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Failure to demonstrate pluripotential hemopoietic stem cells in mouse brains

(colony-forming units, spleen)

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ABSTRACT **Hemopoietic stem cells as defined by the capacity to produce spleen colonies in lethally irradiated recipients were reported by P. F. Bartlett [(1982) *Proc. Natl. Acad. Sci. USA* 79, 2722–2725] to be present in high frequencies in mouse brain. He also reported similar numbers of colony-forming units, spleen (CFU-s), in the brains of W^f/W^f mice, the bone marrow of which lacks detectable spleen colony-forming cells. To verify these observations, single cell suspensions were produced from murine brains by incubation with trypsin and DNase, followed by removal of myelin by Percoll gradient centrifugation. Two to 13 CFU-s were detected per brain. This low number suggested contamination of the brains by either blood or bone marrow leaking from the skull bones during dissection. When the isolated, intact brains were washed carefully in balanced salt solution, the recovered number of CFU-s decreased to 0.1–0.4 per brain. No CFU-s could be detected in the brains of W/W^v mice. It is concluded that the CFU-s observed by Bartlett in preparations of mouse brain did not originate from the brain tissue.**

The presence of hemopoietic stem cells in the brains of mice has been suggested in a study by Bartlett (1), who found 15 colony-forming units, spleen (CFU-s), per 10^5 dissociated viable adult brain cells. Bartlett also recovered substantial numbers of CFU-s from brains of W^f/W^f mice, of which the bone marrow stem cells fail to produce macroscopic spleen colonies. The presence of hemopoietic precursor cells in the brain would provide interesting new clues to the origin and maintenance of nonneurogenic cells in the central nervous system—e.g., microglia (2–5). In addition, this would offer possibilities for elucidating the pathogenesis of primary lymphomas and leukemia in the central nervous system (6–9). Bartlett's finding had obvious significant implications for the current attempts to treat patients suffering from severe neurological complications of various hereditary lysosomal enzyme deficiencies by bone marrow transplantation (10). For example, it is suggested by Rappeport and Ginns (11) that these cells, if transplanted within the brain parenchyma, could secrete the missing enzyme. Therefore, efforts were started in our laboratory to study these brain hemopoietic stem cells in normal as well as stem cell-defective W/W^v mice (12). W/W^v is the more commonly used combination of mutations at the *W* locus. The anemia is more profound than that of W^f/W^f mice, whereas the latter homozygotes are, in contrast to W/W^v mice, not sterile (13).

MATERIALS AND METHODS

Mice. Specific pathogen-free inbred BCBA-F_1, BD2-F_1, BD2-W/W^v, and CBA/T6 mice of both sexes, aged 8–13 days, were used. Isogeneic 12- to 14-wk-old female mice served as recipients.

Table 1. CFU-s detected in brains of different mouse strains

Donor mouse	Irradiated recipient mouse	No. of brains used	Total no. of CFU-s detected	CFU-s detected per brain (range)
BCBA-F_1	BCBA-F_1	48	213	4.4 (2.5–8.2)
BD2-F_1	BD2-F_1	23	132	5.7 (1.7–13.2)
CBA/T6	CBA/Rij	16	42	2.6
BD2-W/W^v-F_1	BD2-W/W^v-F_1	32	0	0

Cell Suspensions. Single cell suspensions were prepared from the brains of 8- to 13-day-old mice killed in CO_2. In each experiment, 8–16 mice were used. The brains were finely sliced and incubated for 30 min at 37°C in enriched Dulbecco's modified Eagle's medium (MEM) containing 0.1% trypsin and 1 mg of DNase (Sigma) per ml. Fetal calf serum was added to stop trypsinization. The brain suspension was washed in Dulbecco's MEM containing 1% fetal calf serum and 1 mg of DNase per ml. The pellet was passed through double-layer nylon gauze and the resulting suspension was subjected to continuous Percoll (Pharmacia) density-gradient centrifugation (Beckman J21B; 45 min; 5000 × *g* at 4°C) to remove the myelin. The cells were washed with Dulbecco's MEM to remove Percoll. This procedure was performed exactly as recommended by Bartlett (1) when our initial attempts to detect brain CFU-s failed. Bone marrow suspensions were obtained by flushing femoral shafts with Dulbecco's MEM. To test the toxicity of the procedure of removing myelin from the brain suspension to CFU-s, bone marrow cells were mixed with brain suspensions and subjected to the described fractionation procedure, including the incubation in 0.1% trypsin.

Spleen Colony Assay. Cells were injected into 12- to 14-wk-old female isogeneic mice that were lethally irradiated (9.0-Gy γ irradiation) at 2–4 hr prior to injection. After 9 days, the recipient spleens were removed and placed in Tellyesniczky's fixative, and surface colonies were counted as described by Till and McCulloch (14).

RESULTS

CFU-s in Single Cell Suspensions of Mouse Brains. Eight-day-old mice were used as recommended by Bartlett (10) because viable brain cells are obtained more readily from these young mice than from older mice. Bartlett reported that the variation in CFU-s content per 10^5 viable cells with age is minimal at between 3 and 40 days of age (1).

About 2–13 (mean, 4.4) CFU-s were determined per brain; the number of viable cells per brain varied between 10^6 and 3×10^6, and 2–10 CFU-s per 10^6 viable nucleated cells were

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Abbreviation: CFU-s, colony-forming unit(s), spleen.

Table 2. Effect of washing the intact isolated brains of BCBA-F_1 mice on the number of CFU-s detected per brain

	No washing			Washing			
Exp.	No. of brains used	Total no. of CFU-s detected	No. of CFU-s detected per brain	No. of brains used	Total no. of CFU-s detected	No. of CFU-s detected per brain	Loss due to washing, %
1	8	15 ± 4	1.9	16	1 ± 1	0.1	93
2	10	24 ± 5	2.4	10	4 ± 2	0.4	83
3	9	35 ± 6	3.9	10	4 ± 2	0.4	89

Totals are presented ± SD.

collected from each brain. Brain cell suspensions irradiated *in vitro* with 20-Gy γ irradiation prior to injection produced no spleen colonies, which indicates that the spleen colonies were of donor origin. Experiments performed with different mouse strains yielded similar numbers of spleen colonies (Table 1). In W/W^v mice, no CFU-s could be detected in bone marrow or brain (Table 1).

In another set of experiments, the isolated brains were carefully washed in balanced Hanks' solution following dissection and the results were compared with the procedure used by Bartlett (no washing). These experiments were designed to determine whether the observed CFU-s were due to contamination with the bone marrow from the bones of the skull, which have to be cleft to collect the brains. Washing the brains reduced the number of CFU-s by 85–95% to 0.1–0.4 CFU-s per brain (Table 2). The total cell number recovered from the Percoll gradient decreased by 50% (range, 43–57%) as a result of the washings and the number of CFU-s fell to 0.1–0.8 per 10^6 viable cells. From the wash fluid, 0.6–1.5 CFU-s were recovered per washed brain. In one experiment, the removed skull bones were broken two or three times and rinsed briefly with Hanks' solution to determine the number of CFU-s released by cutting the skull bones. The resulting suspension was subjected to the same isolation procedure as the brain suspensions. The number of CFU-s recovered amounted to 11 ± 2 per skull. Although the experiment was not stringent in determining the total number of CFU-s present in skull bones, it demonstrates that CFU-s readily leak out in numbers exceeding those found in unwashed brain cell suspensions.

Toxicity of the Methods to Bone Marrow CFU-s. To test the toxicity of the dissociation procedure to bone marrow CFU-s, mixtures of bone marrow and brain tissue were processed and analyzed. The overall recovery of CFU-s after the complete procedure was 70% (range, 55–90%). A similar loss occurred in bone marrow that had not been mixed with brains but was subjected to the brain cell isolation procedure.

DISCUSSION

It is obvious from our data that normal mouse brain tissue does not contain CFU-s. The CFU-s measured in the brain tissue suspension originate from contamination with bone marrow of the skull bones. If the intact isolated brains are washed carefully, CFU-s can be recovered from the wash fluid. There is a discrepancy between the number of CFU-s recovered from the wash fluid and the number calculated from the differences between the number detected in the unwashed and washed brains. This may be explained by loss of cells during the centrifugation of large volumes of a dilute cell suspension. We suggest that Bartlett's findings can be explained by the fact that his isolation procedure fails to remove contaminating bone marrow cells from the brain tissue. In the report of Bartlett (1), all results are expressed per 10^5 viable brain cells without accounting for the number of brains used to obtain the cells and the CFU-s. As the number of cells recovered from the brain tissue per se is very small according to our counts, most of the cells in Bartlett's preparations must have been derived from contaminating bone marrow and blood. This explains the high proportion of CFU-s, which, in fact, is not much lower in the Bartlett report than that normally observed in the bone marrow of mice.

The results of our mixing experiments demonstrate that the isolation procedure itself does not cause significant losses of bone marrow cells.

Seemingly strong support for the brain origin of the CFU-s recovered by Bartlett was his finding that the cell surface phenotype of the brain CFU-s ($B2A2^+$) was different from that of bone marrow ($B2A2^-$). Bartlett failed to report on control experiments with bone marrow cells incubated in trypsin solution prior to exposure to the B2A2 antibody. It has not been excluded, therefore, that trypsinized cells become more susceptible to inactivation by this particular antibody. We have no explanation for Bartlett's finding of spleen colony-forming cells in the brain and not in the bone marrow of W^f/W^f mice; however, we venture to challenge the validity of this claim, since no convincing evidence such as karyotyping has been provided to establish the donor origin of these CFU-s.

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