allow the protein to enter the export pathway.

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10. Mature maltose-binding protein was purified from *E. coli* strain MC4100 by affinity chromatography by using cross-linked amylose as the resin [T. Ferenci and U. Klotz, *FEBS Lett.* **94**, 213 (1978)]. The maltose was removed from the pure protein by extensive dialysis against 10 m*M* tris-HCl, *p*H 7.6. To obtain precursor maltose-binding protein we grew *E. coli* strain MM18 [K. Ito, P. J. Bassford, Jr., J. Beckwith, *Cell* **24**, 707 (1981)] in M9 minimal salts medium supplemented with 0.4% glycerol. When the density reached 1.5×10^8 cells per milliliter, maltose was added (0.2%) and growth was continued for 3.5 hours. Cells were harvested by low-speed centrifugation, converted to spheroplasts [B. Witholt *et al., Anal. Biochem.* **74**, 160 (1976)], and the periplasm was removed. After the spheroplasts were disrupted by sonication, the suspension was centrifuged at 20,000*g* for 20 minutes. The pellet was suspended in 10 m*M* Hepes, *p*H 7.6, 3% Triton X-100, and 5 m*M* EDTA. After incubation for 15 minutes on ice, the suspension was centrifuged at 20,000*g* for 10 minutes. The pellet was solubilized in 10 m*M* Hepes, *p*H 7.6, 4*M* guanidinium hydrochloride (GuHCl) and incubated at 22°C for 15 minutes. The GuHCl was diluted to 0.15*M* and the solution was incubated at 22°C for 30 minutes. After the solution was centrifuged at 16,000*g* for 10 minutes, the supernatant was removed and concentrated to a minimal volume in an Amicon ultrafiltration cell with a PM10 membrane. The precursor was purified from this sample by affinity chromatography as described above for the mature species. Maltose was removed from the precursor maltose-binding protein by repeated dilution and volume reduction in an Amicon ultrafiltration cell (PM10 membrane).

    To facilitate purification of mature ribose-binding protein and the precursor form, the appropriate alleles of *rbsB* were cloned under the lambda P_L promoter on the plasmid vector pPLC2833. The procedures for cloning and purification are described in detail elsewhere [S. Park, thesis, Washington State University, Pullman (1987)]. The mature protein was purified from the periplasmic fraction obtained by osmotic shock of cells and the precursor was obtained from lysed spheroplasts. The purification procedure involved two sequential steps of ion-exchange column chromatography (DEAE-Sephadex and CM-Sephadex) followed by chromatofocusing (Pharmacia, Mono P). We were unable to obtain quantities of wild-type precursor sufficient for these studies. Therefore we used a precursor with a mutated but functional leader sequence [see (*11*)].
11. A. Iida *et al.*, *EMBO J.* **4**, 1875 (1985). The precursor used in this study carries a functional leader that differs from the wild type at two positions. The substitution of a leucyl residue at position −17 by a prolyl residue caused a defect in export, and the second substitution of a seryl residue at −15 by a phenylalanyl residue restored export. The amount of mature protein in the periplasm of the revertant strain is equal to that in the wild type; however, the export of precursor in the revertant strain is slightly slower than normal.
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Donor-Derived Cells in the Central Nervous System of Twitcher Mice After Bone Marrow Transplantation

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The twitcher mouse is an animal model of galactosylceramidase deficiency, comparable to Krabbe's disease, a lysosomal storage disease in humans. As in most lysosomal storage diseases, neurological deterioration is a prominent feature of the disease in these mice. Transplantation of enzymatically normal congenic bone marrow was earlier found to result in prolonged survival and increased levels of galactosylceramidase in the visceral organs of twitcher mice. It is now reported that bone marrow transplantation results in increased galactosylceramidase levels in the central nervous system (CNS). Concomitantly, the levels of psychosine, a highly toxic lipid that progressively accumulates in the CNS of untreated twitcher mice, stabilized at much lower levels in the CNS of treated twitcher mice. Histologically, a gradual disappearance of globoid cells, the histological hallmark of Krabbe's disease, and the appearance of foamy macrophages capable of metabolizing the storage product were seen in the CNS. By immunohistochemical labeling it was demonstrated that these foamy macrophages were of donor origin. The infiltration of enzymatically competent, donor-derived macrophages was accompanied by extensive remyelination in the CNS. It is concluded that after bone marrow transplantation, donor-derived macrophages infiltrate the affected brain tissue and are capable of inducing a partial reversal of the enzyme deficiency.

SINCE THE FIRST BONE MARROW transplantation (BMT) for lysosomal storage disease by Hobbs *et al.* (*1*), many studies on the effect of BMT in patients and animal models with lysosomal storage diseases have been reported (*2–7*). After BMT, the enzymatically normal donor-derived blood cells and tissue macrophages served as a continuous source of enzyme and led to increased enzyme levels in leukocytes, plasma, and various visceral organs (*2–7*). The effects of BMT on the neurological symptoms, which are very prominent in many lysosomal storage diseases, vary with the type of disease. For instance, in patients with metachromatic leukodystrophy, BMT led to improved psychomotor development in comparison with siblings receiving no treatment (*2, 8*), whereas in patients with Sanfilippo's disease, BMT did not lead to beneficial effects (*9*). Increased enzyme levels after BMT were reported in the central nervous system (CNS) of a dog suffering from fucosidosis, but without alleviation of the neurological symptoms (*5*). In β-glucuronidase–deficient mice and in a feline model of arylsulfatase-B

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deficiency (Maroteaux-Lamy syndrome), enzyme activity in the CNS did not increase after BMT (*6, 7*). This has been explained on the basis of the blood-brain barrier, which prevents circulating enzyme from entering brain tissue. The presence of donor-derived cells, which contribute to the increased enzyme activity in lung and liver tissue, for example (*10*), has not been reported in brain tissue so far.

The twitcher mouse, which was used in our studies, is a neurologically affected mutant with a genetically determined deficiency of the lysosomal enzyme galactosylceramidase (*11*), a deficiency that results in a disease pattern similar to human Krabbe's disease (globoid cell leukodystrophy) (*12*). Briefly, the mice, which appear normal at birth, develop a severe tremor and hind-leg paralysis within 3 to 4 weeks after birth and die at an age of approximately 5 weeks (*12, 13*). At autopsy, severe demyelination, disappearance of oligodendrocytes, and infiltration of pathological globoid cells with characteristic galactosylceramide inclusions were found (*12*). Concomitant with the appearance of neurological symptoms, there was a progressive accumulation of psychosine in the brains and peripheral nervous systems of untreated twitcher mice (*14*). It has been hypothesized that accumulation of this toxic storage product leads to degeneration of oligodendrocytes and myelin sheaths (*14*).

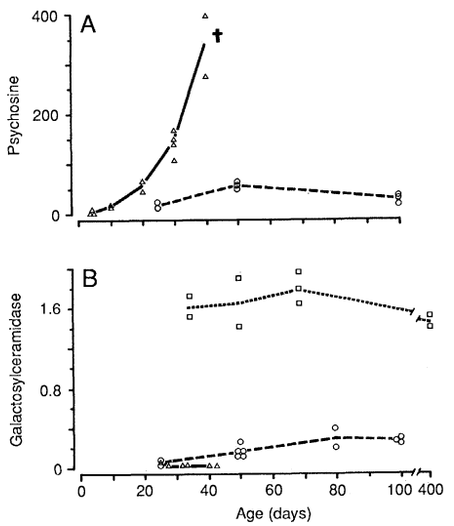

Fig. 1. (**A**) Psychosine (picomoles per milligram of protein) and (**B**) galactosylceramidase (nanomoles per hour per milligram of protein) in the CNS of (○) twitcher mice treated with BMT, (△) untreated twitcher mice, and (□) untreated C57BL-6J control mice. Levels in mice receiving congenic transplants and allogeneic transplants did not differ significantly and were pooled. Galactosylceramidase activity was determined as described (*13*). The activities in the CNS of 50-, 80-, and 100-day-old treated twitcher mice were significantly ($P < 0.01$; Student's t test) increased as compared to untreated twitcher mice. Levels in the CNS of 50-day-old treated twitcher mice were still significantly less ($P < 0.01$) than those of 100-day-old twitcher mice receiving transplants. Galactosylceramidase activity in brains of 25-day-old twitcher mice that received transplants were not significantly different from those of untreated twitcher mice. Psychosine levels were determined by the method of Kobayashi (*28*). Values for twitcher mice treated at ages 25, 50, and 100 days were significantly lower ($P < 0.01$) than those for untreated twitcher mice at ages 30 to 42 days. Values of untreated C57BL-6J controls were 1.2 ± 0.6 pmol per milligram of protein (mean ± standard deviation).

We studied the effect of BMT in homozygous twitcher mice and enzymatically normal control littermates. Asymptomatic twitcher mice were identified when they were 4 to 5 days old by galactosylceramidase levels in clipped tails (*15*), and BMT was performed when they were 9 to 12 days old. The twitcher mice had been mated to C57BL-6J mice for approximately 12 generations. Mice, aged 8 to 10 weeks, of the C57BL-6J strain [H-2K^b allele of the major histocompatibility complex (MHC)] were used as bone marrow donor for the congenic transplantations, and mice of the C3H-Rij strain (H-2K^k allele of the MHC complex) were used as donors for the allogeneic transplantations. BMT was performed 1 day after lethal total body irradiation (9.0 Gy of ^{137}Cs gamma rays). Bone marrow cells (3×10^7 to 5×10^7) were injected intraperitoneally. Antibiotics or other supportive care was not given. BMT resulted in prolonged survival and improvement of locomotor ability (*13*). Similar results were reported earlier by Yeager *et al.* (*16*). Chimerism in mice receiving congenic transplants was confirmed by galactosylceramidase activity in bone marrow and spleen tissue, which rose to donor levels within 2 weeks after BMT and remained at donor level during the observation period of 100 days (*13*). Differences in survival or in galactosylceramidase activity between mice receiving allogeneic and those receiving cogenic transplants were not observed. In the mice that received allogeneic bone marrow, chimerism was confirmed by the presence of the H-2K^k antigen on bone marrow cells, as determined by fluorescence-activated cell sorter (FACS) analysis (*17, 18*). To study the effect of BMT in the CNS, we killed the transplanted mice at various times after BMT and perfused them with saline to remove contaminating blood. Galactosylceramidase activity gradually increased in the CNS of twitcher mice after BMT from 2% to approximately 16% of the activity found in the CNS of untreated controls. Psychosine levels stabilized at a level that was approximately 10% of the levels found in untreated twitcher mice shortly before death (Fig. 1). These data are comparable to those recently reported by Ichioka *et al.* (*19*).

On light microscopy, characteristic globoid cells were abundantly present in brains of untreated, 30- to 40-day-old twitcher mice and in brains of 25-day-old twitcher mice that received a bone marrow transplant. In contrast, the macrophages in the CNS of twitcher mice killed more than 40 days after BMT were heterogeneous. In 40- to 80-day-old twitcher mice that received BMT, globoid cells were still present, but in addition a gradually increasing number of foamy macrophages had appeared (Fig. 2A). In the CNS of twitcher mice that were killed more than 100 days after BMT, globoid cells were extremely rare. In contrast, numerous foamy macrophages were present in the brains of these mice. The foamy macrophages were predominantly found in the white matter of the cerebellum and spinal cord and not in the cerebrum, the region of the CNS that is relatively unaffected in twitcher mice. This localization of foamy macrophages compares well with that of the brain lesions and globoid cell infiltration in untreated twitcher mice (*12*), suggesting that the infiltration of foamy macrophages occurred as a response to tissue damage. Such macrophages are also regularly observed in the brains of patients with various demyelinating diseases (*20*). The foamy macrophages contained neutral lipids as demonstrated by oil red O staining (*21*). Cells stained with oil red O were absent in the brains of untreated twitcher mice, normal C57BL-6J control mice, and C57BL-6J mice that received transplants. On electron microscopy, the foamy macrophages did not show inclusions characteristic for galactosylceramide, suggesting that they were capable of metabolizing this substrate. However, the oligodendrocytes and the Schwann cells in the peripheral nerves still contained some inclusions. The white matter in the cerebellum and spinal cord of mice with transplants contained many thinly myelinated axons suggestive of ongoing remyelination (Fig. 2B), in contrast to that of untreated twitcher mice and twitcher mice killed less than 50 days after BMT, which showed many myelin residues indicative of severe demyelination (*22*).

The absence of galactosylceramide inclusions in the foamy macrophages indicated galactosylceramidase activity in these cells and suggested that they were derived from the enzymatically competent bone marrow graft. This was confirmed by immunohistochemical analysis of frozen sections of brain tissue from twitcher mice that were killed 70 to 100 days after transplantation with allogeneic C3H (H-2K^k) bone marrow (*18*). The H-2K^k–positive cells of donor

origin were found predominantly in the white matter of the cerebellum (Fig. 3) but were rarely observed in the white matter of the cerebrum of treated twitcher mice. This distribution pattern of H-2K^k–positive cells is similar to that of the foamy macrophages. Simultaneous staining with monoclonal antibody to H-2K^k and oil red O identified the oil red O–stained foamy macrophages as H-2K^k–positive and therefore donor-derived cells. Control specimens of brains from untreated twitcher mice and from twitcher mice that received transplants from congenic mice did not react with antibody to H-2K^k.

These results provide definite immunohistological evidence for the presence of donor-derived cells in the CNS after bone marrow transplantation. Although donor antigen has been reported previously in material of murine brains subjected to SDS-electrophoresis after transplantation, the location of the donor antigen in brain tissue was not described (*23*). Others have suggested that the reactive macrophages that infiltrate brain tissue after brain damage are of hematogenous origin, in contrast to nonreactive microglial cells (*24*), but these studies were not performed after allogeneic BMT, so firm conclusions on the bone marrow origin of these cells cannot be drawn.

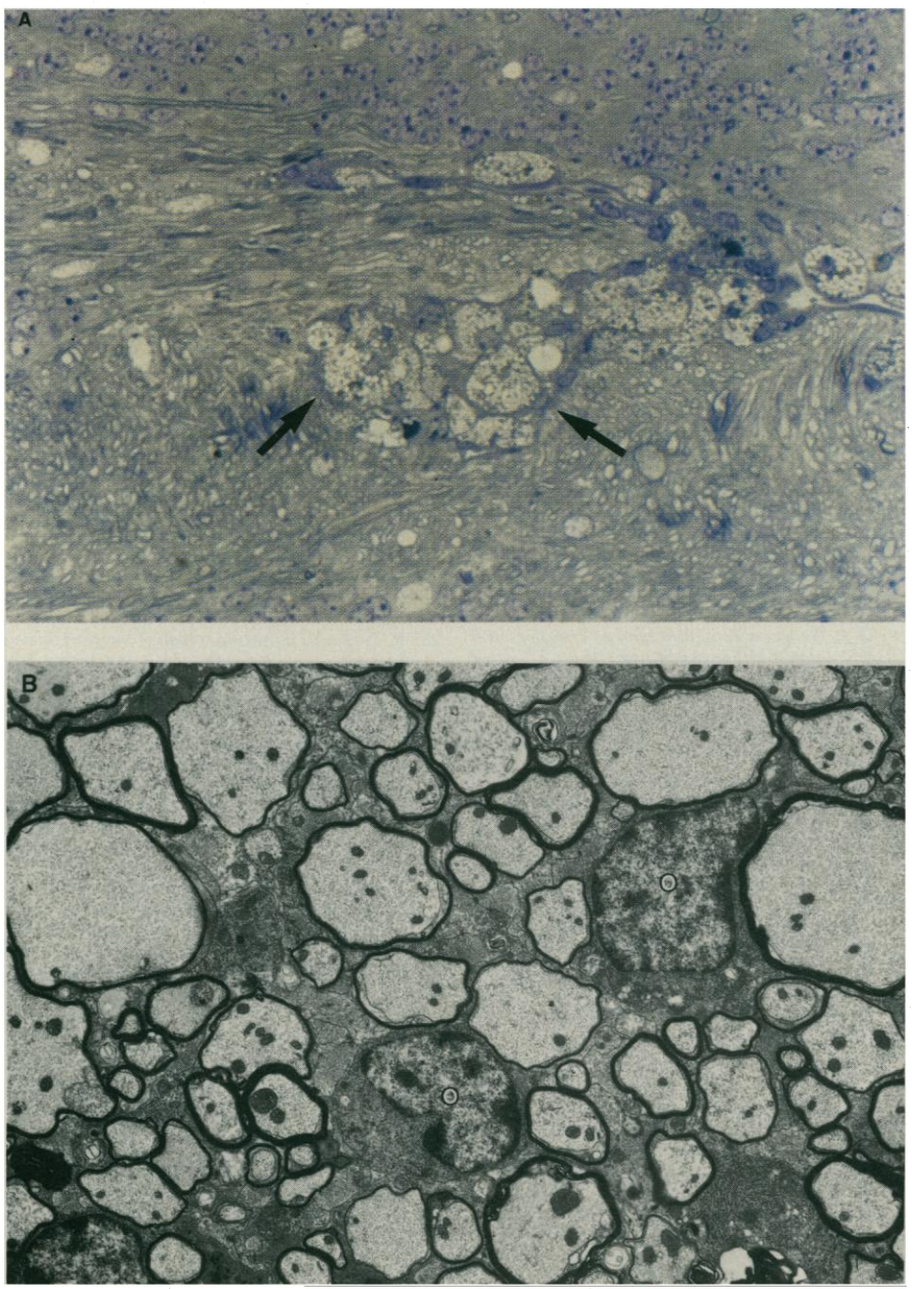

Fig. 2. (**A**) White matter of the cerebellum of a 109-day-old twitcher mouse given allogeneic bone marrow transplant at age 9 days. A cluster of foamy macrophages is indicated with arrows. Section (1 μm) is stained with toluidine blue (×250). (**B**) Electromicrograph of the anterior spinal column of the same animal, showing well-preserved oligodendrocytes (O) and many thinly myelinated axons. There is no evidence for active demyelination in this twitcher mouse 100 days after BMT. Sections were double-stained with uranyl acetate and lead citrate and examined with a Zeiss 10A electron microscope (*22*) (×14,400).

Our data indicate that the infiltration of donor-derived foamy macrophages explains the increased galactosylceramidase activity in the CNS of twitcher mice that received BMT. In 25-day-old treated twitcher mice, infiltration of foamy macrophages was not found, and no increase in galactosylceramidase activity occurred. In contrast, increasing numbers of donor-derived foamy macrophages were evident in brains of 50-, 80-, and 100-day-old twitcher mice that received BMT. In the brains of these mice, galactosylceramidase activity also gradually increased from 2% to between 14 and 16% of donor levels. The increased enzyme activity was accompanied by stabilization of psychosine levels in the CNS of treated twitcher mice at levels that were far below those found in brains of untreated twitcher mice at ages 30 to 40 days.

The alternative explanation for the increased galactosylceramidase levels found in brains of twitcher mice that received BMT is entrance of circulating enzyme into brain tissue. This however is less likely because the level of circulating galactosylceramidase in plasma of the twitcher mice given transplants is very low (*13*), and generally lysosomal enzymes do not cross the blood-brain barrier (*25*), which is intact in twitcher mice (*26*).

Our result also showed that bone marrow transplantation leads to improved myelination in the brain tissue of an animal with lysosomal enzyme deficiency. Yeager *et al.* (*16*), using the same animal model, reported prolonged survival and improvement of the histological lesions in the peripheral nervous system, but their studies done with light microscopy did not reveal improvement in the CNS.

So far, all therapeutic regimens for the treatment of lysosomal storage diseases with CNS manifestations, whether they involved purified exogenous enzyme, organ transplantation, or cellular transplantation, failed to ameliorate the neurological problems as a result of the inaccessibility of the CNS for circulating cells or enzyme (*25*). Our data demonstrate that infiltration of donor-derived cells as a response to tissue damage in the CNS can result in a decrease of the toxic storage product and an improvement of myelination in transplanted twitcher mice. It is our opinion that a similar infiltration of macrophages (*27*) can account for the bene-

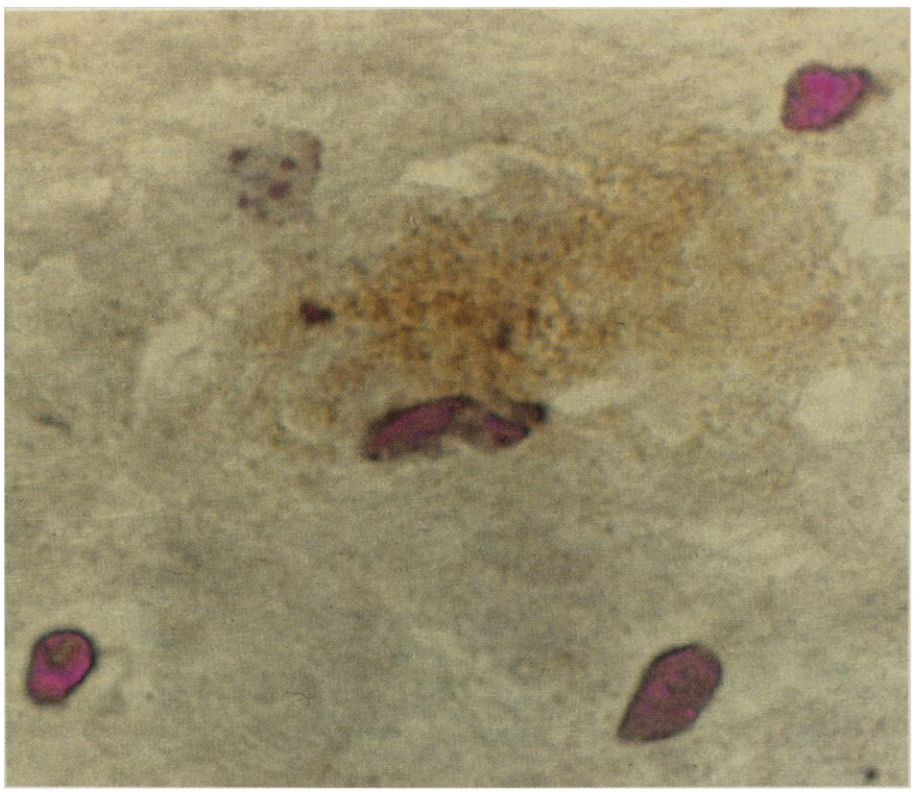

Fig. 3. H-2K^k–positive cell in white matter of the cerebellum of a 108-day-old twitcher mouse given allogeneic bone marrow transplant at age 9 days. The H-2K^k–positive cell is located in the same area as the foamy macrophages indicated in Fig. 2A. Immunohistochemistry was performed as described (*18*). Counterstaining was done with hematoxylin (×1000).

ficial effects in patients receiving bone marrow transplantation for the treatment of metachromatic leukodystrophy (*2*).

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18. Frozen sections (6 to 8 μm) of spleen or CNS were, after fixation in 100% acetone for 15 minutes, incubated with biotinylated monoclonal antibody to H-2K^k (Becton Dickinson, Mountain View, CA), diluted 1:100 in phosphate-buffered saline (PBS) with 5% fetal calf serum, *p*H 7.4, for 2 to 3 hours at room temperature. Subsequently, after three washings in PBS, the sections were incubated for 1 hour at room temperature with streptavidine-peroxidase diluted 1:100 in PBS. After washing, the final reaction product was produced by immersing the sections in a solution of 0.01% hydrogen peroxide and 0.05% diaminobenzidine tetrahydrochloride in 0.1*M* tris buffer, *p*H 7.4, for 10 minutes at room temperature. Chimerism of bone marrow cells was confirmed after incubation of these cells with the biotinylated monoclonal antibody to H-2K^k, with avidin–fluorescein isothiocynate (Becton Dickinson) as a fluorescent marker and by the presence of this antigen on frozen sections of spleen tissue (*18*).
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