Cells Expressing Human Glucocerebrosidase From a Retroviral Vector Repopulate Macrophages and Central Nervous System Microglia After Murine Bone Marrow Transplantation

By Wanda J. Krall, Pia M. Challita, Lynn S. Perlmutter, Dianne C. Skelton, and Donald B. Kohn

Gaucher disease is an inherited lysosomal storage disease in which the loss in functional activity of glucocerebrosidase (GC) results in the storage of its lipid substrate in cells of the macrophage lineage. A gene therapy approach involving retroviral transduction of autologous bone marrow (BM) followed by transplantation has been recently approved for clinical trial. Amelioration of the disease symptoms may depend on the replacement of diseased macrophages with incoming cells expressing human GC; however, the processes of donor cell engraftment and vector gene expression have not been addressed at the cellular level in relevant tissues. Therefore, we undertook a comprehensive immunohistologic study of macrophage and microglia replacement after murine BM transplantation with retrovirus-marked BM. Serial quantitative PCR analyses were employed to provide an overview of the time course of engraftment of vector-marked cells in a panel of tissues. Following reconstitution of hematopoietic tissues with vector-marked donor cells at early stages, GC+ cells began to infiltrate the liver, lung, brain, and spinal cord by 3 months after transplant. Immunohistochemical analyses of PCR+ tissues using the 8E4 monoclonal antibody specific for human GC revealed that macrophages expressing human GC had partially reconstituted the Mac-1+ population in all tissues in a manner characteristic to each tissue type. In the brain, 20% of the total microglia had been replaced with donor cells expressing GC by 3 to 4 months after transplant. The finding that significant numbers of donor cells expressing a retroviral gene product immigrate to the central nervous system suggests that gene therapy for neuronopathic forms of lysosomal storage diseases as well as antiviral gene therapy for AIDS may be feasible.

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cell surface markers reflecting the fact that different types of tissue macrophages maintain distinct functional roles. These observations suggest then, that all tissue macrophage populations will not behave identically in terms of repopulation rates posttransplant.

Although donor-derived cells have been demonstrated in visceral tissues after gene transfer/BMT, the issue of donor microglia repopulation with retrovirus-marked cells has not been previously addressed. The CNS microglia appear to derive from hematopoietic cell invasion of the developing brain during embryogenesis; however, the mechanism of turnover of these cells in the adult is unclear and highly controversial. The paucity of information regarding the lineage of the numerous morphologic subtypes of mononuclear phagocytes in the CNS has complicated this issue. These questions have been addressed using various methods of cell labeling including dyes, carbon particles, radioactive isotopes, and sex-mismatched BMT. Retroviral marking of donor BM cells offers a new approach to this problem since donor cells can be tracked independent of unstable labels or MHC antigens which tend to be down-regulated on resident microglia.

In the studies presented here, we have determined the time course and extent of engraftment of cells of the monocytic lineage in tissues relevant to Gaucher disease. Donor BM cells were marked with a retroviral vector containing the human GC cDNA and detected using a quantitative PCR assay combined with immunohistochemical staining. We show that all macrophage populations, including the CNS microglia, are partially replaced with donor cells which continue to express the vector GC gene at high levels as long as 8 months after BMT.

MATERIALS AND METHODS

Retroviral vectors. The G2 vector was constructed and packaged in the amphotropic packaging line, PA317, in our laboratory as described previously. G2 contains the human GC cDNA driven by the Moloney Murine Leukemia Virus (MMLV) long terminal repeat (LTR) from the N2 vector backbone. The LN vector, in which the MMLV LTR drives the expression of the neomycin resistance gene, was constructed and packaged in PA317 producer fibroblasts in A. Dusty Miller’s laboratory (Fred Hutchinson Cancer Center, Seattle, WA).

BMT. C57/B6 male donor mice (Charles River Laboratories, Wilmington, MA) were treated with 150 mg/kg 5-fluorouracil (Solvay) intraperitoneally 2 days before BM harvest. BM was flushed from the femoral and tibial bone marrows with 0.5 mg/mL proteinase K (GIBCO-BRL) at 56°C for 1 hour; DNA was extracted with phenol/chloroform. For immunohistochemistry, a portion of each tissue was snap-frozen in OCT (VWR) in liquid nitrogen and stored at –80°C.

Quantitative polymerase chain reaction (PCR). A quantitative PCR assay was established using a PA317 clone, PNT-1, containing a single copy of the human GC cDNA to generate a standard curve. DNA from this cell line was diluted with murine DNA such that the total template input was maintained at 100 ng per reaction. Each experiment contained a duplicate set of dilutions of PNT-1 DNA from 100%, 50%, 20%, 10%, 5%, 2%, 1%, 0.5%, 0.2%, 0.1%. Sample DNA at 100 ng per reaction was then subjected to the PCR in 50 μL PCR buffer (1X buffer II, Perkin Elmer-Cetus, Norwalk, CT) 1.25 mmol/L MgCl₂, 20 nmol/L L-gulutamine, and 100 μmol/L pencillin-streptomycin. After washing in BBMM, BM cells were resuspended in BBMM supplemented with 200 U/mL human interleukin-6 (IL-6; Genentech, South San Francisco, CA), 200 U/mL murine IL-3 (Biosource International, Camarillo, CA), 50 ng/mL murine mast cell growth factor (gift of Immunex, Seattle, WA), and 10 U/mL murine leukemia inhibitory factor (GIBCO-BRL). Cells were cultured for 48 hours at 1 × 10⁶/mL before cocultivation with vector packaging fibroblasts.

Packaging cells were irradiated at 40 Gy and plated at 2 × 10⁶ cells per 100 mm² plate in BBMM with growth factors 24 hours before the addition of 8 × 10⁶ BM cells. Cocultivation was conducted for 48 hours with 4 μg/mL polybrene, after which BM cells were removed from plates by vigorous pipetting. Cells were washed in Hanks’ Balanced Salt Solution (HBSS), and resuspended in HBSS with 50 U/mL heparin immediately before injection. Female syngeneic recipient animals were irradiated at 10.5 Gy from a cobalt 60 source in split doses of 6.5 and 4 Gy separated by 24 hours. Animals were injected intravenously with 3 to 4 × 10⁶ nucleated cells transduced with either the G2 or the LN retroviral vector. Tetracycline (~100 μg/mL) was added to the drinking water of transplant recipients to control mortality from sepsis. All procedures involving animals were approved by and in accordance with the guidelines of the Animal Care Committee at Childrens Hospital Los Angeles.

Tissue processing. Animals were sacrificed at various intervals after transplantation. Immediately after lethal CO₂ inhalation, animals were perfused via the left cardiac ventricle with 20 mL warm phosphate-buffered saline containing 50 U/mL heparin; blood was collected at the time of perfusion. Tissues including the spleen, thymus, BM, liver, lung, kidney, brain, and spinal cord were removed. A portion of each was minced and digested with 0.5 mg/mL proteinase K (GIBCO-BRL) at 56°C for 1 hour; DNA was extracted with phenol/chloroform. For immunohistochemistry, a portion of each tissue was snap-frozen in OCT (VWR) in liquid nitrogen and stored at –80°C.

Immunohistochemistry. Tissues were cryosectioned at 4 μm, air dried to 48 hours, and then fixed in acetone for 15 minutes at 4°C before storage at –80°C. Human GC was detected with the 8E4 mouse monoclonal antibody (Dr. Aerts, University of Amsterdam, The Netherlands) which had been conjugated with biotin. Incubation with the primary antibody was followed with streptavidin-alkaline phosphatase (Biogenex, San Ramon, CA). Staining was developed with either the Vector Blue III substrate for alkaline phosphatase (Vector Laboratories, Burlingame, CA), or the New Fuschin substrate (Biogenex). In some cases, after development of the 8E4 reaction, tissues were subsequently incubated with rat MoAbs to CD18, CD11b (McC-1), GR-1, or Thy-1 (PharMingen, San Diego, CA). These antibodies were followed by peroxidase-conjugated mouse-antirat IgG which was developed with 3-amino-9-ethyl-carbazole (AEC; Sigma Chemical Co, St Louis, MO) (in combination with Vector Blue), or nickel-enhanced 3,3’-diamino benzidine (DAB; Vector) (in combination with New Fuschin). Macrophages were identified with the Mac-1 antibody, except in the brain where a combination of Mac-1 and anti-CD18 antibodies was used to identify microglia.

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OH). A standard curve was generated from the PNT-1 dilutions and sample values were calculated by linear regression. Human GC-specific primers and the H3A oligomer have been previously described.\textsuperscript{31} Neo gene primers were described by Carter et al.\textsuperscript{32}

RESULTS

Genetic marking of donor BM with human GC. Donor BM was genetically marked with an MMLV-based retroviral vector bearing the human GC cDNA as a reporter. Genetic marking was accomplished by in vitro stimulation of donor marrow cells with growth factors to induce proliferation which is required for transduction with the retroviral genome, followed by cocultivation of BM with vector packaging cells. After transplantation into lethally irradiated recipient mice, retrovirus-marked donor cells were detected by a PCR assay specific for the human GC cDNA or by immunohistochemical methods to detect production of human GC protein in cells. In all experiments, a control group of animals was injected with donor BM infected with the LN vector, which contains only the neomycin resistance gene. In each experiment, the efficiency of gene transduction was evaluated by PCR amplification of GC (or neo in the case of the LN vector) in 12- to 14-day colony forming units in the spleen (CFU-S). CFU-S derived from BM transduced by both the LN and G2 retroviral vectors were 90% to 100% vector positive in all experiments (data not shown) as has been previously published by our laboratory.\textsuperscript{31} Remaining animals from each of three separate experiments were maintained long-term and then sacrificed at various intervals and perfused with saline before removal of tissues.

Quantitative PCR. To assess the extent and progression of engraftment of retrovirus-marked donor cells in tissues, a quantitative PCR assay was established. In each experiment, a standard curve was generated using DNA prepared from a cell line containing a single integrated copy of the neo and GC genes (PNT-I). PNT-I DNA was titrated from 100% to 0.1% (1 to 0.001 copies per genome) by dilution with negative control DNA. Electrophoresed products were hybridized and exposures (Fig 1A) were quantitated by densitometric analysis. The titration was performed in duplicate and used to generate a standard curve (Fig 1B). Each sample was tested in two separate experiments, and copy number values for each sample were calculated by linear regression.

Sequential analysis of tissues indicates progressive repopulation with donor cells. Twenty-two animals from three separate experiments were harvested at 2 weeks (n = 4), 1 to 2 months (n = 6), 3 to 4 months (n = 9), and 6 to 8 months (n = 4) posttransplant and subjected to the PCR to determine vector copy number (Fig 2). Tissues from each mouse are represented by a patterned bar corresponding to the animal number. The mean copy number per cell was also calculated for each tissue from all animals from a given time point and is shown in Fig 3. At 2 weeks posttransplant, donor cells were predominantly localized to the CFU-S (Fig 2A), with a mean copy number of 0.8 copies per cell (Fig 3A). This indicated that each focus probably contained cells derived from a precursor transduced with a single copy of the vector. Contamination of the foci with neighboring endogenous, nontransduced cells probably accounts for the failure to achieve a measurement of 1 copy per cell. A substantial signal was also observed in the lung and liver at this time; however, this may be attributable to irradiated vector-producing fibroblasts which lodge in these tissues after intravenous injection. In support of this hypothesis, the signal decreases by 1 month, and then increases again by 3 to 4 months as donor cells begin to repopulate the tissue macrophages (Fig 3, E and F).

Tissues from mice sacrificed 1 to 2 months posttransplant showed significant engraftment of donor cells in the spleen, thymus, blood, and BM (Fig 2B and Fig 3, A-D), with a very small number of cells detected in remaining tissues. By 3 to 4 months after transplant, nonhematopoietic tissues including the liver, lung, kidney, brain, and spinal cord began to contain increasing numbers of vector-positive cells (Fig
Lineage of splenic GC+ cells was identified by double-label immunohistochemistry. Vector-marked cells of donor origin were identified in tissues on the basis of antibody staining with the antihuman GC MoAb, 8E4. In the early stages of engraftment (less than 1 month), 8E4+ cells were restricted primarily to the spleen and BM. Day 12 to 14 CFU-S were brightly stained with 8E4 (Fig 4A). CFU-S from control animals transplanted with cells transduced with the LN vector were not stained with the 8E4 antibody (data not shown). At 1 to 2 months after transplant, 8E4+ cells in the spleen remained clustered in groups of 20 to several hundred cells (Fig 4B). At this time, in concert with the restoration of the normal architecture of the spleen, the restriction of 8E4+ cells to the red pulp became more pronounced. By 3 to 4 months posttransplant, 8E4+ cells had redistributed to form a relatively random pattern of donor cells throughout the red pulp of the spleen (Fig 4C). The lineage of these cells was identified by double immunohistochemical staining for macrophages (Mac-1) (Fig 5A), granulocytes (anti-GR-1) (Fig 5B), and T cells (anti-Thy-1) (Fig 5C). 8E4+ cells were predominantly of the macrophage and granulocyte lineages. Mac-1+ cells in the spleen were 30% repopulated with 8E4+ cells at 6 months after transplant. In 3/19 animals, 8E4 staining was observed in the white pulp of the spleen (Fig 5D). These cells were identified as germinal center cells by double staining with peanut agglutinin-fluorescein isothiocyanate (data not shown). The lack of expression of vector-derived sequences in splenic T cells was accompanied by the absence of staining in thymocytes, despite the presence of vector-marked cells indicated by PCR analysis. Occasional 8E4+ cells were observed in the thymus (Fig 6A); these cells were identified as macrophages by Mac-1 staining (data not shown).

Macrophages expressing human GC were detected in visceral tissues by immunohistochemical staining. Most nonhematopoietic visceral tissues did not contain detectable donor cells by PCR or by immunohistochemical staining in the first 2 months of engraftment. However, by 3 to 4 months
posttransplant, $8E4^+$ cells began to appear in the lung (Fig 6B) and liver (Fig 6C). These cells were identified as resident tissue macrophages by double staining with the Mac-1 antibody. $8E4^+$ cells per square millimeter were counted in several sections of tissue from several animals at 6 to 8 months after BMT, and the percentage of total macrophages expressing GC was tabulated by counting double-stained cells (Table 1). These data indicate that liver macrophages were 10% replaced by cells expressing GC by 6 months, whereas 70% reconstitution with $8E4^+$ cells was achieved by 6 months in the pulmonary macrophages.

Perivascular cells and small numbers of parenchymal microglia are repopulated by $8E4^+$ donor cells. Extremely rare $8E4^+$ donor cells were visible in the brain as early as 2 weeks posttransplant; however, the number of these cells was below the detectable limit for PCR (Fig 3 G). GC$^+$ cells were not detected in the CNS by PCR until 1 to 2 months (Fig 3, G and H), with a copy number of 0.003 per cell. The mean copy number increased nearly tenfold to 0.02 by 3 to 4 months posttransplant. This level was then maintained in animals measured at 6 months after BMT. There was a wide degree of variability in the number of $8E4^+$ cells observed in individual 4 µm coronal sections ranging from one to several hundred. Nonetheless, cell counts of $8E4^+$ cells in brains revealed an increase over time with a mean of $1 \pm 0.5$ cell/mm$^2$ observed at 2 weeks posttransplant, $9 \pm 3$ cells/mm$^2$ at 1 to 2 months, $14 \pm 5$ cells/mm$^2$ at 3 to 4 months, and $15 \pm 6$ cells/mm$^2$ at 6 to 8 months (Table 2). Although the reported density of microglia varies in different areas of the CNS, an average number of $70$ cells/mm$^2$ has been measured. This value was used to calculate the percent replacement with $8E4^+$ cells at each time point (Table 2). By 6 months after transplant, an average of 21% of the total microglia were replaced with $8E4^+$ donor cells.

The majority (~90%) of $8E4^+$ donor cells appeared to be perivascular cells based on their morphology or obvious association with the vasculature (Fig 7, A, C, G and H). Perivascular microglia, which reside outside of the basement membrane of the blood vessel, but remain in close proximity to it, were also observed (Fig 7, B and F; Fig 8, A and F). In general, the cryopreservation and acetone fixation of these tissues that was required to visualize GC by immunohistochemistry yielded poor preservation of the delicate microglial processes. However, a number of $8E4^+$ cells (~5% to 10%) appeared to be parenchymal microglia which occasionally displayed apparent ramified morphology (Fig 8, B, D, E, and F). Parenchymal $8E4^+$ microglia were usually distributed as isolated cells, while perivascular cells were frequently observed as clusters of $8E4^+$ cells stretching along the vasculature for some distance (Fig 7C). Double-label immunohistochemistry revealed that nearly all $8E4^+$ cells were also positive for Mac-1/CD-18, indicating a common
MACROPHAGE AND MICROGLIA REPOPULATION

Fig 4. Immunohistochemical staining of the spleen from animals transplanted with BM cells marked with human GC. Expression of the vector-derived GC protein was detected by the 8E4 MoAb (blue). (A) An 8E4+ CFU 12 days after BMT (animal no. 1 from Fig 2A), hematoxylin counterstain. (B) GC expression in clusters of cells in the red pulp 1 month after BMT (animal no. 5 from Fig 2B). CD18+ cells are stained red. (C) Cells expressing GC (8E4+) distribute throughout the red pulp 3.5 months after BMT (animal no. 12 from Fig 2C). Gran-1+ cells are stained red. W, white pulp; R, red pulp.

Fig 5. Immunophenotyping of donor cells expressing GC (8E4+) with double-label immunohistochemistry. Splenic 8E4+ cells (blue, A-C; red, D) are usually found in the red pulp (R), and were double-labeled with (A) Mac-1 (red), and (B) GR-1 (red). (C) Anti-Thy-1 staining (red) showed that the majority of T cells did not express GC. (D) In some animals, 8E4+ cells were also found in the white pulp (W).

macrophage lineage (Fig 7F; Fig 8, A, D, E, and G). 8E4+ donor cells were detected in numerous regions of the brain, including the caudate nucleus (Fig 7A), the hippocampal formation (Fig 7, B and E), the choroid plexus (Fig 7D), the cortex (Fig 8, C and E), cerebellum (Fig 8, B and F), and the brain stem (Fig 7, C and F; Fig 8, A, D, and G).

DISCUSSION

We examined the temporal pattern of macrophage engraftment in 22 murine recipients after BMT with cells transduced with the human GC cDNA. The studies described here were initiated to evaluate the ability of donor-derived cells to replace the host mononuclear phagocytes in relevant tissues after BMT. Further, a distinct goal of this work was to show the migration of donor macrophages to the CNS and their potential for differentiation to microglia to determine whether transduced patient BM cells could repopulate the CNS in various disease states. Finally, we strove to document the long-term persistence of expression of vector sequences in the macrophages of all tissues in situ in transplanted animals to demonstrate the sustained effects of such genetic manipulation.

Our studies used PCR to track the tempo of vector-marked donor cell migration into relevant tissues, while immunohistochemical staining of these same tissues provided information regarding vector gene expression and the cellular distribution and phenotype of these cells. We observed the rapid reconstitution of hematopoietic tissues including the spleen, thymus, blood, and BM with donor cells. Vector copy number in these tissues (except in the thymus) rose in the interval from 3 to 4 months to 6 to 8 months after BMT. Macrophages in the lung, liver, and CNS were also repopulated by GC+ donor cells, but at a much slower rate with slight increases seen in the first 1 to 2 months, and more dramatic increases by 3 to 4 months posttransplant.

Table 1. Mean Percentage of Macrophages Expressing Human GC 6 to 8 Months After BMT

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<th>Tissue</th>
<th>% 8E4+ Macrophages*</th>
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<tr>
<td>Spleen</td>
<td>32 ± 11</td>
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<tr>
<td>Liver</td>
<td>10 ± 4</td>
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<tr>
<td>Lung</td>
<td>67 ± 19</td>
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<tr>
<td>Brain</td>
<td>21 ± 6</td>
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Sections from animals sacrificed at 6 to 8 months after BMT were double labeled with the 8E4 and Mac-1 antibodies.

* Percent of total macrophages that were also 8E4+ was calculated by dividing the number of 8E4+/Mac-1+ cells by the total number of Mac-1+ cells. Percentages are mean ± SD.

Phenotype of 8E4+ cells. The product of the retroviral vector, human GC, was predominantly expressed in macrophages and granulocytes, but was not expressed in thymocytes or mature T cells, nor B cells (except in 15% of recipients, where expression was found in splenic germinal centers). This restriction may be caused by differentiation-associated transcriptional regulation of the retroviral LTR. Alternatively, it may reflect cell-specific differences in ability to accumulate detectable levels of GC protein in the lysosomes.

8E4+ cells in the lung, spleen, and liver. Immunohistochemical analysis of these tissues suggested that the extent of macrophage repopulation was profoundly affected by the tissue type. Although this observation was supported by the PCR, it is important to note that expression of the GC protein may also be differentially regulated in various types of mature macrophages. In general, macrophage repopulation is slow due to the radiosensitivity and longevity of tissue macrophages.

Splenic macrophages were also replaced by 8E4+ cells in significant numbers, with 30% repopulation by 6 months. We note that the frequency of cells expressing GC appeared to decrease in older (4 to 8 months post-BMT) animals (data not shown), despite a sustained vector copy number. A loss in GC immunoreactivity may correlate with the emigration of 8E4+ macrophage precursors from the spleen in the first 1 to 2 months after transplant, and their subsequent replacement with vector-transduced cells that do not express GC. Retrovirus-transduced committed progenitor cells, which generate the CFU-S and then differentiated mature hematopoietic cells at early stages after transplantation, express genes from the Moloney LTR at a significantly higher level than cells derived from more primitive transduced populations. Thus, as the progeny of stem cells begin to contrib-
ute to the macrophage population several months after transplant, a rise in vector DNA+ cells would be observed, but would not be accompanied by an increase in 8E4+ cells.

The highest level of donor macrophage repopulation was observed in the pulmonary macrophages. These cells were the earliest to be replaced in the nonhematopoietic tissues, and showed a progressive increase in numbers over time as shown by quantitative PCR analyses. Alveolar macrophages represent approximately one third the cellular content of the lung; thus, our mean measurement of 0.25 copies per genome indicates that by 3 to 4 months, the lung macrophages were nearly completely repopulated by donor cells. This was confirmed by immunohistochemical staining which showed that 70% of alveolar macrophages were expressing GC at 6 months. BMT studies in humans have similarly reported a lifespan of 3 months for lung macrophages. 13

The repopulation of liver macrophages, or Kupffer cells, with vector GC+ cells was comparable with that observed in the lung. A mean copy number per cell of 0.1 was observed by 6 to 8 months after transplant, indicating that a majority of Kupffer cells (which represent approximately 15% of liver cells) had been replaced by vector-marked cells. Immunohistochemical staining of liver indicated that only 10% of Mac-1+ Kupffer cells were replaced by 3 to 4 months after transplant. Thus, expression of GC from the retroviral vector may be down-regulated in liver macrophages. The failure to observe higher numbers of 8E4+ cells might alternatively be attributable to the high background observed in immunohistochemical staining of the liver which prohibited the longer substrate development times used in other tissues.

8E4+ cells in the CNS. BMT studies performed in animal models for lysosomal storage diseases 36-41 have addressed the efficacy of BMT for the reversal of the neurologic effects of the disease. These studies noted a reduction in substrate storage in the brains of treated animals, and in some cases the influx of donor cells into areas of damage. Hickey and Kimura 26 and colleagues 42 showed that CNS perivascular cells, as well as a very small population of parenchymal resident microglia are derived from incoming donor monocytes in MHC congenic animals. Using retroviral marking of donor marrow, we have identified regions throughout the brain in which numerous donor-derived cells were shown to populate perivascular cells and some parenchymal microglia.

PCR analyses of the brain and spinal cord indicated the presence of vector-positive donor cells in the CNS by 1 to 2 months after transplant at a frequency of 0.003 copies per cell (1 in 330 cells). The frequency of vector-positive cells increased nearly tenfold by 3 to 4 months, and was sustained at this level up to 6 to 8 months after BMT. Microglia were identified by double immunohistochemical techniques combining antibodies to murine CD18 and Mac-1 with the 8E4 antihuman GC monoclonal. 8E4+ microglia were observed as early as 2 weeks after transplant and comprised 1% of the total number of microglia; these cells became significantly more abundant by 1 month after transplant (13% of total microglia), and leveled off between 3 and 4 months and 6 and 8 months, at 20% of the total microglia. Continued high-level expression of human GC was seen at least 8 months. The dramatic increase in donor-derived cells predicted by the PCR (tenfold at 3 to 4 months) was not mirrored by similar increases in 8E4+ cells, which increased less than twofold between 1 and 2 and 3 and 4 months. This may reflect the effect of transcriptional silencing from the viral LTR promoter in the progeny of transduced stem cells.

The relative contributions of in situ mitoses versus monocyte immigration to the turnover of resident microglia is a controversial issue. In situ mitoses have been observed in ramified cells, but the frequency of these events is extraordinarily low. 20 Some additional evidence suggests that the monocyte-derived perivascular cells break through the basement membrane and enter the brain parenchyma in response to lesions and disease state. 34, 43 Thus, it is probably a dual contribution of both of these processes that generate the resident microglia. 20 The data presented here suggest that blood monocytes enter the CNS as perivascular cells, and transform to parenchymal ramified microglia at a slower rate. The majority of 8E4+ cells observed were perivascular cells, although perivascular microglia and parenchymal ramified microglia represented about 5% to 10% of the total 8E4+ cells in the CNS. An alternative explanation is that differentiation into ramified cells is accompanied by transcriptional silencing of the G2 retroviral LTR, thus resulting in the failure to observe larger numbers of donor-derived cells of this type.

Concluding remarks. These data have important implications for the treatment of disease in hematopoietic cells using gene therapy. In addition to the role of CNS microglia in lysosomal storage diseases, these cells harbor the human immunodeficiency virus type 1 (HIV-1) in patients infected with monocyteotropic strains of HIV, and may play a role in acquired immune deficiency syndrome (AIDS) encephalitis. 44, 45 The mode of entry of HIV into the CNS microglia is unknown, but has been postulated to occur via migration of infected monocytes. 45 Therefore, if monocyte immigration is a normal mechanism of microglia turnover, genetic manipulation of BM cells offers a mechanism to express therapeutic genes in an environment that has generally been considered inaccessible.

In this study, we reported a novel quantitative analysis of the engraftment of retrovirus-transduced donor cells in visceral tissues and the CNS after allogeneic BMT. From this work, it has been shown that significant numbers of

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<th>Table 2. Density of Cells Expressing GC in the Brain With Time After BMT</th>
<th>Time After BMT</th>
<th>8E4+ Cells/mm²</th>
<th>% of Total Microglia*</th>
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<tr>
<td>2 wks (n = 3)</td>
<td>1 ± 0.5</td>
<td>14</td>
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<tr>
<td>1-2 mos (n = 6)</td>
<td>9 ± 3</td>
<td>13</td>
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<tr>
<td>3-4 mos (n = 9)</td>
<td>14 ± 5</td>
<td>20</td>
<td></td>
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<tr>
<td>6-8 mos (n = 4)</td>
<td>15 ± 6</td>
<td>21</td>
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8E4+ cells were counted in 2 to 4 4-μm coronal brain sections from each animal and divided by the total area of the section. A mean ± SD was calculated for each group of animals from a given time point.

* Based on an average of 70 cells per mm² according to Lawson et al. 26
Fig 7. Donor-derived perivascular cells expressing human GC (8E4⁺) in the brain at various times after BMT were identified by immunohistochemistry. Perivascular cells were identified by elongate morphology and association with blood vessels (small arrows). Some photographs also contain perivascular microglia (hollow arrows), which were identified by morphology and proximity to blood vessels, as well as parenchymal microglial cells (arrowheads), which were identified based on morphology and the absence of nearby blood vessels. (A) 8E4⁺ cells (blue) were found in the caudate nucleus 1 month after BMT (animal no. 4). (B) An 8E4⁺ (red) perivascular microglial cell and an 8E4⁺ parenchymal microglial cell were found in the hippocampal formation 2 months after BMT (animal no. 8). (C) 8E4⁺ cells (red) were found in the brainstem 6 months after BMT (animal no. 11). (D) 8E4⁺ cells (red) were found in the choroid plexus 1 month after BMT (animal no. 5). (E) 8E4⁺ cells were found in the thalamus 2 months after BMT (animal no. 9). (F) Double labeling of 8E4⁺ cells (red) was seen with Mac-1/CD18 (brown) in the brainstem 6 months after BMT. Note the stained process of the perivascular microglial cell (hollow arrow) (animal no. 20). (A-E) were counterstained with hematoxylin.
cells are capable of infiltrating the CNS and expressing a retrovirally engineered gene product in situ for long periods of time. These data predict that the amelioration of gene-transduced cells.

ACKNOWLEDGMENT

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Fig 8. Donor-derived parenchymal microglia expressing human GC (BE4+) were found in the brain at various times after BMT. Parenchymal cells were identified based on morphology and the absence of nearby blood vessels (arrowheads). Some photographs also contain perivascular microglia, which were identified by morphology and proximity to blood vessels (hollow arrow), as well as perivascular cells which were identified by elongate morphology and association with blood vessels (small arrow). (A) Double-label immunohistochemistry of BE4+ cells (red) and Mac-1/CD18+ cells (brown) found in the brainstem 6 months after BMT. The hollow arrow points to a double-labeled perivascular microglial cell in close proximity to a blood vessel (V). The solid arrow points to a double-labeled parenchymal microglial cell (animal no. 20). (B) BE4+ (red) parenchymal microglial cells were found in the hippocampal formation 2 months after BMT (animal no. 9). (C) BE4+ parenchymal microglial cells (blue) were found in the cerebral cortex 4 months after BMT (animal no. 11). (D) Double-label immunohistochemistry of BE4+ (red) Mac-1/CD18+ (brown) perivascular cells found in the brainstem 6 months after BMT. A putative double-labeled parenchymal cell with radial processes is indicated by a solid arrow (animal no. 20). (E) Double-labeling of BE4+ (blue) Mac-1/CD18+ (red) parenchymal microglial cells found in the cerebral cortex 7 months after BMT (animal no. 10). (F) A single BE4+ parenchymal microglial cell (blue) was found in the gray matter of the cerebellum 7 months after BMT (animal no. 10). (G) Double labeling (BE4+ (red) Mac-1/CD18+ (brown)) of a parenchymal microglial cell was found in the brainstem 6 months after BMT (animal no. 21). (A, B, and F) were counterstained with hematoxylin.
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