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Pluripotential hemopoietic stem cells in adult mouse brain

(colony forming units, spleen/W^f/W^f mice/cell surface markers/blood–brain barrier/microglia)

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ABSTRACT **Single cell suspensions of adult mouse brain were shown to contain large numbers of pluripotential hemopoietic stem cells as detected by the ability to form hemopoietic colonies in the spleens of irradiated hosts. These colony forming unit, spleen (CFU-s) cells derived from brain gave rise to colonies identical in morphology and histology to those of bone marrow-derived CFU-s. The average number of CFU-s obtained per 10^5 dissociated adult brain cells was 14, whereas other adult tissues such as lung, kidney, liver, heart, and thymus contained insignificant CFU-s levels when tested. As the level of CFU-s in adult blood is <1 per 10^6 nucleated cells, blood contamination does not contribute to the high levels found in adult brain. Individual spleen colonies isolated from irradiated CBA (H-2^k) recipients injected with (BALB/c × CBA)F_1 (H-2^d × H-2^k) brain cells were shown by immunofluorescence to contain cells bearing surface H-2^d molecules, thus indicating that the colonies arose from the brain cell inoculum and were not endogenously derived. The surface phenotype of brain- and bone marrow-derived CFU-s was found to differ in that brain CFU-s could be inhibited by prior incubation with a monoclonal antibrain antibody B2A2, whereas bone marrow CFU-s were not. Further differences were found between brain and bone marrow CFU-s in the congenitally anemic W^f/W^f mice. These mice were shown to have very few CFU-s in the adult bone marrow, whereas the brain contained normal adult levels. The large number of hemopoietic stem cells in the brain may indicate an essential requirement for the continual generation of cells such as microglia or phagocytic cells, without the disruption of the blood–brain barrier.**

The idea that hemopoietic stem cells may reside in the brain arose from two independent concepts. First, the brain contains many identifiable cell subpopulations in addition to neurons, which are collectively known as glial cells (1). The microglia are thought to be macrophage precursors and have been shown *in vitro* to have surface receptors for the Fc piece of immunoglobulin and to phagocytose (2). The origin of these brain phagocytic cells is still controversial with evidence for both a source within the brain as well as an exogenous blood source (see refs. 1, 3, and 4 for reviews). The usual mode of replacement of tissue macrophages is from blood-borne precursors derived from bone marrow (5); however, this turnover appears unlikely to occur in the brain as under normal physiological conditions the blood–brain barrier—formed by the endothelial cells—would prevent such cell migration. Thus, the maintenance of levels of tissue macrophages would appear to be dependent upon a progenitor or stem cell population residing within the brain parenchyma.

The second observation that relates hemopoietic cells to brain tissue is the extensive antigenic crossreactivity between these two tissues in the mouse. Antigens such as Thy 1 (6) are known to be expressed in brain as well as on lymphohemopoietic cells, and many antibrain antisera crossreact with hemopoietic cells (7–9). Furthermore, antigenic crossreactivities have been reported between brain tissue and a pluripotential hemopoietic stem cell that give rise to hemopoietic colony formation in the spleen (CFU-s) (7). These latter observations may indicate that the microenvironment of the brain is similar to that of bone marrow or that it may indeed possess several of the cellular elements found in bone marrow.

In this report it is shown that the murine brain contains a large number of pluripotential hemopoietic stem cells as detected by the spleen colony-forming assay. The surface phenotype of brain-derived CFU-s and the presence of such cells in stem cell-deficient mice have also been investigated.

MATERIALS AND METHODS

Mice. Specific pathogen-free inbred CBA/CaHWehi and (CBA × BALB/c)F_1 mice aged 8–10 wk were used. Congenitally anemic W^f/W^f mice were obtained from BIOpdW^fW^+ × DBA/2W^fW^+ matings. These mice were originally obtained from G. Cudkowicz (Buffalo, NY) and have subsequently been bred at The Walter and Eliza Hall Institute.

Cell Suspensions and Antibody Treatment. Single suspensions were prepared from whole brains of mice of various ages as well as from lung, liver, kidney, heart, and thymus of adult animals. The tissues were sliced finely with a scalpel blade and then were incubated for 20 min at 37°C in Hepes-buffered Eagle's (HE) medium containing 0.1% trypsin and 0.08% EDTA. An equal volume of HE medium containing deoxyribonuclease I at 40 mg/ml (DNase, 82,000 Dornase units/mg, Calbiochem) and 10% fetal calf serum was then added and the suspension was mixed briefly and centrifuged (200 × *g* for 2 min). The pellet was resuspended in 1–2 vol of DNase/HE medium and triturated 20–30 times with a 1-ml pipette to produce a suspension containing very few cell clumps. The suspensions were then passed through progressively finer gauged needles—starting with no. 19 and ending with no. 26—to ensure suitability for intravenous injection. Bone marrow suspensions were obtained by flushing femoral shafts with HE medium. Cell suspensions were reacted with various antisera to test for CFU-s inhibition. This was carried out by incubating 5×10^5 bone marrow or brain cells in 1 ml of appropriately diluted antibody for 1 hr at room temperature. The cells were then washed and 10^5 cells were injected into individual irradiated hosts as described below.

Antibodies and Immunofluorescent Staining. Monoclonal anti-Thy 1.2, anti-Ly 1, and anti-H-2K^k antibodies were supernatants from the cell lines 30H12, 53-7-13, and 11-4.1, respectively, which were originally obtained from J. Ledbetter and L. A. Herzenberg of Stanford University. The specificity of these reagents has been described (10, 11).

B2A2 is a monoclonal antibody prepared against mouse brain

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Abbreviations: CFU-s, colony forming units, spleen; HE medium, Hepes-buffered Eagle's medium.

and has extensive reactivity with bone marrow cells and thymocytes (unpublished observations). Anti-H-2^d serum was a polyspecific CBA anti-BALB/c antiserum extensively absorbed with CBA lymphocytes. Immunofluorescent staining was carried out as previously described (12) on freshly dissected spleen colonies by using a second-stage sheep anti-mouse Ig coupled with fluorescein isothiocyanate.

Spleen Colony Assay. This assay was performed essentially as described by Till and McCulloch (13) and has been shown to be a clonal assay (14) to detect hemopoietic stem cells. Graded numbers of viable cells were injected intravenously into adult (8–10 wk of age) CBA mice that had received 850 rads (1 rad = 0.01 gray) of whole body irradiation 2–4 hr prior to intravenous injection (Philips RT50 machine at 250 kV, 15 mA; the filter used was 0.2-mm Cu and the source skin distance was 50 cm with full backscatter conditions). After 9 days the recipients' spleens were removed and placed in Bouin's fixative. Surface colonies were counted, the spleens were sectioned and stained with hematoxylin/eosin, and the colony type was assessed microscopically.

RESULTS

CFU-s in Single Cell Preparations of Whole Mouse Brain. The preparation of single cell suspensions from whole mouse brains—especially those from adult brains—suitable for intravenous injection into irradiated hosts was somewhat difficult and DNase was found to be an essential ingredient of the dissociation procedure to obtain workable cell populations. The viability of the cell inoculum was always at least 85% and contained both neuronal and glial cell populations. However, it was found that the injection of greater than 5×10^5 brain cells resulted in frequent death of the host animal, probably due to emboli from lipid material contained in the cell suspension. Thus, the results reported here are largely from animals that received an inoculation of between 10^4 and 10^5 brain cells.

Experiments that used brain preparations from animals of different ages revealed that all preparations contained significant number of CFU-s (Table 1). The variation in CFU-s content from 3 days of postnatal life to 40 days appeared to be minimal with adult animals containing ≈14 CFU-s per 10^5 viable brain cells. By comparison, 10^5 bone marrow cells obtained from the same animals gave rise to ≈22 CFU-s. However, thymus from the same animals contained no significant number of CFU-s per 10^5 thymocytes. Suspensions prepared from other adult tissues resulted in undetectable levels of CFU-s, even when a dose of 10^6 cells was given (Table 2). Similarly, the injection of 10^6 leukocytes resulted in only 1–2 CFU-s, whereas 10^5 brain cells contained 15.1 CFU-s when tested under the same experimental conditions. This latter result indicates that direct blood contamination could not account for the observed CFU-s frequency in brain tissue.

Origin of CFU-s in Brain-Injected Animals. The observation that colonies form in irradiated recipients after brain cell injection does not necessarily indicate that they are derived from the cell inoculum, as it has been previously observed that endogenous colonies derived from radioresistant stem cells form colonies under certain stimulatory conditions. Several experimental approaches were made to test this possibility. First, it was found that brain cell inoculum irradiated with 2,000 rads prior to injection resulted in no significant spleen colony formation above that observed with uninjected animals. In addition, such irradiated brain cell suspensions did not significantly enhance the level of CFU-s when injected concurrently with bone marrow cells. The second approach was to use the cell surface marker H-2 to identify the origin of the cells comprising the spleen colonies. For this purpose, (CBA × BALB/c)F_1 (H-2^k × H-2^d) cells were injected into CBA (H-2^k) hosts, the individual colonies were dissected out with needles, and a single cell suspension was obtained which was subsequently stained by immunofluorescence for the presence of surface H-2^d. As shown in Table 3, 98% of the colonies contained greater than 20% H-2^d-positive cells. Thus, the colonies observed in the spleens of brain-injected animals were derived from the donor brain tissues.

Table 1. Number of spleen colonies detected in brain, bone marrow, and thymus from animals of different ages

Age, days	Viable cell dose: Brain 5×10^4	Brain 10^5	Bone marrow 5×10^4	Bone marrow 10^5	Thymus, 10^5
3	4.5 ± 2.2	8.9 ± 4.1	7.0 ± 2.3	18.0 ± 5.1	0.25
10	5.4 ± 3.2	14.3 ± 4.2	9.1 ± 3.6	21.2 ± 4.6	0.01
20	6.4 ± 3.2	13.2 ± 5.1	8.2 ± 4.2	20.4 ± 4.8	0.01
40	7.2 ± 3.6	14.2 ± 4.1	10.3 ± 2.0	22.0 ± 5.2	0.01

Colonies were scored 9 days after inoculation. At least six animals are represented in each determination of the mean ± SD. Animals injected with diluent alone had 0.01 ± 0.02 colonies.

Morphology of Spleen Colonies. The colonies formed in the spleen of irradiated animals after brain cell inoculation had similar surface dimensions to those observed after bone marrow cell inoculation.

Microscopic examination of spleen sections revealed that all types of colonies were represented—the predominant type being the pure erythroid; however, pure megakaryocyte and granulocytic colonies were also observed as well as mixed colonies and blastlike colonies (Table 4). None of the colonies was of neural morphology.

Surface Phenotype of Brain- and Bone Marrow-Derived CFU-s. It was of interest to determine whether the surface phenotype of the CFU-s in brain was identical to that of the CFU-s found in bone marrow. Therefore, antibody inhibition studies were undertaken in which suspensions of cells were reacted with antibody prior to injection. It has been found that just antibody binding to cells is sufficient and *in vitro* complement lysis is not required for CFU-s removal. By this method, it was shown (Table 5) that the surface phenotype of bone marrow-derived CFU-s was H-2^+, B2A2$^-$, Thy 1.2$^-$, Ly 1$^-$, whereas that of the brain-derived CFU-s was H-2^+, B2A2$^+$, Thy 1.2$^-$, Ly 1$^-$—the distinguishing feature being that the brain

Table 2. Spleen colony content of adult tissues

Cell dose	Brain	Liver	Kidney	Heart	Blood*
5×10^4	7.2 ± 3.6	0.01 ± 0.01	ND	ND	ND
1×10^5	15.1 ± 4.8	0.02 ± 0.05	0.01	0.01 ± 0.05	ND
5×10^5	ND	0.1 ± 0.1	ND	ND	ND
1×10^6	ND	ND	0.1 ± 0.5	0.1 ± 0.1	1.2 ± 2.1

Single cell suspensions were prepared as described. Each value represents the mean ± SD of five or more spleens. ND, not determined.
* Cell count refers to blood leukocytes.

Table 3. Origin of CFU-s in brain-injected animals

Colonies examined, no.	Donor	Recipient	% individual colonies with H-2^d + ve cells
36	CBA × BALB/c	CBA	98
42	CBA	CBA	0

The colonies were scored + ve if >20% of cells in an individual colony were stained.

CFU-s appeared to bind the monoclonal antimouse brain antibody B2A2 whereas the bone marrow CFU-s did not, even though ≈95% of bone marrow cells have been shown by immunofluorescence to bind this reagent (unpublished results).

CFU-s Content of the W^f/W^f Mouse. The finding that the CFU-s in brain may not be identical to those found in bone marrow prompted the examination of a system in which there is a disturbance or a defect in the hemopoietic system. Thus, the CFU-s content of W^f/W^f mice that are congenitally anemic and known to have very low levels of bone marrow-derived CFU-s was examined. In agreement with McCulloch *et al.* (15), it was found that the CFU-s content of bone marrow from W^f/W^f mice is very low and no detectable levels above controls were found even when 10^6 W^f/W^f bone marrow cells were injected. In marked contrast, brain content of CFU-s of W^f/W^f mice was similar to that obtained with normal B10 × DBA/2 animals (Table 6). Thus, it appears that the CFU-s deficiency observed in the bone marrow is not associated with a brain CFU-s deficiency, further indicating the unrelated nature of these pluripotential hemopoietic stem cell compartments.

DISCUSSION

This study has shown that the adult mouse brain contains a considerable pool of cells that are capable—when injected into an irradiated recipient—of giving rise to hemopoietic colonies in the spleen that are morphologically indistinguishable from colonies obtained with a bone marrow inoculum. This presence of CFU-s in large numbers in the brain while absent from other nonhemopoietic adult tissue raises many questions as to their origin and possible functional significance *in vivo*.

The hemopoietic stem cells measured by the spleen colony assay of Till and McCulloch (13) has been shown to be heterogeneous with respect to their capacity to generate further stem cells and progenitors of hemic cells within the spleen (16, 17). Although the CFU-s appear to be pluripotential in their capacity to generate multiple cell lineages, Hodgson and Bradley (18) have shown that a more "primitive" hemopoietic stem cell that appears to give rise to CFU-s goes undetected in the spleen colony assay because it preferentially homes to the bone marrow. Thus, it would seem that the CFU-s may be partly committed, and consequently the assay may not reflect the true capacity of a cell population to repopulate the hemopoietic series. Therefore, it will be important in future experiments to determine the self-renewal capacity of the brain-derived stem cells.

Table 4. Histological analysis of hemopoietic spleen colonies

	% of total colonies				
Tissue inoculum	Erythroid	Granulocytic	Megakaryocyte	Blast	Mixed*
Bone marrow	62	20	8	2	8
Brain	81	6	7	4	2

Spleens were examined at 9 days after injection and a total of 250 colonies was examined for each tissue.
* Mixed colonies contained >5% of another cell type.

Table 5. Surface phenotype of bone marrow- and brain-derived CFU-s

Antibody pretreatment	Spleen colony, no. per 10^5 cells	
	Bone marrow	Brain
—	21.5 ± 3.1	14.3 ± 3.6
B2A2	30.6 ± 4.2	1.3 ± 1.4*
Anti-Thy 1.2	19.6 ± 4.8	15.6 ± 4.1
Anti-Ly 1	23.1 ± 3.2	10.4 ± 4.2
Anti-H-$2K^k$	2.2 ± 2.8*	2.3 ± 3.1*

Brain and bone marrow cell suspensions were reacted with antisera prior to inoculation (see *Materials and Methods*). Each value represents the mean ± SD of six or more spleens.
* Statistically significant $P < 0.05$ compared to untreated controls.

The origin of the CFU-s within the adult brain is of great interest, and initial speculation would be that they are derived from blood-borne stem cells. However, the low level of CFU-s in blood (≈1 CFU-s per 10^6 leukocytes) makes it extremely unlikely that a passive accumulation of such cells in the adult brain could account for the frequency recorded (14 CFU-s per 10^5 brain cells). There would need to be a specific recognition between the brain endothelium and such stem cells if this were to occur, and—even if this existed—the passage of such stem cells between the tight junctions found in the brain endothelium seems unlikely. The experiments that used the congenitally anemic W^f/W^f mice seem to suggest that the brain CFU-s compartment and that of the bone marrow are not interconnected in that a deficiency in the bone marrow compartment is not reflected in the brain. Nevertheless, the brain CFU-s may be originally derived from blood-borne stem cells during fetal development when higher levels of CFU-s are found in the circulation. Indeed, Barg *et al.* (19) have found significant numbers of CFU-s in fetal thymus at 15 days. Thus, it may be that during the early period prior to formation of the blood–brain barrier, hemopoietic stem cells are able to migrate into the brain and are then restricted from leaving because of formation of the endothelial tight junctions that comprise the blood–brain barrier soon after birth in the mouse. This mechanism also implies a recognition mechanism by which stem cells are selected to migrate, and the observed antigenic similarities between lymphohemopoietic and brain tissue may be reflective of such an attractive mechanism. The antigenic similarities may also imply a suitable milieu in which the hemopoietic stem cell can reside without degeneration.

The other more highly speculative origin of CFU-s is that they are derived from a stem cell that gives rise to both neural cell types and hemopoietic cells. No embryological evidence exists to suggest that these two tissue lineages have a common ancestry. Cells of the central nervous system—both neuronal and glial cells—are derived from neuroepithelium (1), whereas the hemopoietic cells are mesenchymally derived. Neverthe-

Table 6. CFU-s content of W^f/W^f mouse bone marrow and brain

	Bone marrow		Brain	
Cell dose	W^f/W^f	B10 × DBA/2	W^fW^f	B10 × DBA/2
		Experiment 1		
1×10^5	0.20 ± 0.5	20.2 ± 4.2	8.4 ± 6.1	6.1 ± 2.3
1×10^6	0.6 ± 1.7	ND	ND	ND
		Experiment 2		
1×10^5	0.25 ± 0.7	ND	16.4 ± 7.2	15.2 ± 6.8
5×10^4	ND	ND	5.2 ± 4.6	ND

Spleens were examined at 9 days after injection. Each value represents the mean ± SD of five or more determinations. ND, not determined.

less, the surface phenotypes of the brain-derived CFU-s (H-2^+, $2A2^+$, Thy 1^-, Ly 1^-) and the bone marrow CFU-s (H-2^+, $2A2^-$, Thy 1^-, Ly 1^-) differ, and this may indicate that such cells arise from different embryological sources. However, it is more likely that such differences are attributable to microenvironmental differences and stage of stem cell differentiation. It has been observed that CFU-s from blood appear to lack an antigen—detected by polyspecific anti-brain antisera—that is expressed on bone marrow CFU-s (20). Results of experiments with the congenitally anemic W^f/W^f mice may be attributable to a genetic defect affecting one lineage of stem cells but leaving the brain CFU-s precursors untouched. However, until the nature of the developmental defect is more clearly understood, such conclusions are mere speculation.

Whatever the origin of the CFU-s, it is evident that the brain is unique in the large number of these cells it contains. The exact size of the brain CFU-s pool is unknown because the seeding efficiency studies of such cells have not been performed due to difficulty in injecting large numbers of brain cells intravenously. The question that arises from this observation is What function do such cells play in brain physiology? CFU-s are known to give rise to progenitors of red cells, granulocytes, macrophages, and platelets. Do any of these cell types occur within the brain? The brain contains cells called microglia that are capable, upon stimulation, of phagocytosis and expression of a surface receptor for the Fc piece of immunoglobulin. The origin of these cells within the brain has been disputed. It has been thought that, like other tissue macrophages, they are derived from a blood-borne precursor, or that they are derived from endogenous elements (3, 4). The concept of these cells being continually renewed from blood-borne precursors implies either that they only enter upon blood vessel damage or that they are capable of crossing the blood–brain barrier. The idea that such tissue macrophages are derived from a progenitor cell that in turn is derived from a stem cell residing in the brain appears to be an attractive proposition. This system would provide a virtually inexhaustible supply of tissue macrophages without the disturbance of the blood–brain barrier. Such cells may play a key role in the normal homeostasis of brain function by removing cellular debris such as myelin fragments.

Another hemopoietic cell type commonly observed in the brain during disease states such as multiple sclerosis is the lymphocyte (21). Elevated levels of IgG in the cerebrospinal fluid and antibody to myelin basic protein are associated with multiple sclerosis (22). The observation that the antibody secreted in the cerebrospinal fluid of multiple sclerosis patients is of an oligoclonal pattern (23)—implying that it is derived from a few clones of B lymphocytes and not heterogenous in nature as normally observed in serum—may suggest that such secreting B lymphocytes within the central nervous system also may have an origin other than that of circulating B cell precursors. The further isolation and characterization of the brain-derived CFU-s is essential to an understanding of the possible function of such cells in this unique position.

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