported with reculture titers of at least 10^4 [5], in our studies the dilution with repeated reculturing (always 1 culture of 75 μ l volume gives 5 new cultures of ≤ 75 volume, – thus possibly losing the potentiation effects of crowding) may account for the limited passaging success. The peak of cluster-forming cells may be interpreted as descendance of cluster-forming cells from colony-forming cells.

The half-time of CFU-C survival of 26.5 hours in capillaries is very similar to results of others [13] using petri dish cultures. The fact that there is a straight loss of CFU-C without CSF (MLCM) indicates that the numerical increase measured with two kinds of experimental design is mediated by the conditioned

media. Either CSF itself may be mediating or some other factor in the conditioned medium.

Indication of possible self-renewal of CFU-C has already been obtained by use of suspension cultures [16], diffusion chambers [17] and adherent cell layers [18], although in these systems the contribution of CFU-S to the CFU-C population is not quite clear. It has also been shown that the 2 daughter cells of granulocytic/macrophage precursor cells and even the 4 granddaughter cells form separate clones in CSF-stimulated agar culture following separation by micromanipulation [19, 20]. Reculturing of macrophage aggregates for more than 20 passages (but not reculturing of granulocytic aggregates) from primary CFU-C derived colonies has also been reported; petri dish cultures, initial cell inocula of 10^5 nucleated cells/ml (same as in this study), and reculture of a minimum of 10^4 nucleated cells/ml were used by these authors [5]. Long-term survival (3 months) of CFU-C derived colonies may have additionally pointed to some potential for self-renewal of CFU-C [21]. Besides this, to our knowledge only replating of multipotential colonies [2, 3] as well as leukemic blast colonies [22] has been successful. Also, some indications of possible *in vivo* self-renewal of CFU-E, a precursor cell type of the erythroid lineage, have been found [23].

The possibility of an extensive *in vivo* self-renewal of CFU-C has earlier been discussed and rejected [24]. But, from the results reported here (capillary as well as petri dish data), some self-renewal of CFU-C *in vivo* should be regarded possible. It is a matter of conjecture, whether for the entire compartment of cytologically unidentifiable precursor cells a hierarchy of more and more restricted self-renewal parallels the restrictions of differentiation from the non-assayable but evident myeloid stem cell [24, 25] or pre-CFU-S [26] to the CFU-S or multipotential stem cell and further to the committed progenitor cells.

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