*EVIDENCE FOR A RELATIONSHIP BETWEEN MOUSE HEMOPOIETIC STEM CELLS AND CELLS FORMING COLONIES IN CULTURE**

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The spleen colony technique[1] has proved to be a useful tool for the enumeration of hemopoietic stem cells in the mouse and for the study of their properties. However, since the method depends on colony formation in the spleens of heavily irradiated[1] or genetically anemic[2] mice, it has certain inherent limitations. For example, events occurring early in the growth of colonies cannot be observed, since they are obscured by the splenic environment; further, the spleen-colony method has not been applied successfully to the study of human cells[3, 4] and, therefore, the information obtained by use of this method cannot be applied directly to clinical problems. These limitations might be avoided by the use of cell-culture methods. Recently, Pluznik and Sachs[5] and Bradley and Metcalf[6] have reported that cells derived from mouse hemopoietic tissue will form colonies in culture if they are suspended in dilute agar over a firm agar base containing a source of suitable "conditioning factor."[7–9] If a relationship were established between the cells that give rise to these colonies in culture and the hemopoietic stem cells responsible for colony formation in the spleen, the two techniques might complement each other. Results obtained by the use of the culture technique might be related through the spleen-colony method to normal and abnormal hemopoiesis *in vivo*, and the information gained in the mouse by using the spleen-colony technique might be extended to other species, including man, by using the culture method.

The existence of a relationship between hemopoietic stem cells and the cells that give rise to colonies in culture is indicated by the properties of the latter: they are capable of extensive proliferation,[10, 11] self-renewal,[5] and granulopoietic differentiation.[6, 10] Further, they are found in the same organs as hemopoietic colony-forming cells and in approximately the same relative numbers.[5, 6] In order to establish a relationship more directly, we have employed two approaches. First, we have used a chromosome marker technique[12] to determine whether or not the cells that give rise to colonies in culture can belong to the same clone as cells that form colonies *in vivo*. Second, we have used both techniques in assaying cell suspensions derived from individual spleen colonies for their colony-forming activity. Previous work had shown that the distribution of spleen colony-forming cells among spleen colonies is very heterogeneous, with a 1000-fold difference between colonies containing little and those containing much colony-forming potential.[13, 14] If individual colonies yielded similar results in both assay systems, a close relationship between the cells responsible for each activity might be assumed.

The results of these experiments are presented in this report. They provide

proof that the cells responsible for colony formation in culture and in the spleen can belong to the same clone and give evidence for a close relationship between them.

Materials and Methods.—Mice used in transplantation experiments were F_1 hybrids between mice of genotype WB-$W/+$ and C57BL/6-$+/W^v$. This cross[15] yields normal F_1 hybrids (genotype WB B6-$+/+$), mice heterozygous at the W locus (genotype WB B6-$W/+$ and WB B6-$+/W^v$), and mice of genotype WB B6-W/W^v. For colony formation in culture, kidney tubules to be used as a source of "conditioning factor" were obtained from C57BL mice.

Assays of colony formation: The technique used to obtain colonies in cell culture was a modification of that of Bradley and Metcalf.[6] Cultures consisted of two layers in a 35-mm Falcon plastic Petri dish. The bottom layers contained approximately 3×10^4 mouse renal tubules immobilized in medium CMRL 1066[16] with 10% fetal calf serum and 0.5% agar (Difco Bacto-agar). The top layers contained the hemopoietic cells under test immobilized in the same medium but containing 0.3% agar. Cultures were incubated at 37°C in a humidified atmosphere continuously flushed with 5% CO_2 in air. After seven days of incubation, colony counts were made by using an inverted microscope. Spleen colonies *in vivo* were obtained by injection of an appropriate number of hemopoietic cells into lethally irradiated or genetically anemic mice, as described previously.[1, 2] Spleen colonies were counted 10 days later.

Preparation of cell populations with chromosomal markers: Large clones of hemopoietic cells whose members could be identified by the presence of characteristic, unique, abnormal chromosomal markers were prepared as described previously.[12] The markers were induced by ionizing radiation in single hemopoietic stem cells, which then were allowed to proliferate from 1 to 6 months in the tissues of unirradiated mice of genotype W/W^v. After this time, animals bearing suitably marked clones were selected by examining marrow for the presence of abnormal metaphase cells.[17] When these were found, the marrow served as starting material for further experimentation.

Cytological techniques: The chromosomes of cells from marrow, spleen, or spleen colonies were examined using the cytological technique of Bunker,[18] with minor modifications.[14] To obtain suitable preparations from the small populations present in the cultures, further modification was required. Suitable cultures were chosen after 5–10 days of incubation and colcemid (0.5 ml of 10^{-4} gm % dissolved in CMRL 1066) was added. Twelve hours later, individual colonies were picked and placed in 0.5 ml of ice-cold CMRL 1066 and freed of agar. Thereafter, the colonies were either pooled or kept separate. The cells were pipetted onto microscope slides lying in Petri dishes and allowed to settle and adhere to the slides. After 40 min at room temperature, the slides were flooded with sodium citrate solution (40 ml of 1.12% solution at 37°C) and left for 40 min. Next, the preparations were fixed by immersing them in another dish containing fixative (3 parts absolute methanol and 1 part glacial acetic acid) for 1 hr at room temperature. The slides were then chilled, ignited, dried in air, and stained with 4% Giemsa stain.

Preparation of cell suspensions from single colonies: Well-separated 14-day colonies were dissected from the spleen and suspended as described previously.[13]

Results.—Studies using chromosome markers: The objective of these experiments was to utilize hemopoietic cells bearing radiation-induced chromosome markers to test for the presence of the same marker chromosome in spleen colonies and in colonies in culture.

The starting material consisted of marrow in which 80 per cent of the metaphase cells contained a characteristic metacentric marker which was generated as described in *Materials and Methods.* This marrow was injected into heavily irradiated normal coisogenic recipient mice. After 14 days, some of these recipients were killed and a cell suspension was prepared from approximately 50

colonies found in their spleens. All 50 metaphase cells examined from this suspension contained the metacentric marker, and it was used for a trial of the cytological techniques by producing colonies from it both *in vivo* and in culture. Of the colonies in culture, a satisfactory cytological preparation was obtained only from one, but this contained two metaphase cells with the metacentric marker and no cells with normal karyotypes. Four spleen colonies derived from the same cell suspension were examined, and of these, three were found to contain the marker in all of ten metaphase cells, while the fourth colony contained only cells with normal chromosomes.

Some of the irradiated recipients of the marked marrow suspension had received 5×10^5 cells, and the spleen of one of these was used after a month as a source of marked cells for a further test. The results of a cytological analysis of the spleen-cell suspension and the colonies derived from it are given in Table 1. In a sample of 25 metaphase cells obtained from the spleen-cell suspension (first line of Table 1), 21 contained metacentric markers, 3 were normal, and 1 contained long and minute marker chromosomes not seen previously in this material. A pool of cells from spleen colonies derived from the same spleen-cell suspension contained a high proportion of cells with the metacentric marker, but no examples of the long and minute marker were encountered (second line of Table 1). Two colonies in culture derived from the same spleen-cell suspension were examined individually. A total of six well-spread metaphases were found (lines 3 and 4 of Table 1), and all contained the characteristic metacentric marker. In the same experiment, all of the colonies on a single petri dish were pooled and subjected to cytological examination. As may be seen from lines 5 to 8 of Table 1, both the metacentric marker and the long and minute markers were found in cells of these colonies. Thus, the same characteristic metaphase marker demonstrated in suspensions of hemopoietic cells was also found in the cells of both colonies *in vivo* and colonies in culture. The observation of the long and minute markers in colonies in agar and not in spleen colonies might be taken as evidence that a marker present in colonies in culture need not always be found also in colonies *in vivo*. However, it is possible that a more extensive analysis of spleen colonies derived from cells carrying the long and minute markers would have revealed their presence in colonies *in vivo*. Unfortunately, these markers were observed unexpectedly, late in a series of cell transplants, when it was not possible to extend the experiments in order to confirm the presence of the markers in one type of colony but not the other.

TABLE 1. *Analysis of karyotypes of cells from colonies in spleen and in culture.*

	Number of cells analyzed	Number of Cells with Particular Karyotype		
		Metacentric	Long and minute	Normal
Initial spleen cell suspension	25	21	1	3
Pooled spleen colonies	50	44	0	6
Single colonies in culture	2	2	0	0
"	4	4	0	0
Pooled colonies in culture	51	5	41	5
"	25	7	0	18
"	18	10	0	8
"	4	2	0	2

Studies on individual spleen colonies: The object of these experiments was to compare the colony-forming ability of cells derived from individual spleen colonies tested both *in vivo* and in cell culture. Individual well-separated spleen colonies were suspended in 1 ml of medium and divided into two 0.5-ml aliquots. From one aliquot, 0.05 ml was removed and diluted to 0.5 ml. The diluted sample and the 0.45-ml remainder were each injected intravenously into W/W^v hosts which had been irradiated with 150 rads of Cs^{137} gamma rays prior to injection of the cells. From the other 0.5-ml aliquot, samples in the range of volumes from 0.01 to 0.2 ml were taken and plated in agar as described in *Materials and Methods.*[19] A number of blank areas of spleen, not containing detectable colonies but of the approximate size of colonies, were also dissected out and tested in the same way.

A total of 96 colonies was tested, and a close correlation between the results obtained by both assays was observed. Figure 1 presents the results obtained

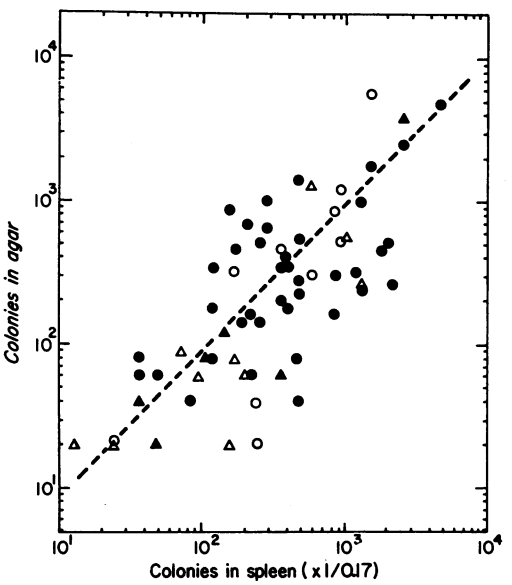

FIG. 1.—Comparison of the number of colonies in agar cultures and the number of colonies in the spleen, for cells obtained from individual 14-day spleen colonies derived from marrow. Each point represents the results for cells derived from a single colony. The different symbols represent data from separate experiments. Colony counts in the spleen have been multiplied by a factor of 1/0.17 to correct for those cells which, when injected intravenously, fail to reach the spleen.[13] Results for 65 out of a total of 96 colonies are shown. The dashed line is the straight line which best fits the data for all 96 colonies on a linear plot.[21]

from 65 colonies in which we found cells able to give rise to both colonies in culture and colonies *in vivo*. The values obtained by the spleen-colony method have been multiplied by a factor of 0/0.17, since it is known that only approximately 17 per cent of injected cells with potential for colony formation lodge in the spleen.[13] Although a correction probably should be also applied to the counts of colonies in agar, this could not be done as no information is available on the plating efficiency of cells forming colonies in culture. If this plating efficiency is low, as it usually is when cells are freshly explanted, then the apparent similarity between the average numbers of the two types of colony-forming cells (dashed line in Fig. 1) is fortuitous, and individual spleen colonies probably contain more cells able to form colonies in culture than those able to form colonies in the spleen.

In 31 colonies, one or the other of the assays failed to demonstrate the presence of cells with colony-forming ability; these data could not be included in the log-log plot shown in Figure 1. Of these 31 colonies, 10 were negative by both criteria, 7 showed only activity in culture, and 8 contained cells able to form spleen colonies but not colonies in culture. The failure to find colony-forming cells within a colony may not be interpreted to mean that they were absent, since only half of each colony was tested for colony-forming cells of either type. In addition, the spleen-colony assay probably detects only about one in five potential colony-forming cells, while the efficiency of colony formation by potential colony-forming cells cultured in agar is not known.

For the data from the other 65 colonies, the close correlation observed over a 1000-fold range of values is evident from Figure 1. The correlation coefficient for the data from the 65 colonies shown in the figure is 0.72. Inclusion of the data not shown in the figure does not change the correlation, since the correlation coefficient for all 96 of the colonies tested is 0.74. The probability of such an association occurring by chance is extremely small ($P \ll 0.001$).[20] The dashed line in Figure 1 was fitted to the data obtained from all 96 colonies.[21]

Eighteen areas of spleen that did not contain colonies visible to the eye in unfixed material were also tested. Of these, nine contained no detectable colony-forming cells of either type, eight contained small numbers of cells able to form spleen colonies but no detectable cells able to form colonies in agar, and one contained a few cells able to form colonies in agar but no detectable cells capable of forming spleen colonies.

Discussion and Conclusions.—Numerous attempts have been made to grow cells derived from marrow in culture (for a review, see ref. 22). Such work, however, has had limited application to the study of differentiation in the hemopoietic system either because quantitation was lacking or because the relationship of the findings to events *in vivo* was not clear. The soft-agar culture technique of Pluznik and Sachs[5] and Bradley and Metcalf[6] appears to be free of both these difficulties. This method yields quantitative data because the colonies in cell culture may be counted and because their number is linearly related to the number of cells plated. The fact that the colonies contain cells morphologically identified as granulocytes[6, 10] provides evidence for a possible relationship between the cell of origin of the colonies and granulopoiesis. In this present communication, we provide more direct evidence for a relationship between colony formation in culture and hemopoiesis *in vivo*.

The finding of the same radiation-induced chromosomal marker in cells in colonies *in vivo* and in colonies in culture provides proof that the cells of origin of each type of colony may belong to the same clone. This result, however, yields no information about relationships within that clone; the cells responsible for colony formation in the spleen might be identical with cells forming colonies in culture, or the one type might be a progenitor of the other.

The results presented in Figure 1 provide evidence for a close relationship between the cells that form colonies in the two locations. A high degree of correlation was found between the contents of the two cell types in individual spleen colonies. This correlation appears to be specific for these particular cells.

In previous experiments, we found that although a parent-progeny relationship exists between spleen colony-forming cells and both granulocytes and erythroblasts,[12] there is no strong correlation between the numbers of such cells in spleen colonies.[14] Preliminary assays of spleen colonies for cells with colony-forming capacity in culture and for granulocytes have indicated similar lack of correlation, again in spite of a known parent-progeny relationship.

The evidence for a close relationship between cells capable of forming colonies in the spleen and those capable of forming colonies in culture derived from the correlation shown in Figure 1 becomes more compelling when the numerical values are considered. When the values obtained by the spleen-colony method are corrected for the dilution that occurs on injection of cells into the mouse (the f value of 0.17 in ref. 13), the numbers of the two types of colony-forming cells in individual spleen colonies are very similar (dashed line, Fig. 1). This finding is compatible with the view that the two classes of progenitor are in fact a single class, and that any cell detected as a colony-forming cell by one technique will also be detected by the other. However, if the plating efficiency of cells in agar is indeed less than 100 per cent, then the results in Figure 1 indicate that single spleen colonies contain, on the average, more cells able to form colonies in culture than cells able to form colonies in the spleen. The observation of the long and minute markers in colonies in agar culture and not in spleen colonies, although unconfirmed, also indicates the existence of cells able to form colonies in culture and not *in vivo*. It may be concluded that our data demonstrate a relationship between the two types of colony-forming cells, but that the exact nature of this relationship remains to be determined.

Experiments in the mouse, such as those reported here, may serve as a guide for experiments in man. It has recently been demonstrated[23] that cells derived from human marrow can form colonies in agar under cell-culture conditions similar to those employed in the studies reported here. If these colony-forming human marrow cells are identical with, or closely related to, the hemopoietic colony-forming cells of the type detectable in mice, then animal experiments may provide a valid precedent for meaningful studies of the role of stem cells in human disease.

Summary.—Marker chromosomes present in hemopoietic cells and in spleen colonies derived from them have been identified in colonies formed when the same hemopoietic cells were cultured in agar. When cells from individual spleen colonies were assayed for their content of cells able to form colonies in culture, a strong correlation between the numbers of the two types of colony-forming cells was found. These results indicate that the cells which give rise to colonies in agar cultures are closely related to the hemopoietic stem cells which give rise to colonies in the spleen.

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[17] In general, where more than 50% of the metaphase cells of the marrow of any W/W^v recipient contained a characteristic chromosome aberration, the same marker was found in 100% of the spleen colonies derived from that marrow.

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[21] Linear regression analysis of the data was carried out using the General Electric computer time-sharing program COLINR. The straight line fitted to the data from all 96 colonies had a slope of 0.91 (95% confidence limits 0.74–1.08) and an intercept not significantly different from zero ($P > 0.5$).

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