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# DEVELOPMENT OF HEMOPOIETIC STEM CELL GENE THERAPY FOR NIEMANN PICK DISEASE USING THE ACID SPHINGOMYELINASE KNOCK-OUT MOUSE MODEL

by

SHAI S. ERLICH

A dissertation submitted to the Graduate Faculty in Biomedical Sciences in partial fulfillment of the requirements for the degree of Doctor of Philosophy, The City University of New York

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10/22/98
Date

Robert J. Desnick, Ph.D., M.D.
Chairman of Examining Committee

10/22/98
Date

Terry A. Krulwich, Ph.D.
Executive Officer

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George Atweh, M.D.

Hans Snoeck, Ph.D.

Adriana Haimovitz-Friedman, Ph.D.

Examining Committee

THE CITY UNIVERSITY OF NEW YORK

# Abstract


DEVELOPMENT OF HEMOPOIETIC STEM CELL GENE THERAPY FOR NIEMANN PICK DISEASE USING THE ACID SPHINGOMYELINASE KNOCK-OUT MOUSE MODEL

By

Shai S. Erlich

Advisor: Edward H. Schuchman, Ph.D.

Type A Niemann Pick disease (NPD) is a severe, neurodegenerative storage disorder that generally leads to death before 3 years of age. Type B NPD is a milder form of the disease with little or no neurologic involvement, but progressive hepatosplenomegaly and pulmonary inflammation which generally leads to death in the second or third decades. Both diseases are allelic variants and are caused by mutations in the acid sphingomyelinase (ASM) gene, resulting in the deficient activity of the lysosomal enzyme, ASM.

Although untested, current theories suggest that both forms of NPD are caused by the accumulation of sphingomyelin in the cells of affected patients. The accumulated

lipid causes impairment of cell functions and ultimately leads to cell death, followed by inflammatory reactions in the involved organs. This hypothesis is based on clinical and pathological observations in NPD patients. However, no large scale experiments to test this hypothesis have been conducted, since the disease is relatively rare in the general population, and it has been difficult to obtain a large enough group of patient materials for complete analysis.

In 1991, our laboratory isolated the full-length cDNA and genomic sequences encoding human ASM. Shortly thereafter, the full-length mouse sequences were isolated and used to construct an ASM knockout mouse model (ASMKO mice). The NPD mouse model has no residual ASM activity, and presents with a clinical and pathological phenotype that is intermediate between Types A and B NPD. We intend to vigorously investigate the disease progression in the mouse model, including complete characterization at the molecular, pathological, and clinical levels. We anticipate that these experiments will shed important light on the specific mechanisms associated with the development of the NPD phenotype.

Currently, no therapy is available for Types A and B NPD. Reports describing procedures such as bone marrow transplantation (BMT), liver transplantation, and amniotic membrane transplantation have been scarce, and their results ambiguous. These approaches are also complicated by problems such as the lack of suitable donors and graft rejection. Because of these problems, gene therapy has been suggested as an alternative

to the standard transplantation treatments. The ASMKO mouse model provides an excellent system to test such therapies in statistically significant numbers of animals.

The overall goal of my research project was to develop hemopoietic stem cell gene therapy for Types A and B NPD using the ASM knockout mouse (ASMKO) as a model system. The specific hypotheses underlying this research were:

A. That a primary cellular site of pathology in NPD is the bone marrow-derived macrophage (NPD foam cells).

B. That BMT in ASMKO mice will produce normal macrophages and other bone marrow-derived cells, there by altering the non-neurologic disease course, and perhaps altering the neurologic disease as well.

C. That retroviral vectors which include the normal human ASM cDNA can be used to transduce pluripotent murine hemopoietic stem cells from the ASMKO mouse.

D. That retrovirally transduced ASMKO hemopoietic stem cells will express normal ASM and give rise to enzyme-expressing blood cells.

E. That methods can be developed to rapidly and efficiently isolate transduced ASMKO hemopoietic stem cells on the basis of ASM expression alone, without the use of a marker gene.

F. That transplantation of the retrovirally transduced, metabolically corrected hemopoietic stem cells in affected mice will alter the disease course.

In order to test these hypotheses and achieve our goal, several specific aims have been established:

**Characterization of the ASMKO mouse phenotype.** A PCR screening test was developed to allow a quick determination of the mouse genotype. This was essential for experiments involving large numbers of newborn animals. Homozygous knock-out and heterozygous mice were analyzed and compared to normal animals on three levels: (A) Biochemical analysis: quantification of enzyme activity and substrate accumulation in various tissues and cell types: (B) Pathological analysis: light and electron microscopy of the involved organs, and measurements of plasma lipoproteins; (C) Clinical analysis: monitoring growth and behavioral patterns. The baseline data obtained from these studies was used later to determine the success of the gene therapy and BMT experiments.

**BMT into ASMKO mice.** In order to provide the rationale for the development of hemopoietic stem cell gene therapy for NPD, we first transplanted normal bone marrow cells into newborn and adult ASMKO mice. These studies were conducted in two stages. In the first stage a larger number of animals were transplanted using varying conditions (i.e., donor cell number, radiation dose). Successful engraftment was determined by WBC enzyme activity reconstitution and Y-chromosome *in situ*

hybridization of transplanted cells (see methods) and a safe and effective protocol for BMT was established. In the second stage, a group of animals was transplanted using this protocol. Successfully engrafted animals were analyzed biochemically, pathologically and clinically, and it was found that the extra-neuropathic disease phenotype can be treated successfully by BMT.

**Transplantation of genetically corrected hemopoietic cells into ASMKO mice.** Affected mice were also transplanted with retrovirally transduced bone marrow cells. Higher ASM activities could be achieved in these animals compared to the normal allogenic BMT results. Animals showing high engraftment will be further studied biochemically, pathologically, and clinically. The results will be compared to the established baselines in order to determine the therapeutic success of this protocol.

**Development of a fluorescence-based selection system for the isolation of retrovirally transduced, metabolically-corrected hemopoietic stem cells.** A selection method was developed which allows us to select the metabolically corrected hemopoietic cells following retrovirus transduction of the ASM gene into the ASMKO mouse bone marrow. The method utilizes an artificial, fluorescent Sphingomyelin substrate that cannot be metabolized in the absence of the enzyme. By combining this labeling method with antibody selection (based on cell surface markers), we were able to select the corrected cells from various bone marrow sub-populations, including the corrected stem and progenitor cell populations. These cells can now be used for *in vivo* transplantation of

ASMKO mice. In addition, this selection system has proved to be useful as an assay for the *in vitro* assessment of gene transfer into hemopoietic stem cells.

This work is dedicated

in loving memory

of my father,

Menachem Erlich

# Acknowledgements

Many thanks to:

To Dr. Silvia Miranda, my partner on this project, for a true and exceptional collaboration, for all the help and support, the useful discussions (on science and other matters) and for putting up with my mood swings.

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To Prof. David Bishop and Prof. George Atweh for serving on my advisory committee.

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Chapter 5

# List of Abbreviations

| | |
|---|---|
| ASM | Acid Sphingomyelinase |
| NPD | Niemann-Pick Disease |
| LSD | Lysosomal Storage Disease (s) |
| BMT | Bone Marrow Transplantation |
| HSCGT | Hemopoietic Stem Cell Gene Therapy |
| WBC | White Blood Cell (s) |
| CNS | Central Nervous System |

# Introduction

## 1. Background

The first case of Niemann-Pick Disease (NPD) was described in 1914 by the German pediatrician Albert Niemann (Niemann 1914), but it was only in 1927 that Ludwick Pick identified it as a unique clinical identity, distinct from Gaucher disease (Pick 1927). Based primarily on clinical observations, in the 1960s NPD patients were classified into five distinct clinical subtypes (Crocker 1961). Type A NPD presents as a severe neuropathic disorder that usually causes death by 3 years of age. In contrast. Type B NPD patients suffer from various degrees of hepatosplenomegaly and pulmonary infiltration (Brady 1966; Brady et al. 1966), but the neurologic system is not affected and patients can survive into late adolescence or adulthood. Types C, D and E patients present with various degrees of neurologic damage and usually do not survive past the first or second decades of life (Pentchev et al. 1985; Vanier et al. 1991; Vanier et al. 1991; Vanier et al. 1991). Because of the clinical and pathological similarities among the different types of NPD, they where originally thought to be caused by different mutant alleles of the same gene (allelic heterogeneity). We now know, however, that the different clinical types are caused by mutations in at least two different genes. Types A and B NPD are due to mutations in the gene encoding acid sphingomyelinase (ASM; sphingomyelin phosphodiesterase; E.C. 3.1.4.12) (Schuchman and Desnick 1995), while Types C, D and E are caused by defects in a gene(s) involved in cholesterol transport (Pentchev, Comly et al. 1985).

## 2. The Lysosome

*Lysosomal Enzymes-General Properties.* The lysosomes are intracellular organelles which contain at least 40 different hydrolytic enzymes that are used to digest macromolecules. These enzymes are all acid hydrolyses and can only function in the low pH (~5.0) of the lysosomal lumen. This pH is maintained by an ATP-dependant V-type $H^+$ pump (Mellman et al. 1986).

Lysosomal enzymes are synthesized by ribosomes located on the rough endoplasmic reticulum (ER) (Kornfeld 1987). Following their translocation into the lumen of the ER, these enzymes are glycosylated at specific asparagine residues and transferred to the Golgi apparatus where subsequent trimming of the oligosaccharides occurs (Walter et al. 1984; Kornfeld and Kornfeld 1985). In addition, mannose-6-phosphate (M6P) is added to the oligosaccharides of most lysosomal hydrolases in a two step process (von Figura and Hasilik 1986). First, UDP-N-acetylglucosamine-1-phosphotransferase catalyzes the transfer of phospho-N-acetylglucosamine to specific mannose residues, yielding N-acetylglucosamine-1-phospho-6-mannose. This enzyme recognizes its target by binding to a specific conformation dependant signal patch which only occurs in lysosomal hydrolases (von Figura and Hasilik 1986). In the second step, the N-acetylglucosamine is removed by N-acetylglucosamine-1-phosphodiester-α-N-acetylglucosaminidase to reveal the M6P moieties. The M6P groups are recognized in the *trans* Golgi network by the transmembrane M6P receptor proteins, which bind the lysosomal hydrolases and help package them into specific transport vesicles (Hille-Rehfeld 1995). These vesicles leave the Golgi apparatus and fuse with late endosomes.

Because of the low pH in these endosomes the lysosomal hydrolases are released from the M6P receptor proteins and proceed to the lumen where they begin to digest endocytosed material delivered from early endosomes (von Figura and Hasilik 1986). The unbound M6P receptor molecules are assembled into transport vesicles that bud from the late endosome and return to the membrane of the *trans* Golgi network for reuse. The lysosomal hydrolases remain in the late endosome and continue on their journey to the mature lysosome where further post-translational modifications, may occur.

*Lysosomal Storage Diseases*. The concept of lysosomal storage diseases (LSD) was introduced in 1965 by Hers, who investigated the relationship between Pompe disease and the deficient activity of α-glucosidase (Hers 1965). Based on his observations, Hers defined an inborn lysosomal storage disease as one in which 1) a single lysosomal enzyme is deficient, and 2) abnormal deposits of substrate lie within the lysosomes, resulting in an increase of the size and number of these organelles. Later this definition was broadened to include some rare cases, such as I-cell disease, in which a defect in a single protein can result in the almost complete absence of activity of all of the lysosomal enzymes.

Based on the nature of the accumulated substance, LSD have been classified into several groups which include: sphingolipidoses (Fig. 1), glycoproteinoses, mucolipidoses, mucopolysaccharidoses, and others. However, in many cases the deleterious effects of the accumulated substrate and the exact pathogenesis of the disease remain unknown. To date, many naturally occurring animal models of these diseases have been discovered (Desnick et al. 1982; Patterson et al. 1982; Bedell et al. 1997). More recently, gene targeting techniques have been used to produce knock-out mouse models of LSD. These

include α-L-iduronidase-deficient mice for MPS Type I (Clarke et al. 1997); glycosylasparaginase-deficient mice for aspartylglucosaminuria (Kaartinen et al. 1996), arylsulfatase A-deficient mice for metachromatic leukodystrophy (Hess et al. 1996), arylsulfatase B-deficient mice for MPS VI (Evers et al. 1996), protective protein/cathepsin A-deficient mice for galactosialidosis (Zhou et al. 1995), acid-sphingomyelinase-deficient mice for Niemann-Pick disease, Types A and B (Horinouchi et al. 1995; Otterbach and Stoffel 1995), β-hexosaminidase A deficient mice for Tay-Sachs disease (Yamanaka et al. 1994), β-hexosaminidase B deficient mice for Sandhoff disease (Phaneuf et al. 1996), and glucocerebrosidase-deficient mice for Gaucher disease (Tybulewicz et al. 1992). These animal models can be used to investigate the processes underlying LSD progression and should prove to be valuable tools in the development of treatment options for these diseases.

### 3. Acid Sphingomyelinase

*Biochemical Properties of Acid Sphingomyelinase.* Acid Sphingomyelinase (sphingomyelin phosphodiesterase, E.C. 3.1.4.12; ASM) is the lysosomal enzyme which hydrolyzes sphingomyelin into ceramide and phosphocholine (Kanfer et al. 1966). In 1991 Quintern et al. (Quintern and Sandhoff 1991) purified the enzyme from human urine and spleen and found it to have a molecular weight ranging from ~67 to 75 kDa based on SDS PAGE analysis (Quintern and Sandhoff 1991). Biosynthetic studies in cultured skin fibroblasts have suggested that ASM is synthesized as a 75 kDa precursor that is processed into several smaller molecular weight species (e.g., 72, 67 and 57 kDa)

within lysosomes (Ferlinz et al. 1994; Hurwitz et al. 1994). Small amounts of the precursor form also were secreted into the culture medium, along with the 57 kDa form.

In order to further investigate the biochemical and structural properties of ASM, our lab established an ASM overexpression system using Chinese hamster ovary (CHO) cells (He and Schuchman, Personal Communication). This system has permitted the production and purification of large amounts of recombinant human ASM (rhASM). Studies of this enzyme have shown that: 1) native ASM appears to exist as a multimer and is dependent on intra and/or inter molecular disulfide bonds to maintain its structural and functional integrity, 2) zinc is an essential component of the ASM active site and required for substrate binding and degradation, 3) a fatty acid moiety containing at least 6, and more optimally 12 carbons, is required to achieve efficient binding and degradation of sphingomyelin, and 4) the negatively charged phosphocholine moiety is essential for substrate binding, suggesting that after hydrolysis of sphingomyelin by ASM, the ceramide produced is released rapidly from the enzyme. In addition, the usefulness of rhASM for the development and evaluation of enzyme replacement therapy for NPD is currently being studied in our lab.

*The Sphingomyelinase Enzyme Family.* Several sphingomyelinases have been described. Lysosomal ASM, the subject of my research, is the enzyme deficient in Types A and B NPD. Our laboratory has recently discovered that the ASM gene encodes another, non-lysosomal form of ASM which is $Zn^{+2}$ activated and secreted from many cell types (Schissel et al. 1996). In addition, a $Mg^{+2}$ dependent neutral sphingomyelinase (NSM) has been described. This enzyme is encoded by a distinct gene and is located on the outer leaflet of the plasma membrane (Yedgar and Gatt 1976).

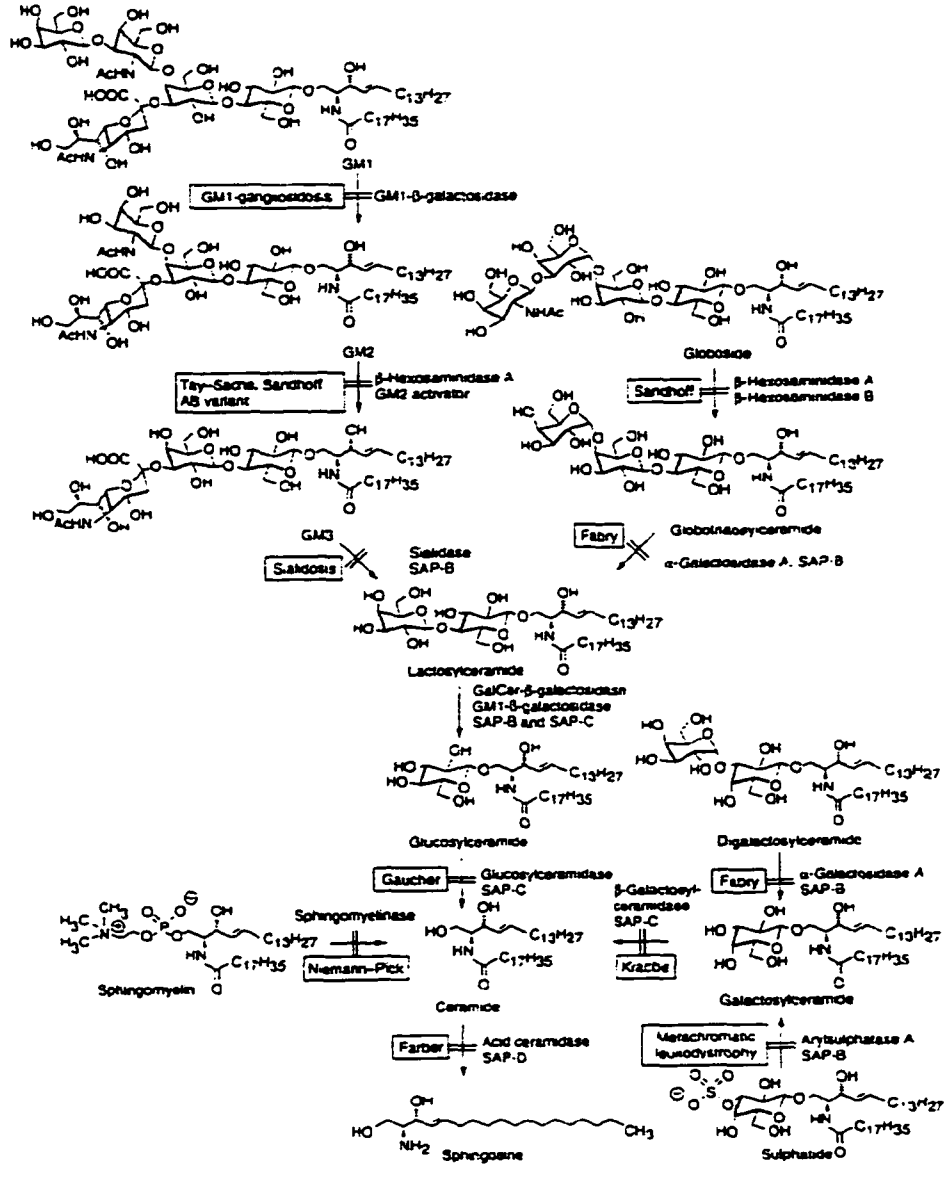


**Fig. 1. Lysosomal glycosphingolipid metabolism.** Sphingolipid activator proteins, exohydrolases and known storage diseases are shown (Sandhoff and Kolter 1996).

*The ASM Gene.* The full-length human ASM cDNA was isolated by our group in 1991 (Schuchman et al. 1991). Five cDNA species were obtained. The full-length cDNA (designated Type 1) was composed of 2347 bp with an open reading frame of 1890 bp encoding for a 629 amino acid peptide. The other cDNA species (Types 2-5) contained internal deletions of the ASM sequence and it was hypothesized that they were products of alternative splicing.

In order to investigate the alternative splicing hypothesis, the genomic sequence of ASM was isolated using the Type 1 cDNA as a probe (Schuchman et al. 1992). The ASM gene has been mapped to the short arm of chromosome 11 (region pl5.l—pl5.4) , and was found to be ~4.5 Kb in length and to contain six exons and five introns (Fig. 2). Comparing the sequence of the cDNA species to that of the genomic DNA revealed that the Types 2-5 cDNAs were indeed the result of alternative splicing. Types 1, 2 and 3 cDNAs were expressed in COS-l cells, but only Type 1 was found to encode a functional protein.

*The Murine ASM Gene.* The human cDNA was used to screen murine cDNA libraries and to isolate the full-length murine ASM cDNA. This cDNA was 2320 bp and contained a 1884 bp open reading frame encoding a 627 amino acid polypeptide (Horinouchi et al. 1993). The overall identities between the human and murine sequences were 81% at the nucleotide level and 83% at the amino acid level. Expression of the full-length murine cDNA in COS-1 cells resulted in the expression of a functional enzyme.

The murine cDNA was used to clone the genomic region of the ASM gene. The gene was found to have a similar size and structure to the human gene: it was ~4.5 kb in

length and contained 6 exons and 5 introns (Fig. 3-A). The only notable difference between the human and murine sequences was in the length of intron 2, which was longer in the human gene (1059 bp) than in the murine gene (510 bp) due to the insertion of an *Alu* repetitive element.

**4. Niemann-Pick Disease**

*Clinical Features.* The main differences between Types A and B NPD are summarized in Table 1 (Schuchman and Desnick 1995). Type A NPD is caused by severe mutations in the ASM gene that abolish almost all of the ASM activity (Levran et al. 1991; Levran et al. 1992; Levran et al. 1993). Like other lysosomal storage diseases, there is no indication of the disease during the pregnancy and affected newborns appear normal at birth. The disease usually progresses undetected until 3 to 4 month post partum, when feeding difficulties develop because of hypotonia and muscular weakness. From this point on, there is a rapid deterioration of neurologic functions (Schuchman and Desnick 1995). The child becomes weaker and hypotonic, and developmental milestones disappear. Visceral involvement includes the liver and spleen, which are mildly enlarged, and pulmonary infiltration that does not cause respiratory difficulties (Grunebaum 1976). In the late stages of the disease, affected infants lose contact with their surroundings and death usually follows by the age of 3 years (Schuchman and Desnick 1995).

**Table 1: Typical clinical features of Types A and B NPD**

| Feature | Type A | Type B |
|---|---|---|
| Age of onset / Diagnosis | Early Infancy | Childhood / Adolescence |
| Neuro degenerative course | + | - |
| Cherry-red macula | 50 % | <10 % |
| Hepatosplenomegaly | + | + |
| Marrow NPD cells | + | + |
| Pulmonary involvement | + / - | + |
| Age at death | 2 .3 years | Adolescence / Adulthood |
| Autosomal recessive inheritance | + | + |
| Ashkenazi Jewish predilection | + | + |
| Acid sphingomyelinase activity | <5% | <10% |

Unlike the uniform presentation of Type A, the Type B phenotype is more variable (Schuchman and Desnick 1995) This phenotype is caused by mutations that result in slightly higher residual ASM activities than those found in Type A NPD (Levran et al. 1991). In general, the Type B phenotype does not involve the neurologic system, although some cases with mild neurologic defects have been described (Sogawa et al. 1978; Elleder and Cihula 1983; Takada et al. 1987). The disease is usually undetected until childhood when the diagnosis is generally made following the incidental observation of hepatomegaly or splenomegaly in a routine medical examination (Schuchman and Desnick 1995). As the child grows, the abdominal distention becomes less obvious. Although rare, in severely affected individuals the hepatosplenomegaly can lead to life threatening conditions such as cirrhosis, portal hypertension, ascites, and pancytopenia (Tassoni et al. 1991). In contrast, in some mildly affected individuals hepatosplenomegaly may be undetected until adulthood (Chan et al. 1977; Dawson and Dawson 1982). The lungs are also involved in most NPD Type B patients, with alveolar infiltration which is usually present at the time of diagnosis. As the disease progresses. this infiltration causes decreased pulmonary diffusion, followed by low arterial $P0_2$ values and exertion dyspnea. In some severely affected individuals, recurrent life threatening pneumonia have been described (Lever and Ryder 1983). These conditions contribute to a shorter life expectancy in patients with Type B NPD compared to normal individuals.

*Pathology and Pathogenesis*. As noted above, Types A and B NPD are caused by the deficient activity of ASM (Brady, Kanfer et al. 1966), secondary to mutations in the

gene encoding this enzyme. As noted above, ASM is the lysosomal enzyme that catalyzes the cleavage of sphingomyelin to phosphocholine and ceramide (Fig. 1) (Kanfer, Young et al. 1966). It has therefore long been thought, although not proven, that the accumulation of sphingomyelin (SPM) in the lysosome, due to the deficient activity of ASM, causes lysosomal dysfunction which leads to cell death, and, ultimately, the disease phenotype (Schuchman and Desnick 1995). This hypothesis is supported by the presence of SPM loaded foam cells ("NPD cells"; see below) and elevated SPM levels in the involved organs. In addition to SPM, other lipids are also accumulated in the affected cells (Schuchman and Desnick 1995). For example, cholesterol is sometimes elevated to a greater extent than SPM. However, the role played by cholesterol (and these other lipids) in the pathogenesis of NPD has not been investigated.

A very common histologic finding in NPD is the "foam cell" or "Niemann-Pick cell" (Golde et al. 1975; Ludatscher et al. 1981) (Fig 4). These cells originate in the bone marrow and are present in almost all tissues (Elleder et al. 1983; Levade et al. 1985). It is of note that in organs of the reticuloendothelial system, namely the spleen and the lymph nodes, the tissue is almost completely infiltrated by the late stages of the disease (Bes et al. 1984). The pathologic involvement of various organ systems in NPD is detailed in Table 2.

*Genetics and Molecular Diagnosis*. NPD is an autosomal recessive disorder. It has a panethnic distribution, but is more prevalent in the Ashkenazi Jewish population (Schuchman and Desnick 1995). Recent studies have shown that the carrier frequency of Type A NPD in this population is ~1:70. The prevalence of Type B NPD in the

Ashkenazi Jewish population is lower then that observed for Type A, although a common Type B mutation has been discovered (Levran, Desnick et al. 1991; Vanier et al. 1993).

Mutation analysis of the ASM gene has shown that 3 mutations R496L (Levran, Desnick et al. 1991), L302P (Levran, Desnick et al. 1992), and fsP330 (Levran, Desnick et al. 1993) account for ~90% of Type A mutant alleles in the Ashkenazi Jewish population (Fig. 2). In contrast, all but one of the Type B mutations have been shown to be private mutations (Fig. 2). The exceptional Type B mutation, ΔR608, has been found to account for >80% of the mutant alleles in Type B NPD patients originating from the Magherb region (Vanier, Ferlinz et al. 1993). It has also been found in all 3 Type B Ashkenazi Jewish patients available in our cell bank (Levran, Desnick et al. 1991). The majority of the ~40 known mutations have been expressed in COS-1 cells. Generally, expression of Type A mutations resulted in little or no ASM activity, while expression of Type B mutations resulted in low, but detectable activity. Correlation of the mutation severity and localization has indicated that the amino acid sequence encoded by exon 3 is an important site for enzyme activity or stability, and may include the enzyme active site (Takahashi et al. 1992).

*Treatment.* To date, no specific therapy is available for NPD Types A and B. Sometimes splenectomy is performed for mechanical reasons and for patients suffering from hemorrhagic diathesis because of low blood platelet count.(Schuchman and Desnick 1995). There are also reports showing that the frequent episodes of pneumonia which occur in NPD Type B patients are more refractory to treatment with standard antibiotic regimens probably because of the massive infiltration of the lungs with NPD cells in these patients (Schuchman and Desnick 1995). The recent cloning of the ASM gene and

cDNA has prompted our laboratory to investigate and develop new therapeutic approaches, such as enzyme replacement therapy, allogenic bone marrow transplantation and hemopoietic stem cell gene therapy, for NPD Types A and B. The work described in this dissertation is part of these efforts.

*Animal Models*. Two naturally occurring NPD models have been described in large animals. These reports were canine (Bundza et al. 1979) and feline (Wenger et al. 1980) NPD models, which presented with similar clinical features as the human patients. However, these were isolated reports, and breeding colonies were never established for these models. In 1995 an "ASM knock-out" (ASMKO) NPD mouse was created in our laboratory using gene targeting techniques (Fig. 3-B & 3-C) (Horinouchi, Erlich et al. 1995). These mice present a biochemical, pathological and clinical phenotype similar to that of the human Types A and B NPD (Horinouchi, Erlich et al. 1995). This mouse model was used extensively in the present research.

**Table 2: Pathologic involvement of various organs in Types A and B NPD.**

| Organ System | Type A NPD | Type B NPD |
|---|---|---|
| Central Nervous System (Landrieu and Said 1984; Schuchman and Desnick 1995) | Brain mass reduction<br>Cerebellum is the main site of involvement<br>"Myelin Bodies"<br>Disappearance of Purkinje cells | Not involved |
| Spleen (Schuchman and Desnick 1995) | Mild splenomegaly<br>NPD cells present | Mild to severe splenomegaly<br>NPD cells present in large numbers |
| Lymph Nodes (Schuchman and Desnick 1995) | Variable involvement<br>Can enlarge to 3-5 times the normal size | Variable involvement<br>Can enlarge to 3-5 times the normal size |
| Bone Marrow (Schuchman and Desnick 1995) | NPD cells compose up to 3% of the BM<br>Normal formation of other blood components | NPD cells in variable number<br>Normal formation of other blood components |
| Lungs (Schuchman and Desnick 1995) | Mild NPD cell infiltration into alveoli | Severe NPD cell infiltration into alveoli<br>NPD cells found throughout lung tissue |
| Liver (Schuchman and Desnick 1995) | Enlarged 1.5-2.5 times | No size change to severe hepatomegaly |
| Kidney (Pick 1927; Schuchman and Desnick 1995) | Moderately involved<br>Some NPD cells present<br>No functional impairment | Moderately involved<br>Some NPD cells present<br>No functional impairment |
| Eyes (Robb and Kuwabara 1973; Howes et al. 1975; Libert et al. 1975; Walton et al. 1978) | Lipid deposits in the retina, choroid, cornea and lens epithelium | Not involved |

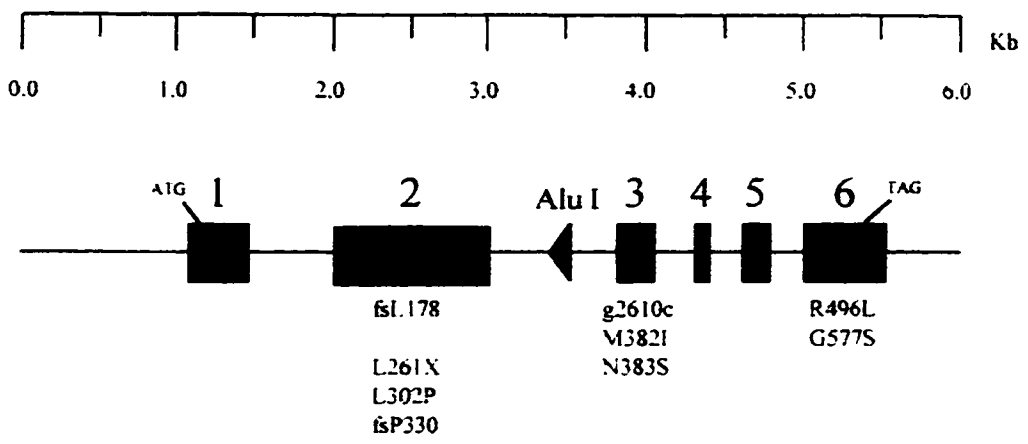


**Fig. 2. The human ASM gene and some mutations.** Type A NPD mutations are denoted in black, and Type B NPD mutation in gray.

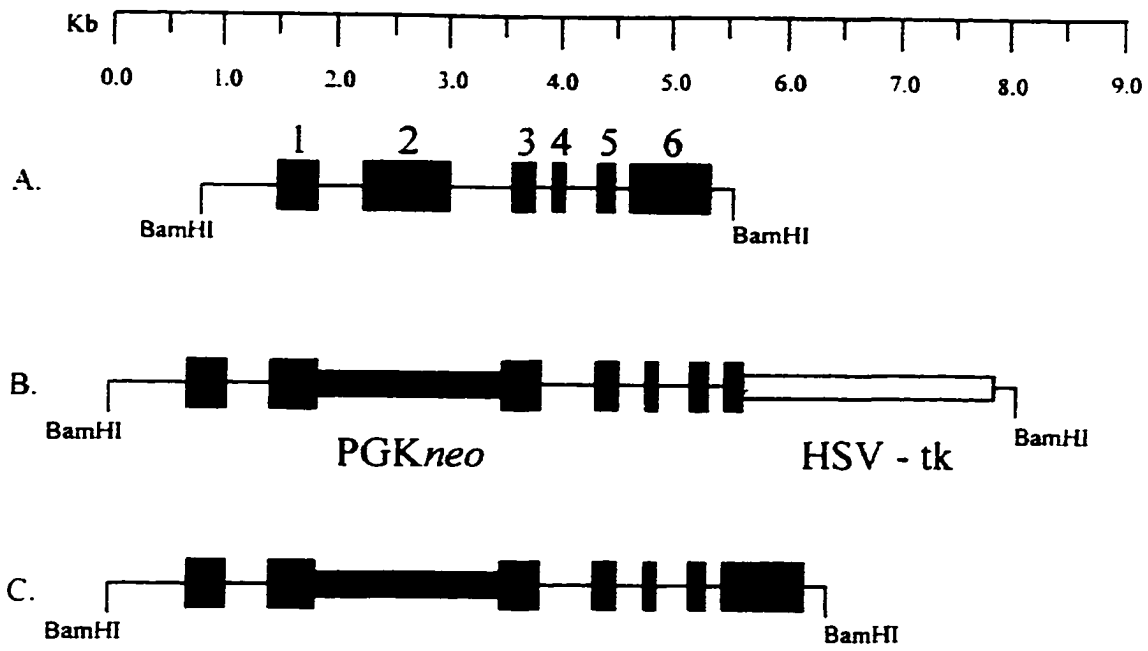


**Fig. 3. ASMKO mouse construction.** A, The normal mouse ASM gene consisting of six exons spanning ~4.5 Kb. B, The ASM replacement vector. C, The disrupted ASM gene.

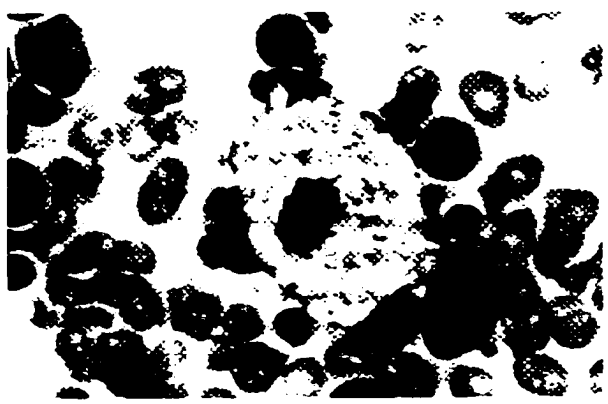

**Fig. 4. NPD foam cell.** Foam cell in a bone marrow aspirate from patient with NPD (Schuchman and Desnick 1995).

**5. Therapy for Inborn Errors of Metabolism-General Concepts.**

With the advent of molecular biology techniques and their application to human genetics, a large number of genes involved in the "inborn errors of metabolism" have been isolated. Much research has been done to characterize the mutations leading to these disorders, and for many of them, prenatal diagnosis is being routinely offered to families at-risk. On the other hand, advances in offering treatment to patients have been painfully slow. Several conservative treatment strategies, such as dietary restriction, pharmacological elimination of substrates, and cofactor supplementation have been tried usually with mixed results (Valle 1991). In recent years, however, several new therapeutic strategies have been developed which may have a greater impact on disease progression and in some cases may even prove to be completely curative. The following is a short review of these techniques.

*Enzyme Replacement Therapy (ERT).* ERT was first suggested by deDuve in 1964 as a new therapeutic strategy for LSD, based on the hypothesis that exogenous enzymes which would be infused into the blood stream of patients would be taken up by the affected cells and delivered to the site of substrate accumulation. This theory was later confirmed by numerous *in vitro* cross correction studied involving the co-culture of normal and affected cells (Hug et al. 1973; Kihara et al. 1973; Kresse and von Figura 1974; Sifers et al. 1983; Eto et al. 1987). These studies have shown that enzyme secreted to the media by normal cells could be endocytosed by the affected cells and reduce the amount of accumulated substrate. These *in vitro* studies were followed by *in vivo*

experiments (Rietra et al. 1974; Desnick et al. 1976; Desnick and Grabowski 1981) which were usually unsuccessful because of problems such as immunological responses, short circulation time of the enzyme and the lack of consistent catabolic effect (Rietra, Bergh et al. 1974; Desnick, Thorpe et al. 1976; Desnick and Grabowski 1981). Another major problem was the inability to produce large quantities of the enzyme prior to the advent of recombinant DNA techniques. The failure of these trials prompted the establishment of a consensus list of the following requisites that must be met before human ERT experiments could continue: 1) The injected enzyme must be available in large quantities, 2) The enzyme must be sterile and non-immunogenic, 3) Methods should be developed to prolong the circulation time of the enzyme as much as possible, 4) animal models of the disease should be used before attempting the therapy on human subjects and, 5) monitoring protocols must be established in order to evaluate the effectiveness of ERT in treating LSD (Rietra, Bergh et al. 1974; Desnick, Thorpe et al. 1976; Desnick and Grabowski 1981). Advances in the fields of genetics and molecular medicine have addressed most of this issues. However despite many efforts the problems relating to prolonging the retention of the injected enzyme in the circulation (Belchetz et al. 1977; Fiddler 1980; Desnick and Grabowski 1981; Wieder and Davis 1983; Coppola et al. 1994) and immunological issues (Richards et al. 1993; Yogalingam et al. 1996; Brooks et al. 1997) have not as of yet been satisfactorily solved. In addition, the cost of this form of therapy is enormous since the enzyme has to be repeatedly administered throughout the life of the patient. Nevertheless a few clinical trials of ERT are currently under way (Barton et al. 1991; Ponce et al. 1997), and several products are already commercially available (e.g., Ceredase for Gaucher disease).

Recently, the usefulness of rhASM for the development and evaluation of enzyme replacement therapy for NPD was studied in our lab (He and Schuchman, Personal Communication). Cell culture experiments revealed that the enzyme was rapidly taken up by NPD skin fibroblasts, and that this uptake could be partially inhibited by the addition of M6P to the culture media. The recombinant enzyme also was intravenously injected into ASMKO mice, and its clearance from the circulation was monitored. Within 5 min after the injection virtually all of the activity was absent from the plasma and most of the injected activity could be recovered in the liver, followed by small amounts in the spleen, heart and kidney. Since the Kupffer cells of the liver (and to a lesser degree the hepatocytes), are an important site of pathology in NPD, these results suggest that enzyme replacement may be a useful approach for the treatment of patients who do not have major central nervous system involvement (i.e., Type B NPD).

*Allogenic Bone Marrow Transplantation.* In order to avoid the problems associated with ERT it was suggested that allogenic bone marrow transplantation (BMT) could be used as an alternative therapy for LSD (Hobbs et al. 1981). The principal underlying this approach is that the normal transplanted marrow will produce normal blood cells, which express the normal gene products and can correct the metabolic disorders (Kaye 1995). Thus, the normal bone marrow tissue serves as a constant and natural source of enzyme. To test this approach several BMT experiments were carried out in animal models of LSD and human patients (Haskins 1996), where it was found that different diseases responded very differently to this treatment . Moreover, it was also found that the improvement (or lack of improvement) seen in diseases treated by BMT

appeared to be consistent in animals and humans. For example, following BMT, corneal clearing did not appear to occur in cats and children with mucopolysaccharidosis VI, but did in dogs and children with mucopolysaccharidosis I (Haskins 1996). In addition, it was observed that BMT has the largest therapeutic effect on LSD when performed as early as possible. Generally, BMT appears to be effective in storage diseases that primarily affect systemic organs, such as non-neuropathic Gaucher disease (Ringden et al. 1988; Hoogerbrugge et al. 1995). In mucopolysaccharidosis I and II , BMT appears to stabilize the disease, but skeletal lesions are relatively unaffected by transplantation (Hoogerbrugge, Brouwer et al. 1995). It remains controversial whether storage diseases involving the central nervous system can be treated effectively with this method, since the effects of BMT on neurological symptoms of these various diseases varies wildly (Kaye 1995).

Although for some diseases allogenic BMT may prove curative there are still several practical problems associated with it. The main problem is the lack of fully compatible sibling donors in most families. Other problems include complications due to lethal total body irradiation, immuno-suppression, infections, and graft vs. host disease (GVHD), which are often associated with this procedure. Hemopoietic stem cell gene therapy (HSCGT) provides a valuable alternative to BMT (Anderson and Fletcher 1980). Using this approach, which is discussed in detail bellow, bone marrow cells are removed from the patient and the hemopoietic stem cells are isolated or enriched. These cells are genetically corrected by inserting the normal gene via a vector (viral or other). The corrected cells are then returned to the patient to give rise to metabolically corrected hemopoietic lineages. Since this approach involves only autologous BMT, the risks of

rejection and GVHD are minimized. Also, the problem of needing normal donors is eliminated.

*Gene therapy.* A new approach for the treatment of genetic diseases was suggested by Anderson et al. in 1980 (Anderson and Fletcher 1980). This approach, known as "gene therapy", relied on the introduction of the normal gene into an affected cell to correct the biochemical defect, thereby altering the disease course. The brief review below will summarize some of the advances and limitations of gene therapy and describe some of the strategies taken by the different groups involved in this field.

*Germ Line vs. Somatic Cell Gene Therapy.* Normal genes can be introduced into the germ line or into somatic cells. In germ line gene therapy, the normal gene is introduced into the one cell embryo or the germ cells of the parents so that all of the cells of the affected individuals will be corrected, including the germ line. Furthermore, the gene will be transmitted in a Mendelian fashion to the offspring of the treated individual, and the disease should ultimately disappear from the family lineage. However, because of the ethical problems involved in this approach, such as the genetic manipulation of embryos, as well as its effect on the population gene pool, it has been prohibited in human studies. Germ line gene therapy has been conducted in mouse models of genetic diseases, however, and has demonstrated the feasibility of the gene therapy approach (Costantini et al. 1986).

Somatic cell gene therapy refers to the introduction of the normal gene into somatic cells, without affecting the germ line. This approach is based on the assumption that if enough cells can be genetically corrected, or "transduced", they will produce

sufficient quantities of gene products to correct the metabolic defect. Somatic cell gene delivery can be achieved by either *ex vivo* or *in vivo* approaches. Using the *ex vivo* method, the target cells are taken out of the patient's body, and the normal gene is transferred into them *in vitro*. The cells are then returned to the patient immediately, or following a selection step to isolate transduced cells. The *ex vivo* approach has been used to successfully transfer cDNAs into a variety of cells types, including hemopoietic cells (Eglitis et al. 1985; Hock and Miller 1986; Kantoff et al. 1986; Kwok et al. 1986; Williams et al. 1986; Szilvassy et al. 1989), fibroblasts (Selden et al. 1987; Sorge et al. 1987; Miyanohara et al. 1988; St. Louis and Verma 1988; Palmer et al. 1991), (Ledley et al. 1986; Wolff et al. 1987; Wilson et al. 1988; Anderson et al. 1989), endothelial cells (Zwiebel et al. 1989), keratinocytes (Morgan et al. 1987; Fenjves et al. 1989), etc. The advantage of this technique is that efficient transduction of the target cells can be achieved. The main disadvantage is that in some diseases the target cells cannot be removed from the patient easily (as in diseases affecting the whole body, the brain, the muscular system, etc.). For these diseases, the method of choice is *in vivo* gene therapy. Using this approach, the gene is packaged into a vector (viral or non-viral), and then injected into the blood stream or directly into the affected organ. While simple conceptually, it has proven to be very difficult to achieve stable, high expression of transgenes using this approach, mainly because target cells are not always accessible to the vector or the vector has no specific tropism to the target cells. Nevertheless, the approach has been used in a limited number of human trials with varying success (Ferrari et al. 1991; Welsh et al. 1994).

*Viral and Non-Viral Vectors for Gene Delivery.* Two classes of vectors are available to carry the transgene DNA into the cell: viral and nonviral. The viral vectors that have been developed to date are based mainly on retroviruses and adenoviruses. In the retroviral system a DNA construct is prepared in which the retroviral proteins are replaced by the transgene (Eglitis, Kantoff et al. 1985; Anderson et al. 1986; Yu et al. 1986). The construct is then electroporated into packaging cells that have been genetically engineered to provide the viral structural proteins (Cone and Mulligan 1984; Markowitz et al. 1988; Markowitz et al. 1988; Markowitz et al. 1988). Transcription of the construct furnishes the viral genomic RNA, which together with the viral proteins will produce a viral particle that is able to transfect cells, but unable to replicate independently. These defective viral particles are then used to transduce cells. Using retroviral vectors, many cDNAs have been transduced into various cell types usually with very high efficiency (Morgan et al. 1987; Sorge, Kuhl et al. 1987; Chang and Johnson 1989; Fenjves, Gordon et al. 1989; Callow 1992; Flugelman 1995). It should also be noted that retroviruses integrate the transgene stably into the genome, albeit in a random fashion. The main disadvantage of this approach, however, is that retroviruses can only infect dividing cells, thereby excluding their use with many non-dividing somatic cells Also, the random fashion in which the retrovirus integrates its genome into the cell DNA might cause tumors due to disruption of tumor suppressor genes or activation of pro-oncogenes (Boris-Lawrie and Temin 1994). Nevertheless, because of their high transduction efficiency and stable transfection rate, retroviral vectors have remained the method of choice in most clinical gene therapy trials (Hsueh 1992; Blaese et al. 1993; Merrouche et al. 1995). In, addition, a new class of retroviral vectors, based on the

lentivirus family, has been recently developed (Parolin and Sodroski 1995; Lever 1996; Poeschla et al. 1996). Among the many advantages of this new vector class is the fact that it can transfect even non-dividing cells, thus making it suitable for HSCGT (Parolin and Sodroski 1995; Lever 1996; Poeschla, Corbeau et al. 1996).

The second main viral gene delivery vector currently used is based on the adenovirus. As with the retrovirus system, some viral genes necessary for replication are replaced by the transgene in adenoviral vectors, resulting in replication deficient adenovirus particles. Since the adenovirus does not integrate its genome into the host cells DNA, it can very efficiently transduce non-dividing cells. This property was used by Crystal et al. to deliver the CFTR gene into the airway epithelium of CF patients (Crystal et al. 1995). Adenoviral vectors were also used to transfect myoblasts (Acsadi et al. 1994), hepatocytes (Fang et al. 1994) and in developing gene therapy for cancer (Kleinerman et al. 1995; Yang et al. 1995). During these studies, however, the main disadvantage of using adenoviral vectors has come to light. It was found that in order to maintain the transgene m dividing cells, repeated administration of the virus was required. This in turn elicited immune response in the patients, which interfered with the therapeutic effects. Another risk involved with using these vectors lies in the fact that the wild-type adenoviruses are ubiquitous and may recombine with the recombinant vectors. However, despite these limitations it is likely that adenovirus based vectors will continue to be developed at a rapid pace and become a wildly used gene delivery system.

In addition to retroviral and adenoviral gene delivery systems, other techniques based on viruses are being developed by various groups. The viruses used by these groups include: Adeno-associated virus (AAV) (Berns and Giraud 1995), Herpes simplex

virus 1 (HSV- 1) (Latchman 1994), Vaccinia virus (Peplinski et al. 1995), etc. It is expected that in the next several years the problems involved with using these viruses will be solved, allowing construction of a variety of improved vector systems.

Non-viral vectors such as liposomes (Liu et al. 1995; Trivedi and Dickson 1995; Dalesandro et al. 1996), plasmid vectors (Wolff et al. 1990), etc., also have been used to transfect cells in human gene therapy experiments. Although these vectors offer a less immunogenic alternative to the viral based systems, they have produced disappointing results both in regard to targeting a specific cell type and transfection efficiency.

*Target Tissues for Gene Therapy.* The first diseases for which gene therapy was suggested were those which responded well to BMT (Gatt et al. 1978; Barksdale et al. 1981; Weinthal et al. 1991; Reisner and Segall 1995) The transplanted marrow produces normal blood elements, which express the normal gene products and can correct the metabolic disorders (Bagnis et al. 1995). Therefore, the logical extension is that if a normal gene can be inserted into the patient's pluripotent hemopoietic stem cells (PHSC), these cells will also produce normal blood elements, thereby altering the course of the disease. The main advantage of this technique over BMT is that autologous cells can be used, minimizing the likelihood of graft rejection.

The main obstacle to the use of this therapeutic approach is that few methods are available for the selection and propagation of hemopoietic stem cells, and the transduction efficiency of these cells is very low. However, intense research is being conducted in this field and various protocols have been developed to achieve some degree of success, at least in murine systems. For example, Szilvassy et al. have described a technique to isolate PHSC following treatment of donor mice with 5-

fluorouracil (5-FU) (Szilvassy, Fraser et al. 1989; Szilvassy et al. 1989). The cells that were obtained with this method were shown to be enriched for day 12 CFU-S cells and at least 1% of them were capable of long term marrow repopulation in lethally irradiated animals. Another approach is the use of monoclonal antibodies to positively or negatively select for the PHSC. These studies have shown that the PHSC in the mouse are c-Kit$^-$, Sca-1$^+$, Lin$^-$ (van de Rijn et al. 1989; Zsebo et al. 1990; Spangrude 1992; Uchida and Weissman 1992). In the human system it was long been thought that the CD34$^+$ HLADR$^-$ CD38$^-$ cells also were the PHSC (Brown et al. 1991; Strauss et al. 1991; Egeland et al. 1993; Nakamura et al. 1994). However, recent experiments performed in mice with the DNA binding Hoechst 33342 dye are challenging this assumption (Spain and Mulligan 1992; Wolf et al. 1993). It therefore remains to be seen if CD34$^+$ is indeed a marker of the human PHSC.

Other problems which hinder the use of PHSC for gene therapy are the difficulties related to growing them in culture for long periods of time and genetically transducing them. In clinical gene therapy trials for adenosine deaminase (ADA)-deficient combined immunodeficiency, CD34$^+$ cord blood cells were infected with the LASN-ADA retroviral vector, and transplanted into the patients. The transplanted cells gave rise to mature T cells and granulocytes that expressed the gene, but only at the low frequency of 1/10,000 to 1/100,000 cells (Bordignon et al. 1995). In order to enhance the ability of the retroviral vector to transduce these cells, it has been suggested by several groups that the cells be treated with various hemopoietic growth factors during the transduction. The growth factors that have been tested include interleukin (IL)-3, IL-6, stem cell factor (SCF, kit ligand) and IL-1 (Bodine et al. 1990; Brugger et al. 1993; Koller et al. 1993). These

growth factors have been shown to act synergistically to stimulate division of PHSC, thus allowing retroviral transduction. However, it has also been shown that this treatment may create an engraftment defect in the target PHSC, because the cells are induced to differentiate (Peters et al. 1996). Methods are therefore being sought to overcome this difficulty.

Another organ that has been an active target for gene therapy is the liver. Among candidate diseases for this approach are those in which the deficiency is in an hepatic enzyme, and those in which liver transplantation has proved to be helpful. Such diseases include PKU (Cristiano et al. 1993; Eisensmith and Woo 1994) and familial hypercholesterolemia due to LDL receptor defects (Grossman and Wilson 1992; Grossman et al. 1994). Methods to transduce liver cells have ranged from *in vivo* transfection following partial hepatectomy (to stimulate cells to divide) to *in vitro* transfection followed by re-implantation of the corrected cells via the portal circulation (Ponder et al. 1991; Li et al. 1992; Li et al. 1995). Various vectors have been used, both viral (retrovirus and adenovirus) and non-viral (polylysine based vectors) (Cristiano et al. 1993; Midoux et al. 1993). Although the preliminary results of these experiments were encouraging, further research must be performed before liver gene therapy will become available. Some of the issues that must be addressed include improvement in the efficiency of liver cell transduction and minimization in the use of dangerous surgical procedures.

Other cell types have been used as targets in gene therapy experiments include endothelial cells, myoblasts, neurons, airway epithelial cells etc. several reviews describing the use of these cells in gene therapy experiments are available (Callow 1992;

Dai et al. 1992; Porteous and Alton 1993; Jinnah and Friedmann 1995; Rowland et al. 1995). However since, these cells are not a major site of pathology in Type B NPD they will not be discussed further.

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# Chapter 1

# Characterization of the Acid Sphingomyelinase Knock-Out (ASMKO) Mouse Model

# Abstract

Types A and B Niemann-Pick disease (NPD) result from the deficient activity of acid sphingomyelinase (ASM). An animal model of NPD has been created by gene targeting. In affected animals, the disease followed a severe, neurodegenerative course and death occurred by eight months of age. Analysis of these animals showed their tissues had no detectable ASM activity, the blood cholesterol levels and sphingomyelin in the liver, spleen, lungs and brain were elevated, and atrophy of the cerebellum and marked deficiency of Purkinje cells was evident. Microscopic analysis revealed 'NPD cells' in reticuloendothelial organs and characteristic NPD lesions in the brain. Thus, the ASM deficient mice should be of great value for studying the pathogenesis and treatment of NPD.

# Introduction

Acid sphingomyelinase (ASM; sphingomyelin phosphodiesterase; E.C. 3.2.1.14) is the lysosomal enzyme required to hydrolyze sphingomyelin (SPM) into ceramide and phosphocholine (Kanfer et al. 1966). In humans, an inherited deficiency of ASM activity results in the Type A and B forms of Niemann-Pick disease (NPD) (Brady et al. 1966; Schneider and Kennedy 1967). Type A NPD is a severe, neurodegenerative disorder that routinely leads to death by three years of age (Schuchman and Desnick 1995). In contrast, patients with Type B NPD have little or no neurologic involvement and often survive into adulthood. The nature of this wide phenotypic spectrum is unknown, but is presumably related to the levels of residual ASM activity in affected patients (Schuchman and Desnick 1995).

Historically, two distinct sphingomyelinases have been described, an acidic sphingomyelinase (ASM) which is localized in the lysosomes and deficient in Types A and B NPD, and a $Mg^{+2}$ dependent neutral sphingomyelinase (NSM) which is localized on the outer leaflet of the plasma membrane (Rao and Spence 1976; Yedgar and Gatt 1976). Different genes are thought to encode these two enzymes since there are normal levels of NSM in the brains of patients with Type A NPD (Gatt et al. 1978).

The full-length human and murine ASM cDNA and genomic sequences have been isolated in our laboratory (Schuchman et al. 1991; Newrzella and Stoffel 1992; Schuchman et al. 1992), and over a dozen mutations causing Types A and B NPD have

been described (Schuchman and Desnick 1995). The human and murine genes have been mapped to the short arm of chromosome 11 (region p15.1—p15.4) and 7, respectively (da Veiga Pereira et al. 1991; Horinouchi et al. 1993).

Two naturally occurring NPD models have been described in large animals. These reports were canine (Bundza et al. 1979) and feline (Wenger et al. 1980) NPD models, which presented with similar clinical features as the human patients. However, these where isolated reports, and breeding colonies were never established for these models.

In 1980, Pentchev et al. described a colony of BALB/C mice with clinical features typical of Type A NPD: weight loss, ataxia and a short life span. They also documented a reduction in ASM activity and elevated levels of SPM and cholesterol (Pentchev et al. 1980; Pentchev et al. 1980). Shortly afterwards, a Japanese group described a strain of C57BL/6Js with similar clinical features and biochemical defects (Miyawaki et al. 1982; Sakiyama et al. 1982). Because of the similarities in the clinical and biochemical presentations of these mice to the human NPD phenotype, they where thought to represent naturally occurring animal models of this disease. However, in 1984 Pentchev et al. reported that these mice had a defect in cholesterol transport, and were likely to be a model of Type C NPD (Pentchev et al. 1984). Furthermore, as soon as the murine ASM gene sequence became available, the DNA of these mice was analyzed for mutations, and none were found (Horinouchi, Sakiyama et al. 1993). Therefore, the naturally occurring mouse models have Type C NPD, not Types A or B.

In the absence of naturally occurring models of Types A and B NIPD, our group undertook efforts to create an "ASM knock-out" (ASMKO) NPD mouse using gene targeting techniques. The DNA construct that was used for the gene targeting contained the murine ASM gene, a neo expression cassette inserted into exon 2 in the reverse orientation, and an HSV-TK expression cassette inserted into exon 6 (Fig 1). This chapter describes the characterization of the ASMKO mice on the clinical, pathological, and biochemical levels.

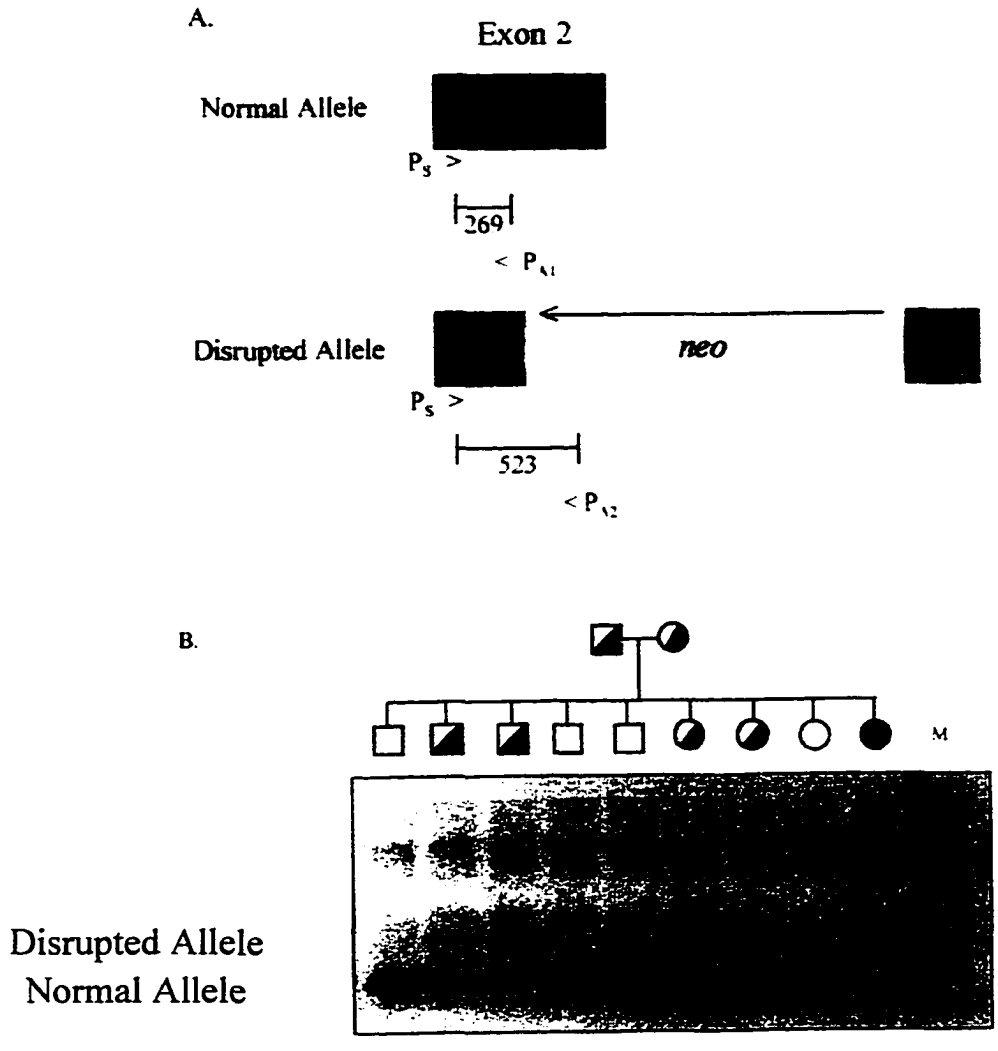



**Fig. 1. Schematic representation of the ASM replacement vector and PCR genotyping of ASMKO mice.** A, The normal mouse ASM gene consisting of six exons spanning ~4.5 Kb. and the disrupted ASM gene; B, Results of a PCR amplification of pups from a heterozygous breeding pair. The marker (M) is 100 bp DNA ladder (New England Biolabs). The locations of the three primers that were used (Ps, PA1 and PA2) in the normal and disrupted ASM alleles are shown.

# Materials and Methods

*PCR Method to Determine Mouse Genotype.* Mouse DNA was purified from small pieces of tail tissue using a commercially available kit (Puregene DNA isolation kit D-5000; Gentra Systems Inc.). PCR amplification (30 cycles, each consisting of 1 mm at 93°C, 1 mm at 58°C, 1 mm at 72°C) was performed on a Minicycler PCR machine (MJ Research) using three primers: an ASM sense primer (Ps; 5'-AGCCGTGTCCTCTTCCTTAC-3') from exon 2, an ASM antisense primer also from exon 2, downstream to the insertion point of the neo cassette (PAI; 5'-CGAGACTGTTGCCAGACATC-3'), and an antisense primer from within the neo cassette (PA2; 5'-GGCTACCCGTGATATTGCTG-3') (Fig 1). Thus, in normal homozygous mice a single band of 269 bp corresponding to the endogenous, undisrupted ASM gene is amplified. In affected, homozygous knock-out mice a single band of 523 bp is amplified from the sense and neo primers. As expected, two bands are amplified from heterozygous mice. (Fig 1). Each amplification reaction (100 µl final volume) contained 100 pmol each of the three PCR primers, 1 µg of genomic DNA, 1 X PCR buffer (Promega), 1.5 mM $MgCl_2$, 5 U of Taq polymerase (Promega) and 200 µM each of dNTPs. To visualize the bands, 25 µl of the reaction mixture was run on a 1% agarose gel and stained with ethidium bromide.

*ASM and NSM Activity Determination - $^{14}C$ Assay.* These assays were performed using minor modifications of previously described methods (Maruyama and Arima 1989;

Quintern and Sandhoff 1991) To determine ASM activity, methyl- $^{14}C$ -labeled sphingomyelin (Amersham; specific activity 54.5 mCi $mmol^{-1}$) was diluted in the presence of unlabeled sphingomyelin to a final specific activity of 0.1 mCi $mmol^{-1}$. The chloroform in this substrate mixture was evaporated and the dried substrate was resuspended in a buffer (15 µl per reaction) containing 0.2 M sodium acetate (pH 5.2) and 0.2% Triton X-100. The resuspended substrate was heated for 1 mm at 68°C to form micelles and placed on ice. 15 µl of each tissue extract (20-40 µg of protein) were added to the substrate preparation and incubated for 1 hr at 37°C. Protein concentrations were determined using the fluorescamine assay (Stein et al. 1973). Radioactive phosphocholine produced from the $^{14}C$-labeled sphingomyelin was extracted using 200 µl of chloroform/ methanol (1:1 v/v) and 90 µl of water. $^{14}C$-phosphocholine production was quantitated using a model 1219 Rackbeta liquid scintillation counter (Pharmacia). 1 U of enzyme activity equals the amount of enzyme that produces 1 nmol of $^{14}C$-phosphocholine /hr/ml. To determine NSM activities, the methods were essentially the same except that the substrate was resuspended in a buffer containing 40 mM HEPES, pH 7.4, 5 mM $MgCl_2$ and 0.2% Triton- X-100. Significance levels (P values) were determined using unpaired Student's t-test (two sided).

*Sphingomyelin Determination.* The initial SPM determinations on the ASMKO mice were performed in Dr. Konrad Sandhoff's lab in Bonn, Germany. However, with the help of Dr. Shimon Gatt of the Hebrew University, I implemented the assay in our laboratory and carried out further analyses. To determine SPM content in mouse tissues,

~200 mg of tissue was homogenized in 600 µl of chloroform/methanol (1:2 v/v). After incubating the sample in 55°C for 15 min the proteins were removed by centrifugation. In order to maximize lipid extraction, the pellets were washed with an additional 400 µl of chloroform/methanol (1:1 v/v). After combining the supernatants, the lipids were extracted by adding 600 µl of water, vortexing and spinning the sample at 3,000 rpm for 10 mm. The upper phase was then removed, and the lower phase solvent (chloroform) was evaporated. For SPM quantitation, most phospholipids were first removed by alkaline hydrolysis in 0.4 N KOH / 90% Methanol for 2 hr at 55°C. To avoid interference from plasmanogen and other phospholipids in the brain extracts, acidic hydrolysis was carried out in these samples using 0.6 M HCl in ethanol for 30 min at 37°C before alkaline hydrolysis. Of the remaining lipids, only sphingomyelin contains phosphorous. The lipid mixture was then digested by perchloric acid (70%) for 40 min at 180°C, followed by treatment with 0.5% ammonium molybdate and Fiske & Subbarow reducer (4 mg/ml; Sigma Chemical, St. Louis, MO) for 10 min at 100°C. Absorbance at 830 nm was measured in a spectrophotometer (model 1201, Milton Roy Spectronic Inc. Rochester, NY). A standard curve was generated using an inorganic phosphorous solution (Sigma Chemical, St. Louis, MO).

*Collaborative Studies*. Full characterization of the ASMKO mouse model on the pathological and behavioral levels require both expertise and equipment which are not present in our laboratory. In order to perform these studies, we established collaborations with experts in the fields of animal pathology (Dr. Mark Haskins, Department of

Pathobiology, University of Pennsylvania School of Veterinary Medicine, and Dr. Daniel Perl, Department of Pathology, Mt. Sinai School of Medicine), plasma cholesterol and lipoproteins (Dr. Ira Tabias, Department of Medicine, Columbia University, and Dr. Charles Bisgaier, Department of Atherosclerosis Therapeutics, Parke-Davies Pharmaceutical Research) and mouse neurology and behavior (Dr Victor L. Freidrich, Jr., Brookdale Center for Molecular Biology, Mt. Sinai School of Medicine). The contributions of these collaborators are described in "Results".

# Results

*PCR Genotyping and Establishment of Breeding Colonies.* When I entered this project, only several heterozygote ASMKO animals existed and no breeding colony for this mouse model was established. At that time, the mouse genotype could only be detected by Southern blotting techniques. In order to facilitate the establishment of a breeding colony for ASMKO mice, I designed a multiplex genomic screening PCR test. (See Materials & Methods). I have determined the accuracy of this PCR test by analyzing the same DNA samples using both the PCR and Southern blotting methods. In all cases (20 DNA samples), the same genotype was determined. In addition, we monitored the development of those animals that were determined to be homozygous for the disrupted ASMKO allele by the PCR test, and no ASM activity could be detected and the characteristic NPD phenotype appeared at 3 months of age (see below). With the help of this quick genotype screening method, I was able to establish a breeding colony of ASMKO mice. We now have more then 50 breeding homozygous ASMKO breeding pairs producing animals for bone marrow transplantation (BMT) and hemopoietic stem cell gene therapy (HSCGT) experiments.

*Inheritance.* The ASMKO phenotype was inherited as an autosomal recessive trait; heterozygous breeding pairs yielded 25% affected offspring (Fig. 1-D). There was no phenotype associated with heterozygosity. Affected males could breed until 20 weeks of age and affected females until 10 weeks. There were no phenotypic differences between males and females. The litter size of affected females was normal, although

there was a higher incidence of neonatal death among the litters of affected mothers. We found that there is a positive correlation between advanced maternal age and newborn mortality in the ASMKO litters. In contrast, this phenomenon was not related to the paternal age and genotype. When the affected mother was replaced by a surrogate normal female or a younger affected female, newborn mortality rates return to normal. Therefore, we believe that this phenomenon is attributed to the inability of severely affected mothers to take care of the pups, most likely due to advanced brain disease.

*ASM and NSM Enzyme Assays.* To determine the biochemical phenotype of ASMKO mice, I sacrificed 35 animals at different ages (3wks, 3mo and 6mo), performed post-mortem surgery, and removed all of the major organs (brain, lungs, heart, liver, spleen, and kidneys). ASM and NSM activities were analyzed in organs from normal, ASMKO, and heterozygous animals (Fig 2). No ASM activity could be detected in ASMKO mice while heterozygous animals showed reduced activity to ~50% of normal (Fig 2-A). In normal animals, $Mg^{+2}$-dependent NSM activity is only present in the brain, and to a lesser degree in the kidneys (Fig 2-B). I found no differences in kidney NSM activity between normal and affected animals ($p>0.93$). However, the affected animals showed significant reduction in their brain NSM activity compared to that found in normal animals ($p<0.003$) (Fig 2-B). The detection of NSM activity in the ASMKO mice implies that this enzyme is encoded by a distinct gene from that encoding ASM. This is supported by the observation that no ASM mRNA could be detected in the organs of ASMKO mice (personal communication from Chi-Ming Li, not shown). We believe that

the reduced NSM activity found in the ASMKO mouse brain is a result of the abnormal cell pathology and lipid accumulation in this organ.

*SPM Accumulation.* Initial lipid analyses were performed (in Dr. Sandhoff's laboratory) on liver and brain extracts from normal and ASMKO mice. After implementing the method in our laboratory, I and Dr. Silvia Miranda (a post-doctoral fellow in our lab) repeated these experiments and also measured the substrate accumulation in the lungs and spleens of affected and normal animals (Fig 3). There was accumulation of SPM in all tissues, although much more substrate could be detected in the liver and spleen than in the brain and lungs.

*Plasma Lipoproteins.* Initial analysis of plasma cholesterol and lipoprotein level in the ASMKO mice were performed by Dr. Charles Bisgaier. He detected an ~80% increase in total plasma cholesterol levels in the affected mice. This increase was associated with elevated HDL levels, while other lipoproteins (e.g., LDL and VLDL) showed little or no change (Fig 4). This finding is similar to that found in human NPD patients (unpublished observations).

*Pathology.* At the time of death, affected animals were about half the weight of normal controls. All of the major organs were smaller and no hepatosplenomegaly was observed (Fig 5). 3 and 6 month affected animals were given to Dr. Daniel Perl for light and electron microscopic analysis. Characteristic 'NPD' cells were found in most tissues, especially in the bone marrow and spleen (Fig 6-A). Electron microscopic analysis demonstrated the presence of numerous multilammellar cytoplasmic inclusions in all

tissues, particularly in the brain (Fig 6-B). A remarkable loss of Purkinje cells and general atrophy of the cerebellum and midbrain also was evident.

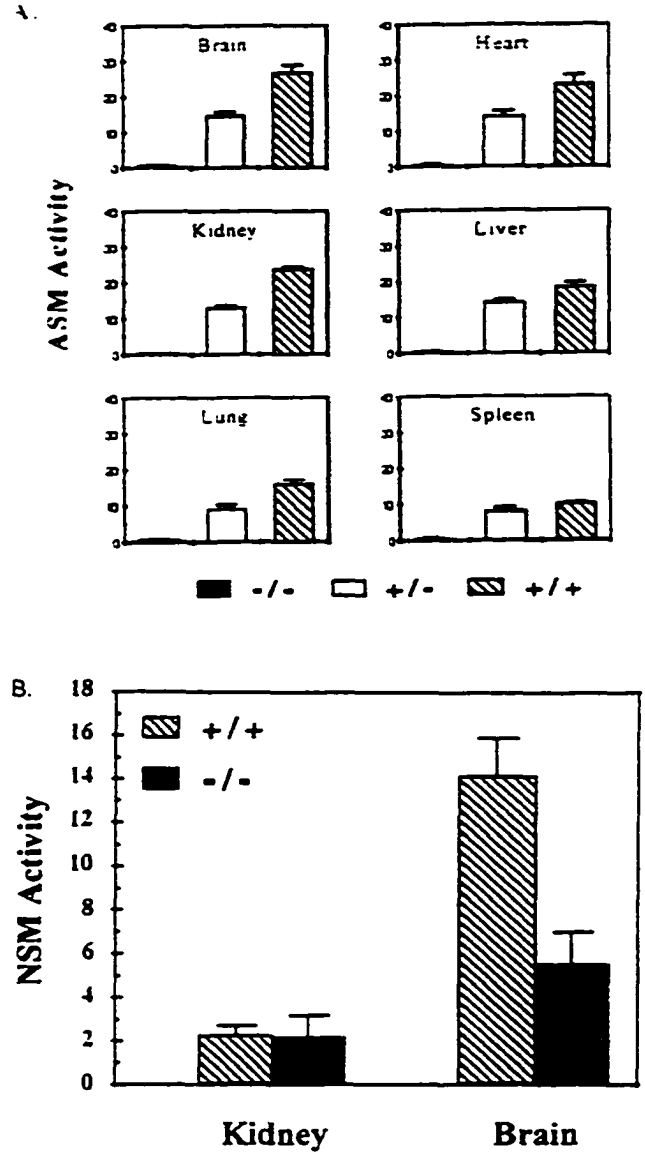



**Fig. 2. ASM and NSM activities in the ASMKO mouse colony.** A, ASM and B, NSM assays were performed as described in the methods. Activities are expressed as units/mg protein (one unit equals that amount of enzyme that produces 1 μmol of $^{14}$C-labeled phosphocholine/hr/ml). For the ASM studies, 5 animals were analyzed for each group (normal, heterozygous and homozygous ASMKO). For NSM, normal (n=12) and affected (n=9) animals were studied. The bars indicate the mean value +/- one standard error.

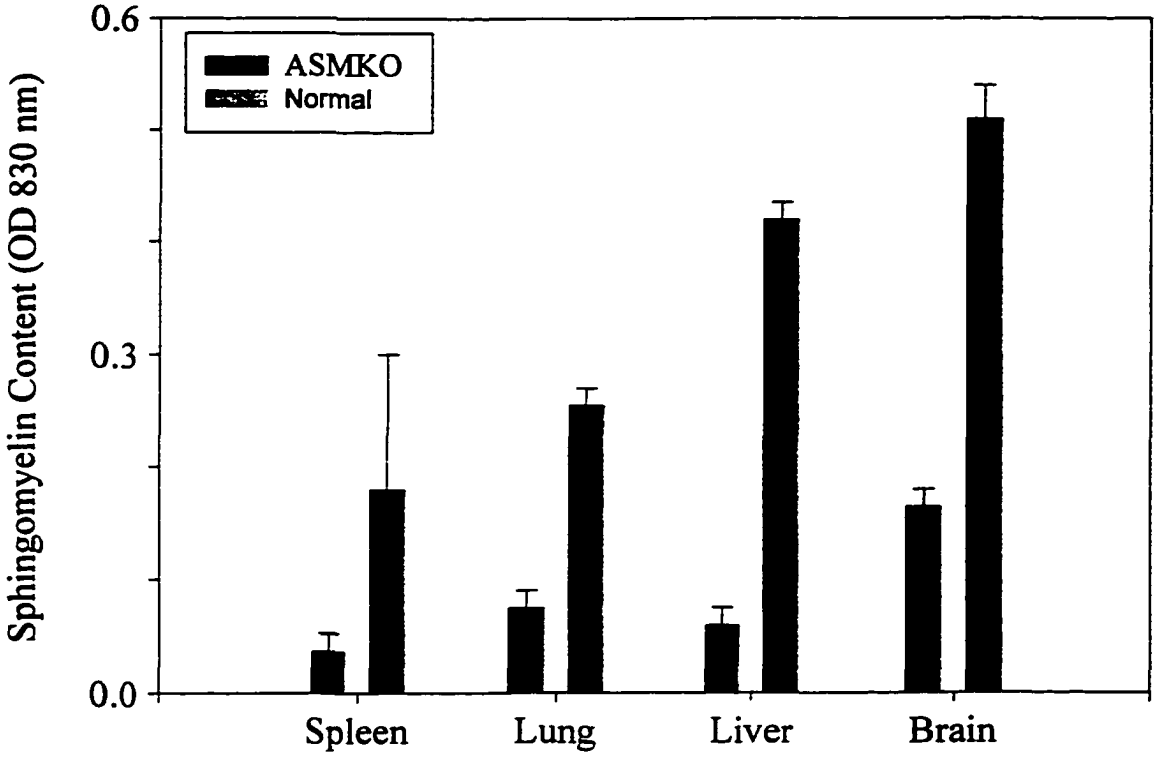



**Fig. 3. Sphingomyelin content in tissues of normal and ASMKO mice.** Sphingomyelin determination was done as described in the methods. Hatched bar represents normal and solid bar represents ASMKO mice. Six animals were used in each group. The bars indicate the mean value +/- one standard error.

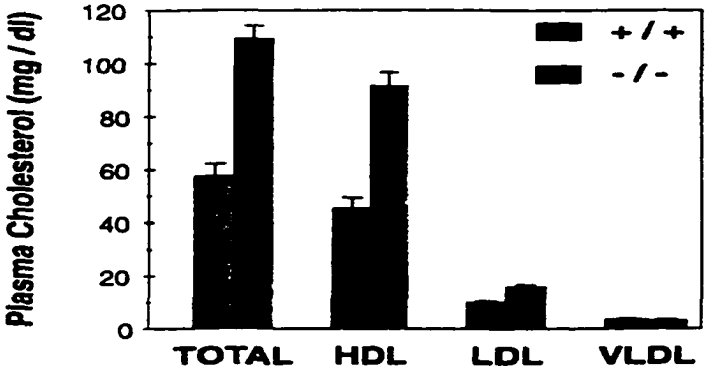



**Fig. 4. Plasma cholesterol levels in the ASMIKO mice.** The cholesterol levels in the VLDL, LDL and HDL lipoprotein fractions were determined (by Dr. Charles Bisgaier). Five ASMKO and twelve normal animals were used. The bars indicate the mean values +/- one standard error.

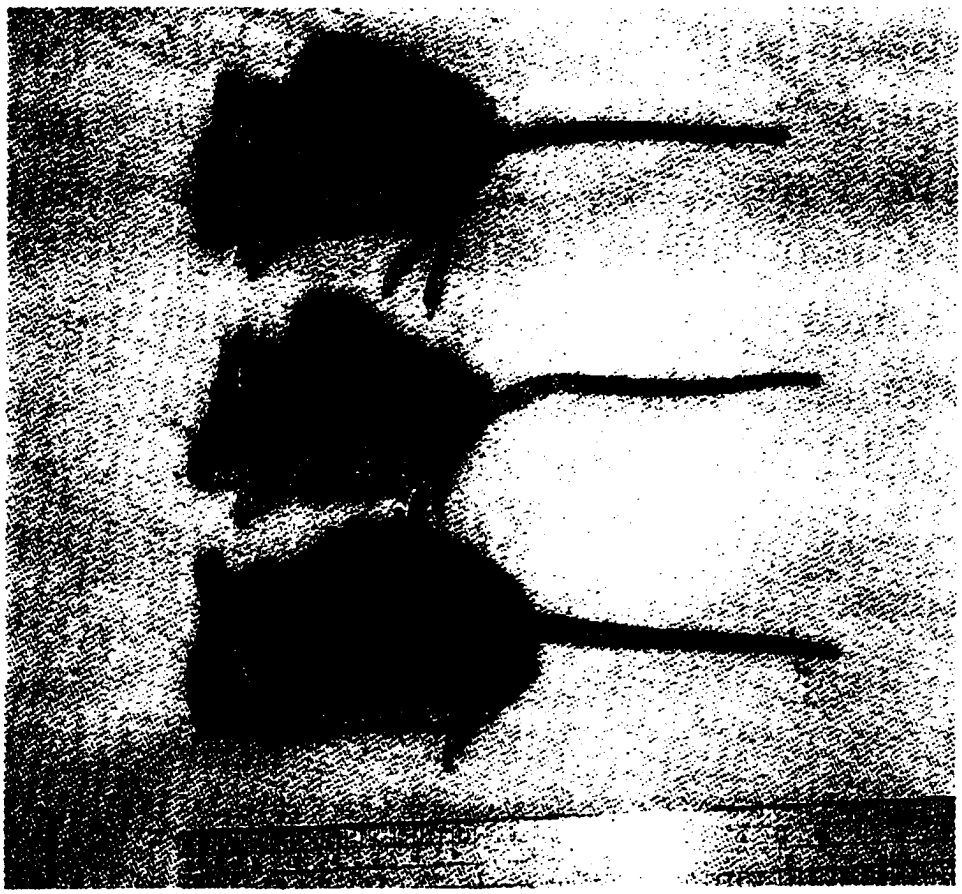
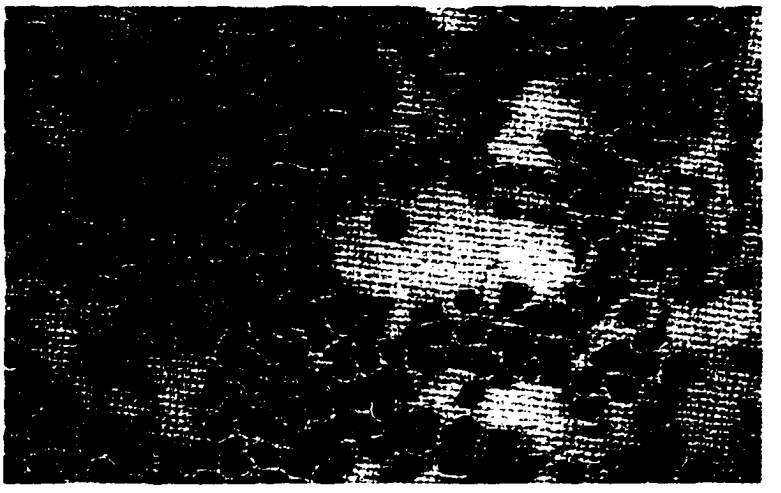


**Fig. 5. Phenotype of the ASMKO mouse.** Note the dramatic size difference and "hunched" appearance of the affected animals (top two animals) compared to a control littermate (bottom) at four month of age.

A.



B.

**Fig. 6. Histology studies in ASMKO tissues.** A, Light microscopy of ASMKO mouse spleen. Note the infiltration of foam cells throughout this representative section. B, Electron microscopy of the ASMKO brain. Electron micrograph of an internal granular layer neuron of the cerebellum containing parallel and concentric intracytoplasmic inclusions (magnification, 12,500X)

*Clinical and Behavioral Observations*. All ASMKO mice are born without any apparent phenotype. Their early development is normal until 8 weeks of age when ataxia and mild tremors become noticeable. From this point there is a rapid neurologic deterioration, and by the age of four months all affected animals suffer from severe ataxia. The animals become lethargic, unresponsive to stimuli and have difficulties eating. Death usually occur between 6 and 8 month of age, probably as a result of starvation.

# Discussion

ASM is a critically important lysosomal enzyme. NPD is a debilitating, fatal disease for which no therapies are available. Among possible therapeutic approaches, enzyme replacement using recombinantly produced ASM, BMT and somatic gene therapy using genetically corrected hematopoietic progenitors for autologous transplantation are viable alternatives (Schuchman and Desnick 1995). However, to effectively evaluate these and other therapies, animal models of the human disorder must be available. Moreover, in recent years the role of ASM in cell growth and differentiation has been intensively investigated and several reports have suggested a role for this hydrolase in various cell signaling pathways (Kolesnick and Golde 1994). Thus, to facilitate these basic and applied research efforts, our laboratory constructed the ASMKO (i.e., NPD) mouse.

The ASMKO mouse is an authentic model of Types A and B NPD. There was no residual ASM activity and massive accumulation of sphingomyelin was evident. Blood cholesterol levels also were markedly elevated, similar to human NPD (Schuchman and Desnick, unpublished observations). The light and electron microscopic abnormalities present in the affected animals were typical of NPD and, in particular, numerous multilammellar inclusions were evident in most organs, including the brain. Furthermore, similar to type A NPD patients, ASMKO mice develop a progressive neurodegenerative disease. However, in contrast to the human patients in which the progression of the brain disease is rapid and usually leads to death by the age of 3 years, the mice survive for up

to 8 month (1/3-1/2 of the normal mouse life span). This is fortunate since it opens a "window of opportunity" in which the ASMKO mice can be used to test novel therapeutic regimens for NPD such as BMT or HSCGT protocols.

Shortly after our publication, Otterbach & Stoffel (Otterbach and Stoffel 1995) reported another ASMKO mouse that was independently derived in their lab. Although the molecular defect of this ASMKO mouse is different then that present in our model (exon 3 is disrupted instead of exon 2) and their genetic background is different (C57BL/6 vs. C57BL/SV129), the two models share virtually the same phenotype including the lack of ASM activity in all tissues, massive SPM accumulation and clinically progressive neurodegenerative course. This independent finding verifies our results and suggests that the observed phenotype is indeed caused by the total elimination of the ASM activity.

The ASMKO mice also provide a unique opportunity to evaluate the individual roles of ASM and $Mg^{+2}$ dependent NSM in ceramide-mediated signal transduction. The levels of NSM were determined in kidney and brain since these are the two tissues with the highest activity in normal animals (Yedgar and Gatt 1976). Kidney had ~25% of the brain NSM activity and the renal levels were essentially the same in ASMKO and control mice. In contrast, the NSM levels in brains of affected animals was ~50% that of controls. Although these results are intriguing, it is likely that the reduced NSM activity is due to the abnormal cell pathology and lipid accumulation in the brains of affected animals, rather than a primary defect in the enzyme. Clearly, these results suggest that at

least half of the $Mg^{+2}$-dependent NSM activity is derived from a gene product distinct from ASM. Future investigations should clarify the relationship between these sphingomyelinases and delineate their specific roles in the ceramide-mediated signal transduction pathway.

Recently, the role of ceramide in signal transduction and apoptosis has been intensively investigated (Hannun 1994; Kolesnick and Golde 1994). Initial observations suggested that stress signals (such as ionizing radiation and treatment with lipopolysaccharide) cause the activation of sphingomyelinase to generate ceramide, which serves as a second messenger in initiating the apoptotic response. Notably, in each case, rapid activation of a sphingomyelinase activity leading to the production of intracellular ceramide proceeded the biological effect. However, conclusive evidence for this paradigm was lacking. In 1996, Santana et al. (Santana et al. 1996), in collaboration with our lab, demonstrated that ASMKO mice and NPD lymphocytes failed to respond to ionizing radiation with ceramide generation and apoptosis, thus proving for the first time that ASM activity takes part in this signaling pathway. More recently it was shown (again using the ASMKO mouse model) that it is the systemic release of tumor necrosis factor (TNF)-alpha that mediates ASM activation (Haimovitz-Friedman et al. 1997).

Furthermore, using peritoneal macrophages derived from our ASMKO mice colony it has been shown that a $Zn^{+2}$ stimulated sphingomyelinase that is secreted by many cell types is actually the product of the ASM gene (Schissel et al. 1996). This isoform of ASM is derived by an unknown post-translational process, is found

abundantly in the plasma and has been implicated in the formation of atherosclerotic plaques (Tabas et al. 1993).

In conclusion, an ASM deficient mouse has been derived. The clinical, biochemical and pathological findings mimic the characteristics of human Types A and B NPD, making them excellent models for studies of the disease pathogenesis and development of novel therapeutic strategies. In addition, these mice provide a unique opportunity to investigate the independent roles of ASM and NSM in ceramide-mediated signal transduction. The role played by ASM in atherosclerotic processes can also be examined using the ASMKO mouse model.

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# Chapter 2

## Allogenic Bone-Marrow Transplantation in Acid Sphingomyelinase Knock-Out Mice: Pilot Study to Determine the Effect of Various Parameters on Blood and Brain Engraftment

# Abstract

In order to evaluate the therapeutic effectiveness of bone marrow transplantation (BMT) for the treatment of NPD, and to establish rationale for the development of hemopoietic stem cell gene therapy (HSCGT) for this disorder, we performed a series of BMT experiments in the ASMKO mice. These experiments were divided into two stages. In stage 1, which is described in this chapter, a pilot study was conducted in which a total of 202 animals were transplanted at different ages (newborn and adult). We varied the number of bone marrow cells injected into the animals and the amount of radiation used to ablate the recipient's own bone marrow. The survival of the animals and the level of engraftment were monitored in order to design and establish an optimal transplantation protocol, which provides efficient engraftment with minimal pathological effects on the animals. In addition, the number of donor cells detected in the brain post-transplantation, and the levels of ASM activity achieved in the brain of transplanted animals were measured in order to evaluate BMT as a possible therapeutic approach for Type A NPD. The overall conclusions of this study were: (1) Newborn ASMKO animals were more susceptible to radiation-induced mortality than normal animals; (2) Engraftment could be improved, at low radiation doses, by increasing the number of donor cells; (3) The engraftment rate of bone marrow cells in the blood of normal animals was better than that achieved in the ASMKO animals using the same transplantation protocols; (4) In newborn animals, the engraftment rate of donor cells in the blood and brain of the transplanted animals was directly correlated. In contrast, there was no engraftment of donor cells in the brains of adult transplants; and (5) The brains of newborn ASMKO

animals were better engrafted by donor cells than newborn normal animals. Base on these results, a uniform protocol was developed and used to assess the therapeutic effects of BMT on the ASMKO phenotype (see chapter 3).

# Introduction

In man, acid sphingomyelinase (ASM) deficiency leads to two types of Niemann-Pick Disease (NPD). Type A NPD is characterized by a rapidly progressive neurodegenerative course that leads to death by 3 years of age, while patients with Type B NPD have little or no neurologic involvement and may survive into early adulthood (Schuchman and Desnick 1995). Mice with NPD (i.e., acid sphingomyelinase knock-out [ASMKO] mice) develop normally until about 4 months of age, when ataxia first becomes evident (see chapter 1). They then follow a severe, neurodegenerative course that leads to death between 6 and 8 months of age. Affected animals have no detectable ASM activity in their tissues or fluids, and blood cholesterol levels and sphingomyelin in the liver and brain are markedly elevated. Atrophy of the cerebellum and a dramatic deficiency of Purkinje cells is evident. Microscopic analysis showed NPD foam cells in reticuloendothelial organs and characteristic NPD lesions in the brain. Thus, the ASMKO mice are an authentic model of human NPD that is useful for the study of disease pathogenesis and the development of therapeutic strategies (Horinouchi et al. 1995; Otterbach and Stoffel 1995).

Similarly to many other lysosomal storage diseases, there is currently no effective treatment for Types A and B NPD. Since in Type B NPD the primary cellular sites of pathology are the resident macrophages of the reticuloendothelial system (Schuchman and Desnick 1995) it is possible that following bone marrow transplantation (BMT) in these patients, the normal bone marrow-derived cells in the blood and other organs (e.g.,

Kupffer cells in the liver) will release sufficient ASM for uptake by affected non-hematopoietic cells thus leading to metabolic cross-correction of the affected cells(Yeyati et al. 1995). While treatment of the non-neurologic, Type B form of NPD by this therapeutic approach should be feasible, BMT in Type A NPD patients will only be successful if the normal bone marrow-derived cells (or ASM itself) migrate across the blood brain barrier (BBB) and release ASM for uptake and cross-correction of neurons.

Based on these principals, several treatment options, such as liver transplantation (Daloze et al. 1977; Gartner et al. 1986), amniotic membrane transplantation (Scaggiante et al. 1987), and BMT (Vellodi et al. 1987; Bayever et al. 1992; Bayever et al. 1992) have been tried in the human patients, but the long term outcome of these procedures has not been properly documented. The failure of these human trials can be attributed in part to the absence of a consensus unified protocol for the transplantation of these patients. However, this kind of protocol could only be established by analyzing the results of large scale experiments in animal models of the disease. Therefore we decided to use the ASMKO mouse model of NPD to design and test BMT and hemopoietic gene therapy strategies for the treatment of NPD. In the first stage of this project, which is described in this chapter, we systematically evaluated the effects of radiation, age, donor cell number, and phenotype on survival, engraftment, and cell migration into the brain following BMT. The results of this study helped us in designing a safe and effective transplantation protocol that we later used on a large number of animals to evaluate the effect of this form of therapy on the phenotype of the ASMKO mice (see chapter 3).

# Materials and Methods

*Mice.* The ASMKO mouse colony was established from heterozygous breeding pairs obtained by gene targeting of 129/Sv embryonic stem cells and subsequent microinjection into C57BL/6 blastocysts (Horinouchi, Erlich et al. 1995). Gender-mismatched BMTs were performed by injecting cells derived from using normal male mice (6 to 12 weeks old) into affected ASMKO female animals. Both donor and recipient animals were obtained from within the ASMKO colony.

*Donor Bone Marrow Preparation.* The methods used for the isolation of bone marrow and BMT were implemented in our laboratory with the help of Dr. Jan Visser of the New York Blood Center. Donor animals were sacrificed by cervical dislocation, and the bone marrow harvested from the medullary cavities of the femur and tibia by flushing the bones with Hank's balanced salt solution (Gibeo-BRL) using a 27-gauge needle. The cells were washed twice in the same solution and single cell suspensions were obtained by passage through a cell strainer (40 μm; Becton Dickenson). At this point, cells were either counted and injected into recipients, or further enriched for mononuclear cells using a density gradient (Lymphocyte Separation Medium; Organon Teknika Corp.) and a 5 mm exposure to hemolytic buffer (5 mM $MgCl_2$, 1 mM EDTA, pH 8.0).

*Transplantation Protocols.* The obtained donor cells were counted, diluted to the desired concentration, and immediately injected into the tail veins of adults or, in the case

of newborns, divided in half and injected into both the temporal veins and peritoneal cavity. Between 1 to 30 million donor cells per gram of body weight were given to the newborn animals while the number of cells given to adults was 50,000-1,500,000. Normal or ASMKO newborns (up to 4 days old) or adults (6 to 12 weeks old) served as recipients. These animals were either non-irradiated or subjected to a single total body irradiation dose of 200, 400, or 800 cGy from a dual $^{137}Cs$ source (dose rate = 80 cGy/min). Age-matched littermates (normal and ASMKO) were used as radiation controls (i.e., irradiated, but not transplanted). No antibiotics or other supportive care were given to any of the animals after transplantation.

*Evaluation of Transplanted Animals.* After transplantation, the animals were monitored daily for survival. Assessment of engraftment was carried out 3 months after BMT by two assays: Y-chromosome in situ hybridization in female recipients (performed by Dr. Silvia Miranda) and ASM activity in white blood cells (WBC).

*Tissue Harvesting and Preparation.* Peripheral blood was obtained by retinal orbit bleeding and WBCs were isolated after lysis of the red cells using hemolytic buffer (5 mM $MgCl_2$, 1 mM EDTA, pH 8.0). The cellular proteins were extracted in 0.2 % Triton X-100 on ice. To detect ASM activity in the brain, recipient animals were anesthetized with Ketamine (Sigma, St Louis, MO; 0.5 g/kg of body weight) and subjected to cardiac perfusion. An incision was made in the right atrium to allow blood to flow out and a cannula was introduced through the left ventricle into the aorta, delivering 50 ml of warm 0.9% NaCl solution. After perfusion, tissues were collected and immediately frozen on dry ice for subsequent sectioning or enzyme analysis (see below). Protein extracts were

prepared from brain tissue by homogenization in 0.2% Triton X-100 on ice using three 10-second bursts of a Potter-Elvehjem tissue homogenizer (Thomas Scientific, Sweedesboro, NJ). Total protein was determined by the method of Stein et al. (Stein et al. 1973).

*ASM Activity Determination - Fluorescence Assays.* The radioactive $^{14}C$ ASM assay is suitable for analyzing enzymatic activity in tissue extracts. However, due to the low activity present in normal white blood cells (WBC), a more sensitive assay was required to detect ASM activity in these cells. With the help of Dr. Shimon Gatt of the Hebrew University, I established an extremely sensitive fluorescence-based ASM activity assay system. Sphingomyelin, to which the fluorescent probe BODIPY was linked covalently (BODIPY dodecanoyl sphingosyl phosphocholine; Bl2SPM), was synthesized essentially as described previously for lissamine rhodamine sphingomyelin (Dinur et al. 1992), except that BODIPY dodecanoic acid (Molecular Probes Inc., Eugene, OR) was condensed with sphingosyl phosphocholine.

The standard 40 μL ASM assay mixture consisted of 30 μL of sample (homogenized cells) and 2 nmol of B12SPM suspended in 0.1 mol/L sodium acetate buffer, pH 5.2 containing 0.6% Triton X-l00 and 5 mmol/L EDTA (for detection of the lysosomal ASM activity) or 0.1 mmol/L $ZnCl_2$ (for detection of the secreted, zinc stimulated ASM activity). After incubating the assay mixture at $37^0C$ (up to 3 hours), the samples were loaded on thin layer chromatography plates (TLC LK6 D Silica gel 60, Whatman, Clifton, NJ) and resolved using chloroform/methanol (95:5, vol/vol). After

resolution, the band containing the fluorescently labeled ceramide was scraped from the TLC plates, extracted in chloroform/methanol/water (1:2:1, vol/vol/vol) for 15 minutes at 55°C, and quantified in a spectrofluorometer (fluorescence spectrophotometer 204-A, Perkin Elmer). The excitation and emission wavelengths of the instrument were set at 505 and 530 nm, respectively.

*In Situ Hybridization.* All of these studies were performed exclusively by Dr. Silvia Miranda. Briefly, the WBCs and brains of recipient female animals were obtained and fixed. This was followed by in situ hybridization with a Y-chromosome specific probe that only labels the donor male cells but not the recipient's cells. Following the hybridization, a total of 300 WBC nuclei from each animal were scored to determine the percentage of donor-derived cells in the blood of the transplant recipients. To estimate the number of donor-derived cells in the brain, half of each recipient brain was surveyed by collecting every $25^{th}$ section, counting the number of Y-chromosome positive cells using a fluorescent microscope, and plotting each location according to illustrations from an atlas of the mouse brain (Sidman et al. 1971). The number of donor cells per section was then multiplied by 50 to estimate the total number of cells in the brain.

*Statistical Analyses.* Analysis of variance was performed using the general linear models (GLM) procedure of the Statistical Analysis System (SAS) software package (SAS Institute Inc., Cary, NC). Data on blood and brain engraftment were available for 38 ASMKO and 95 normal mice, which were divided into several radiation/cell dose groups. This included animals that were transplanted perinatally (n = 91) or as adults (n = 42); all of the transplanted animals were analyzed for engraftment 3 months post-

transplant. For these statistical analyses, the complete data set was analyzed to take into account the individual and combined effects of genotype, radiation, bone marrow cell dose, and age at transplant. As described in the Results, newborn ASMKO mice survived only low doses of radiation. Thus, the data set is substantially unbalanced across age, and the two age groups were therefore separated for the statistical evaluations. Significant effects (Type III SS) are provided (see Tables 2 and 3).

# Results

*Correlation Between Fluorescence-Based ASM Activity Assay And Y-Chromosome In Situ Hybridization Results.* WBCs from 16 female recipients were analyzed using both the fluorescence based ASM activity assay (performed by me) and the Y-chromosome in situ hybridization assay (performed by Dr. Miranda). The results of this double-blind study have demonstrated that the ASM activities and Y-chromosome hybridization results correlate to an extremely high degree ($r=0.99$) (Fig 1). We also found that the in-situ hybridization technique was a less sensitive method, reporting engraftment levels about 91% of those measured by the enzyme assay. This finding allowed us to routinely use the much simpler enzyme assay to determine engraftment in this and future BMT and HSCGT studies. It also allowed us to analyze more animals, since both male and female recipient animals could be used.

*Survival of BMT Mice as a Function of Radiation Dose.* Table 1 shows the survival of transplanted animals as a function of radiation dose and animal age and genotype. These studies revealed that no serious side effects or mortality occurred using radiation doses equal or less than 200 cGy. Higher radiation levels caused increased mortality, especially in newborns. Indeed, 1-day-old ASMKO newborn mice did not survive "sublethal" radiation doses of 400 cGy, while among normal 1-day-old mice the survival rate was 78%. Radiation with a "lethal" dose of 800 cGy caused death in all of the 1-day-old newborn animals (ASMKO or normal). When a second group of 4-day-old animals was transplanted using a radiation dose of 800 cGy, ASMKO animals were

notably more susceptible to radiation-induced mortality showing only a 10% survival rate compared to 71% in normal animals.

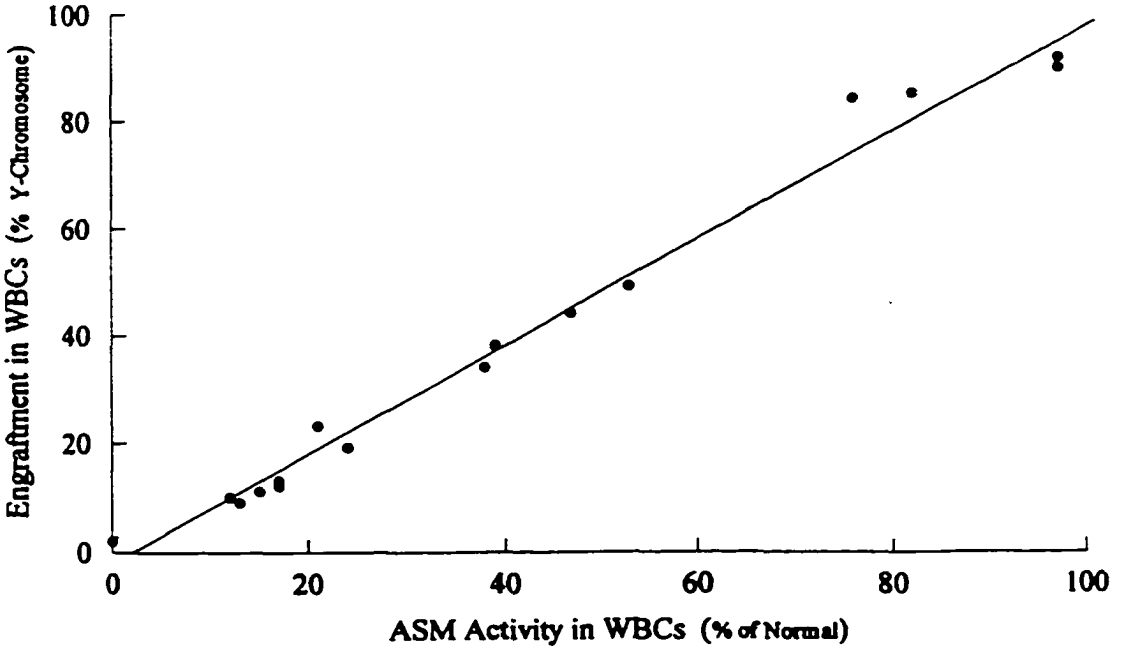


**Fig. 1. Correlation between results obtained by Y-chromosome in situ hybridization vs. ASM assays.** The line represent the linear regression performed (Y=0.90998 1 x X)

**Table 1: Survival of BMT Recipients**

| Radiation Dose (cGy) | Newborn ASMKO | Newborn Normal | Adult ASMKO | Adult Normal |
|---|---|---|---|---|
| 0 | 5/5 (100) | 13/13 (100) | 5/5 (100) | 5/5 (100) |
| 200 | 32/32 (100) | 13/13 (100) | 5/5 (100) | 6/6 (100) |
| 400 | 0/10 (0) | 21/27 (78) | 5/7 (71) | 7/7 (100) |
| 800 | 2/20 (10) | 25/35 (71) | 4/7 (57) | 5/5 (100) |

The number of animals surviving at 3 months post-BMT/total number of animals transplanted is shown. The percentage of surviving animals for each group is indicated in parentheses. A total of 155 newborn animals were transplanted. All of these animals in the 0, 200, or 400 cGy groups were 1 day old. Among the 800 cGy group, the first 25 newborn animals transplanted also were 1 day old. These animals all died within 1 week of the transplant. An additional 30 newborn 4-day-old animals were therefore transplanted in the 800 cGy group and 27 of these animals survived (12 ASMKO, 25 normal). Chi-square analysis showed that: 1) overall survival is significantly reduced at 400 and 800 cGy doses as compared with the 0 and 200 cGy groups ($P < .0001$); (2) that ASMKO mice have greater mortality than normal animals ($P = .0001$); and (3) that newborns have reduced survival as compared with adults ($P = .017$).

In general, adult mice were more resistant to the radiation pathological effects. However, even in this age group we noted that ASMKO mice were more sensitive to radiation then normal mice, although to a much lesser degree than newborn animals, and no statistically significant differences in survival were seen among these different adult age groups.

*Engraftment as a Function of Radiation Dose, Recipient Age, Genotype, and Cell Dose*. To assess the effects of various parameters on the engraftment of donor cells in BMT recipients, a series of gender-mismatched transplants (male into female) were performed. Figure 2 and Table 2 show that in newborn and adult mice, blood engraftment was significantly affected by cell dose, radiation dose, and/or genotype. These data also showed that newborn ASMKO mice engrafted less than normal mice (Fig 2) and had increased mortality at higher radiation doses (i.e., above 200 cGy) (Table 1). Among adult animals, similar trends were evident, but less dramatic.

Among 28 animals that were transplanted without receiving prior radiation (newborn and adult), blood engraftment was < 10% (Fig 2). In contrast, among newborn animals subjected to a single radiation dose of 200 cGy before transplant (n=25), engraftment ranged from 11% to 98%; notably, none of the 200 cGy animals transplanted as newborns failed to engraft. Increasing the radiation dose from 200 to 400 cGy led to moderately increased engraftment in the newborn recipients. In general, engraftment in the newborns was proportional with the donor cell number, particularly within the 200 and 400 cGy groups (Fig 2) and continued to increase up to 8 months post-transplant (not shown).

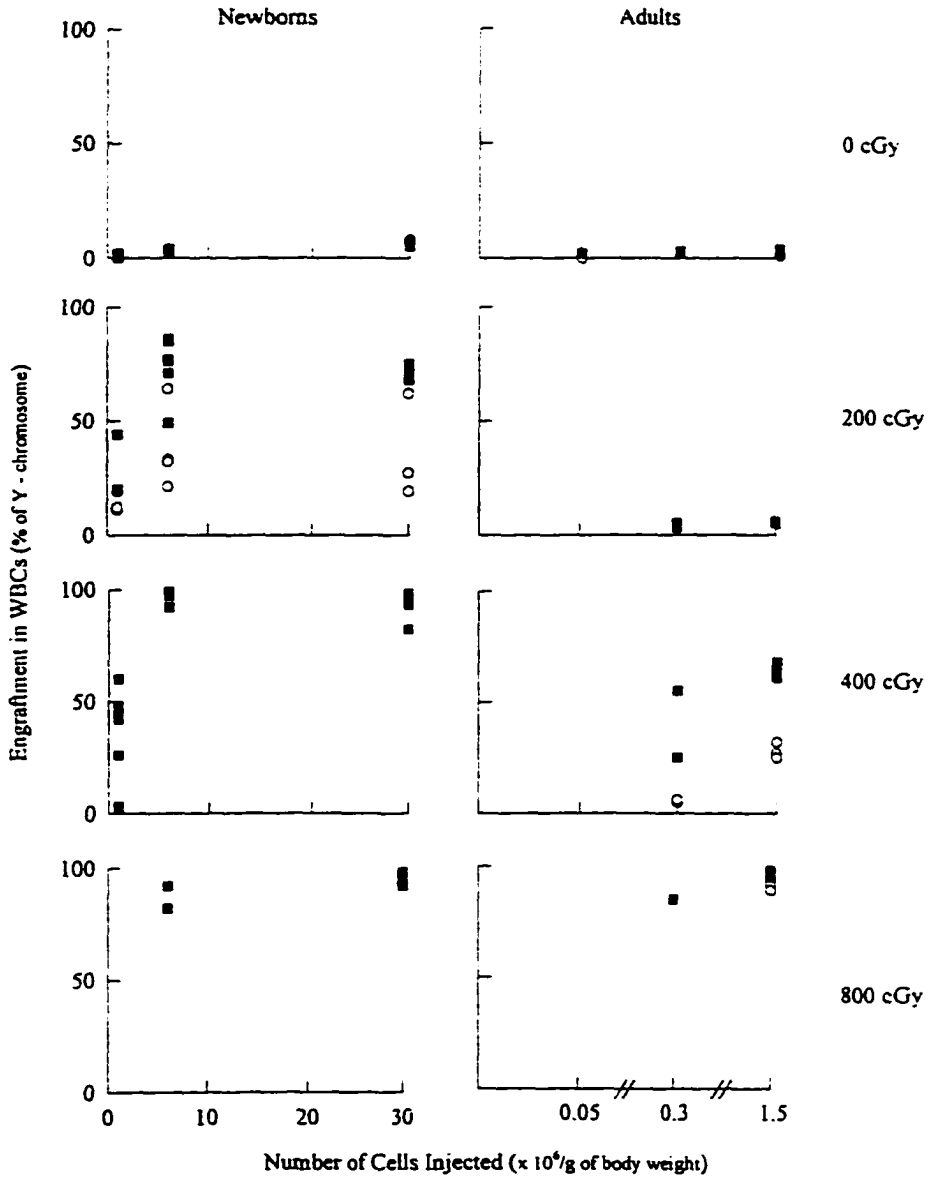


**Fig. 2. Engraftment as a function of radiation dose, age, genotype, and donor cell number.** For the newborn transplants. 18, 25, 16, and 13 female transplant recipients were analyzed for the 0, 200, 400, and 800 cGy groups, respctively. For the adult transplants, 10, 11, 12, and 9 female recipients were analyzed for each group, respectively (■, normal; ○, ASMKO). Engraftment = the number of male cells detected among 300 nuclei analyzed in the female recipients. A t-test analysis showed that engraftment was significantly less in the ASMKO mice as compared with normal mice (see Table 2).

**Table 2: Statistical Analysis of Engraftment**

**Parameters in Newborn and Adult Mice**

| Effect(s) | Newborns F (df) | Newborns P | Adults F (df) | Adults P |
|---|---|---|---|---|
| Cell dose | 9.8 (2) | .0005 | 27.0 (1) | .0001 |
| Radiation | 105.9 (1) | .0001 | 55.4 (1) | .0001 |
| Cell dose X radiation | 6.5 (2) | .004 | 9.7 (3) | .004 |
| Genotype | 9.9 (1) | .004 | --- | --- |
| Genotype X radiation | 11.7 (1) | .002 | --- | --- |
| Genotype X cell dose X radiation | --- | --- | 13.0 (3) | .0001 |

Statistical analysis (analysis of variance) of various engraftment parameters were modeled as described in Materials and Methods. Values are only provided for significant effects. The results indicate that the extent of engraftment is affected by cell dose and by the amount of pretransplant radiation. In addition, engraftment is significantly reduced in ASMKO mice as compared with normal mice (see Fig 2 and text for details). Dashes indicate effects that were not significant. Abbreviation: df, degrees of freedom.

In contrast to the results with newborns, none of the adult animals (n = 11) receiving 200 cGy engrafted more than 10%. However, it should be recognized that although the same number of cells were injected into the newborn and adult recipients. the cell dose (i.e., cell number injected per gram of body weight), was significantly less in the adults than the newborns (Fig 2). To achieve engraftment in the adult animals. radiation doses of 400 cGy or higher were required; indeed, at the 400 cGy dose, nearly all of the adult animals receiving a cell dose greater than or equal to $3 \times 10^3$/g engrafted.

*Effect of Recipient Sex on Engraftment.* An additional set of 20 ASMKO 1-day-old animals (10 each male and female) were subjected to 200 cGy and transplanted with normal male cells. ASM assays of the peripheral blood WBCs showed that there was a slight improvement in the gender-matched transplants as opposed to those that were gender-mismatched (Fig 3), although these differences were not statistically significant.

*Donor-Derived Cells and Enzyme Analysis in the Brain.* To monitor the presence of donor-derived cells in the brain, Y-chromosome in situ hybridization in brain sections was performed by Dr. Miranda. Donor-derived cells were distributed uniformly throughout the brains of newborn recipients and were evident in the cerebral cortex. thalamus, and other forebrain structures, as well as brain stem and cerebellum. No special association was seen with choroid plexus, ependyma or subependymal zones. Notably, no donor-derived cells were detected in the brains of animals transplanted as adults, irrespective of the radiation dose, number of cells transplanted, or degree of engraftment in the blood (Fig 4). In contrast, among newborns, donor cells were detected in the brains of

most engrafted recipients, although the number of donor-derived cells was low and increasing the radiation dose from 200 to 800 cGy did not dramatically improve this result (Fig 4). Figure 5 and Table 3 illustrate that there was a clear correlation between the degree of blood engraftment in the newborn group and donor cell entry into the brain. and that brain engraftment was better in ASMKO than in normal newborns.

In addition to the Y-chromosome analysis, ASM activities in the brains of transplanted ASMKO animals also were measured (by Dr. Miranda). In the absence of zinc, no ASM activities were detected in brain homogenates, while in the presence of 0.1 mmol/L $ZnCl_2$, a small amount of activity (from 2% to 8% of normal) was found in 10 of 19 animals transplanted as newborns (not shown).

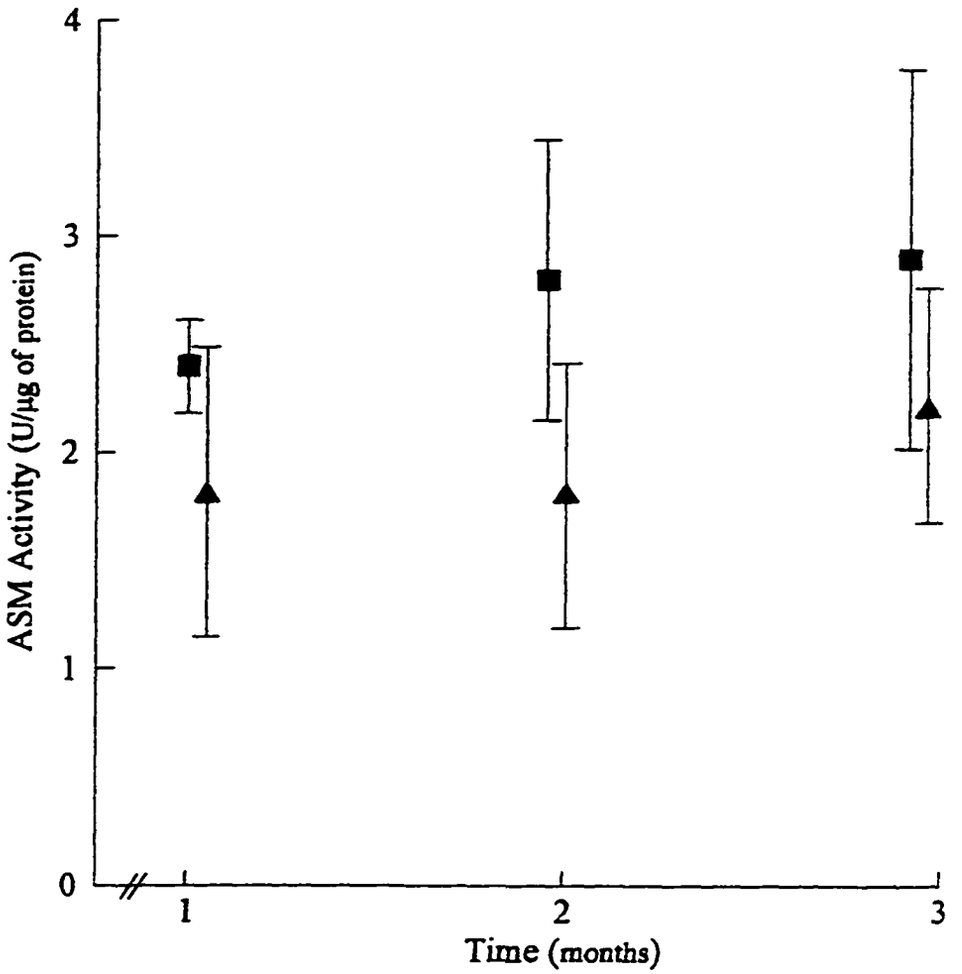


**Fig. 3. Comparison of engraftment in gender-matched and mismatched transplants.** To compare engraftment in the gender-matched (■) and mismatched (▲) transplants. ASM activities were determined. For this comparison, the transplants were performed on 1-day old ASMKO animals subjected to 200 cGy of radiation before the transplant, and receiving 3 x $10^7$ normal cells/g of body weight. 1 U = the amount of BODIPY-ceramide produced/hour/ml. The mean and standard error of the mean are plotted in the graph. Note that although the mean values are higher for gender-matched transplants as compared with gender-mismatched, the differences are not statistically significant. The t-test values for months 1, 2, and 3 were $t$ = 1.84/P = .079, $t$ = 0.847/P = .415, and $t$ = 1.13/P = .272, respectively.

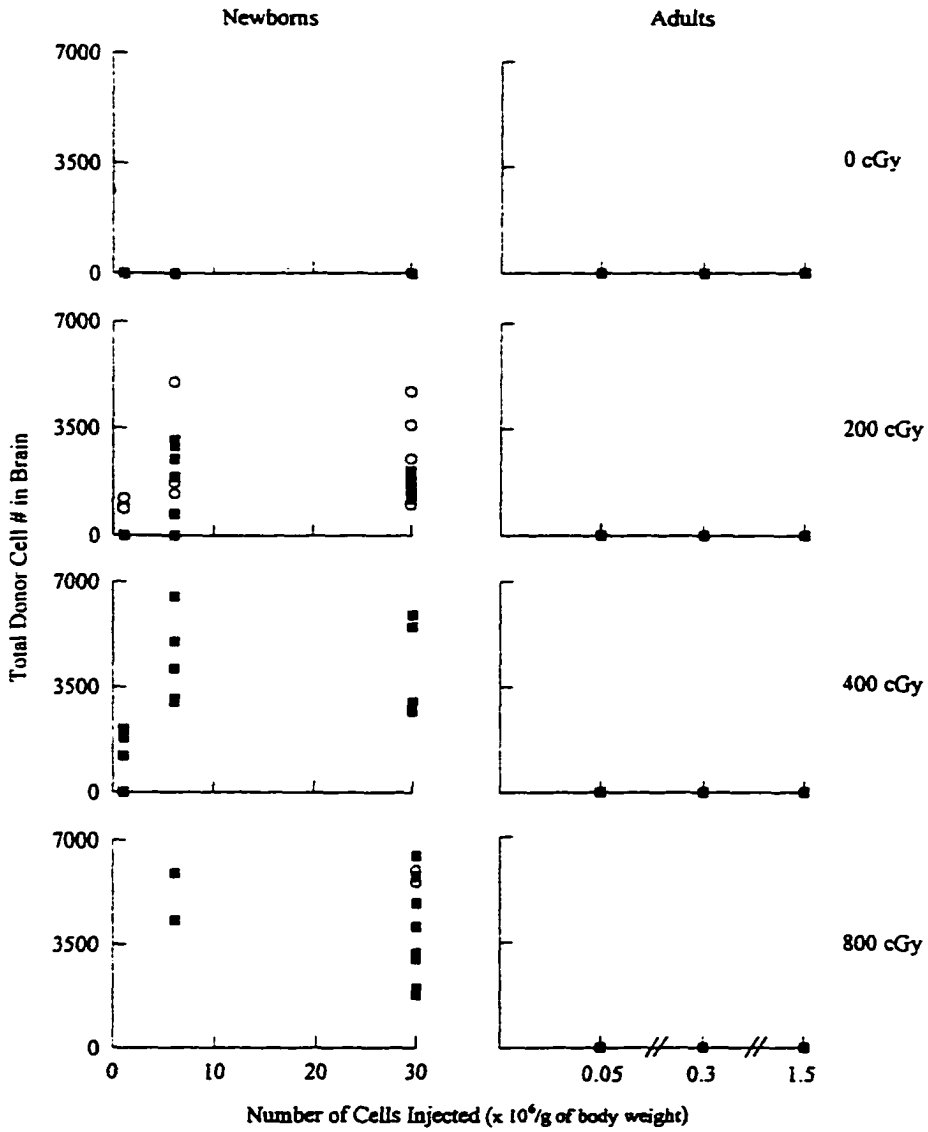


**Fig. 4. Donor-derived cells in the brains of transplanted recipients as a function of radiation dose, age, genotype, and donor cell number.** For the newborn transplants, 18, 25, 16, and 13 female transplant recipients were analyzed for the 0, 200, 400, and 800 cGy groups, respectively. For the adult transplants, 10, 11, 12, and 9 female recipients were analyzed for each group, respectively (■) normal; (○) ASMKO. A *t*-teat analysis showed that brain engraftment was significantly higher in the ASMKO mice as compared with normal mice (see Table 3).

**Table 3: Statistical Analysis of Brain Engraftment Parameters in Newborn Mice**

| Effect | F (df) | P |
|---|---|---|
| Blood engraftment | 136.0 (1) | .0001 |
| Blood engraftment X genotype | 23.9 (1) | .0001 |

The number of donor-derived cells in the brains of newborn mice 3 months post-BMT was modeled to evaluate the effects of blood engraftment, genotype, and their interactions [Model F(3.39) = 23.9; $P$ = .0001]. The number of donor-derived cells in the brain correlates with the number in the blood (see Fig 5) and is significantly higher in ASMKO as compared with normal mice Abbreviation: df, degrees of freedom.

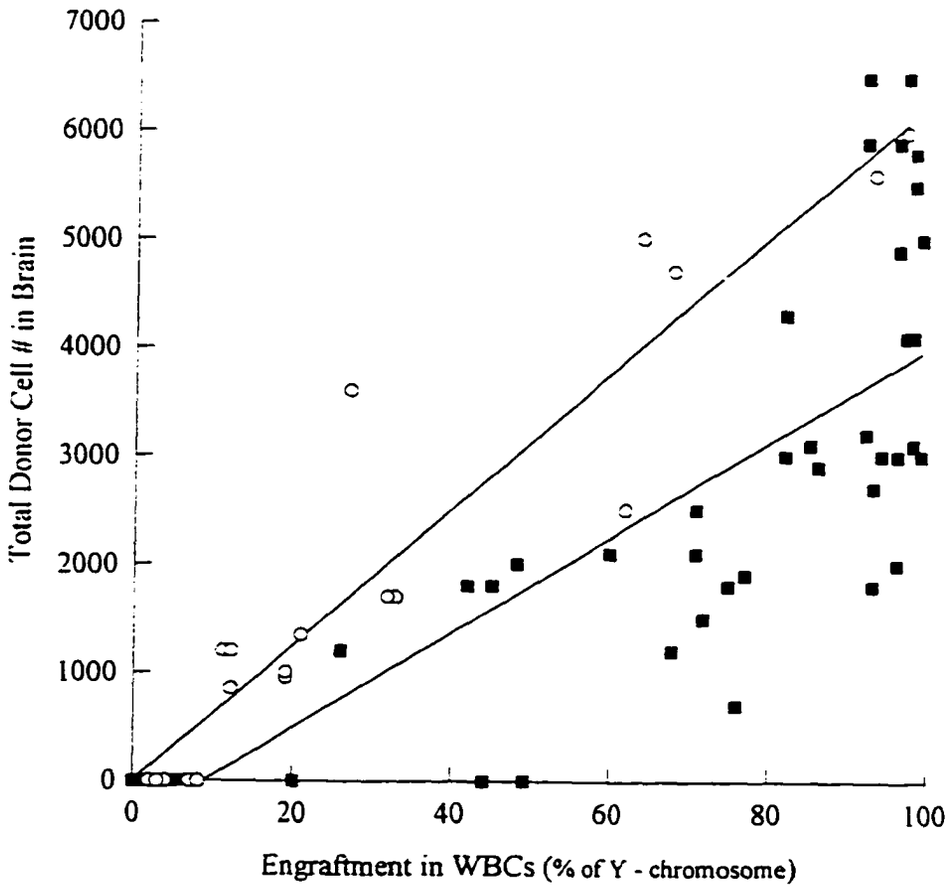


**Fig. 5. Correlation of engraftment in the brain and blood of normal and ASMKO newborn mice.** (■) Represents normal animals and (○) represents ASMKO animals. The correlation coefficient for normal animals was 0.704 and 0.889 for ASMKO animals.

# Discussion

Similarly to many other lysosomal storage diseases, there is currently no effective treatment for Types A and B NPD. Several treatment options, such as liver transplantation (Daloze, Delvin et al. 1977; Gartner, Bergman et al. 1986), amniotic membrane transplantation (Scaggiante, Pineschi et al. 1987), and BMT (Vellodi, Hobbs et al. 1987; Bayever, August et al. 1992; Bayever, Kamani et al. 1992) have been tried in the human patients, but the long term outcome of these procedures has not been properly documented. Therefore, we used the ASMKO mouse model of NPD to develop and evaluate BMT and HSCGT for the treatment of this disorder. Analogous approaches have been evaluated for a variety of other lysosomal storage diseases (Hoogerbrugge et al. 1988; Sands et al. 1993; Walkley et al. 1994). The work in this chapter represents the first step towards this goal, which is the development of an optimal transplantation protocol that will facilitate engraftment in NPD individuals without causing high mortality or pathology. Several parameters were evaluated, including radiation dose, cell dose, and the age and phenotype of the recipient animals. In addition, engraftment of donor cells in the brains of recipient animals was measured, since a major pathologic component of Type A NPD resides in the CNS.

Among the notable results, no engraftment was obtained in any experimental animals without minimal marrow ablation. However, we have found that newborn animals could be engrafted at the relatively low dose of 200 cGy. The 200-cGy dose did not cause mortality in the recipients, nor did it lead to any neural or visceral pathology.

Adults only showed engraftment at doses of 400 cGy and higher. However, the likely explanation for this finding relates to the fact that although the same total number of cells was injected into the newborn and adult animals, the cell dose (i.e., number of cells injected per gram of body weight) was markedly less in the adults than the newborns.

Notably, blood engraftment was significantly higher in normal animals (newborn or adult) than ASMKO animals. This result is intriguing because it was recently shown that certain NPD cell types (e.g., lung epithelia) are resistant to radiation-induced cell death (Santana et al. 1996). Thus, it is possible that we did not achieve the same degree of radiation-induced myeloablation in the ASMKO recipients as we did in the normal recipients, and less "space" was therefore available for donor cell engraftment.

Another striking result obtained from these studies was the fact that newborn ASMKO mice were more susceptible to radiation-induced death than normal newborn mice. This result was not due to the negligence of ASMKO mothers, as only non-affected, heterozygous mothers were used. On the surface, this finding may seem in conflict with the fact that certain cell types in the ASMKO mice are radiation resistant (Santana, Pena et al. 1996). However, it must be noted that the mechanism(s) underlying the increased mortality of the ASMKO mice observed in the present study are unknown, and likely to be complex. Indeed, in the previously published report on radiation resistance (Santana, Pena et al. 1996), only two tissues were analyzed, lung and thymus. Moreover, in that report, the radiation doses and time course were completely different from those used in the present work.

With regards to the CNS, our results confirm the fact that the BBB of newborn animals is more amenable to hematopoietic cell migration than that of adults. Among all of the adult transplant recipient animals analyzed, including those subjected to a high radiation dose of 800 cGy and almost completely engrafted in the blood, no donor-derived cells were found in the brain. In contrast, among newborn recipients, donor-derived cells could be found in the majority of the animals, even those subjected to a low dose of 200 cGy and only partially engrafted in the blood. These donor cells were not due to blood contamination, as cardiac perfusion was performed before harvesting the brain tissue, and only the donor cells found in the brain parenchyma (not in the leptomeninges or blood vessels) were counted.

In general, the degree of brain engraftment in newborns correlated well with the degree of engraftment in the blood, however, significantly more brain engraftment was observed in ASMKO animals as compared with normal animals. It is possible that the neurodegenerative state of the ASMKO brain provides a better engraftment environment than the normal brain because more bone-marrow derived cells may be permitted to migrate into the diseased brain for immunologic surveillance. However, it is important to recognize that although some donor cells were usually found in the brains of animals transplanted as newborns (ASMKO or normal), the total number of donor cells/animal was very low. Despite this finding, up to 8% of normal ASM activity was detected in the newborn ASMKO transplant recipients. Because the detection of this activity was dependent on the addition of zinc, it may be assumed that this represents the secreted

form of ASM (Schissel et al. 1996). Indeed, because the animals were perfused before being killed, it is possible that even higher amounts of ASM activity were secreted by the small numbers of donor cells.

Most of the studies cited in this report describe data obtained 3 months post-transplantation. This time point was chosen for a variety of reasons, including an established literature documenting that engraftment in transplanted mice occurs by 1 month (Yeager et al. 1992), and the fact that maximal infiltration of microglia precursor cells into the CNS takes place by 3 months (Perry and Gordon 1988). However, it is possible that the number of donor-derived cells present in the recipient animals continues to increase beyond the 3-month time point (Ling et al. 1991). Indeed, in a small group of ASMKO animals (n = 10) that were subjected to 200 cGy and analyzed at 8 months post-transplant (not shown), the number of donor-derived cells found in the brain was increased as compared with those analyzed at 3 months. While the explanation for this finding is unknown, it is possible that donor-derived cells continued to migrate into the CNS of these animals throughout the entire 8-month study period, or, more likely, donor cells that had entered the CNS soon after radiation/transplantation continued to divide and provide more enzyme-expressing cells within the brain.

Thus, these studies provide important data concerning the design of BMT and/or HSCGT trials for NPD. Based on our results we have identified an optimal transplantation protocol that was used in a large BMT experiment in ASMKO mice. In this protocol newborn ASMKO mice (1-4 day old) received a single total body irradiation

dose of 200 cGy. Following their irradiation, the mice were injected intra-peritoneally with $1.5x10^7$of freshly prepared normal bone marrow cells. A second dose of $1.5x10^7$ freshly prepared normal cells was injected in the same way at 24 hr post-irradiation. The results of these experiments are described in chapter 3.

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# Chapter 3

## Biochemical, Pathological & Clinical Effects of Normal Bone Marrow Transplantation on the ASMKO Mouse Phenotype.

# Abstract

Acid sphingomyelinase knock-out (ASMKO) mice are a model of types A and B Niemann-Pick disease. In the present study, we evaluated whether bone marrow transplantation (BMT) carried out on newborn ASMKO mice could prevent or alter the Niemann-Pick disease phenotype.

Previous work from our laboratory (Chapter 2) has helped us in identifying and establishing a safe yet effective protocol for BMT in ASMKO mice. In the present study a large group of 1-day-old ASMKO (n=35) mice were transplanted with normal bone marrow after being given total body irradiation at a "sub-lethal" dose of 200 cGy. The transplantation effects were then analyzed by biochemical, pathological, and clinical approaches.

Following BMT 97% of the transplanted animals showed engraftment ranging from 7% to 100%. The effects of BMT on the ASMKO phenotype included: (1) Improved growth, (2) Increased survival from a mean of 5 months to 9 months; (3) Delay in the onset of ataxia; (4) Reduced lipid storage in several organs including the brain. (5) Histologic improvement seen throughout the reticuloendothelial system; and (6) Reappearance of the Purkinje cells in certain areas of the transplanted animals cerebella.

The results demonstrated that although BMT had a significant ameliorating effect on the ASMKO phenotype, it was not, by itself, sufficient to cure the CNS disease.

# Introduction

As with many other lysosomal storage diseased, there is currently no treatment available for Niemann-Pick disease (NPD) (Schuchman and Desnick 1995). Therefore, a major hypothesis of my research is that bone marrow transplantation (BMT) and hemopoietic stem cell gene therapy (HSCGT) could be used to treat this disease. The rationale underlying this hypothesis is that since one of the major cellular sites of pathology in NPD is the bone marrow-derived macrophage ("NPD cell"), then replacement of these cells by transplanting normal hemopoietic cells (or genetically "corrected" NPD hemopoietic cells, see chapter 4) would give rise to metabolically normal macrophages. These macrophages will metabolize the sphingomyelin (SPM) that has been accumulated in the tissues, thereby minimizing the damage to involved organs. Furthermore, it is speculated that donor-derived cells of the monocyte-macrophage system will release normal enzymes at various sites of pathology for uptake by parenchymal cells. Such "secretion-reuptake" has been observed in cell culture systems (Yeyati et al. 1995). This approach should be most effective for Type B NPD where indeed the bone marrow-derived macrophage is the primary site of pathology (Schuchman and Desnick 1995). It is also possible that if BMT were successfully carried out on Type A NPD infants before the onset of the major neurologic symptoms, it could slow the neurodegenerative course by replacement of microglia in the central nervous system (CNS) that would supply sufficient acid sphingomyelinase (ASM) for enzymatic "cross-correction" of neurons. Indeed, based on these principals, BMT experiments in human NPD patients have been performed (Vellodi et al. 1987; Bayever et al. 1992;

Bayever et al. 1992). Unfortunately, only small numbers of patients have been studied and the results were inconclusive. However, the beneficial effects of BMT have been demonstrated in a number of experimental animal models of other lysosomal storage diseases (Hoogerbrugge et al. 1988; Sands et al. 1993; Walkley et al. 1994).

Previously, the ASM "knock-out" (ASMKO) mouse has been characterized in our lab (see chapter 1) (Horinouchi et al. 1995; Otterbach and Stoffel 1995) and was found to mimic the clinical, biochemical, and pathological findings of Types A and B NPD. Therefore, it is an excellent model in which to study basic aspects of the disease pathogenesis and to investigate various approaches to treatment. In chapter 2 a pilot study was described which measured the effects of radiation dose, age, and genotype on engraftment after BMT in ASMKO mice (Miranda et al. 1997). Following that study, a uniform, safe and effective protocol for BMT in ASMKO was implemented in our laboratory. In this chapter, the clinical, biochemical, and pathological results of this procedure in newborn animals that were preconditioned with a low dose (200 cGy) of total body irradiation. These studies were carried out to evaluate the efficacy of BMT for NPD patients, and are part of ongoing efforts to develop HSCGT for this disorder.

# Materials and Methods

*Mice.* The ASMKO mouse colony was established from heterozygous breeding pairs obtained by gene targeting of 129/Sv embryonic stem cells and subsequent micro-injection into C57BL/6 blastocysts (Horinouchi, Erlich et al. 1995).

*Preparation of Donor Cells.* Normal male mice (6-12 weeks old) from within the colony were used as donors. Donor animals were killed by cervical dislocation, and the bone marrow cells were harvested from the femura and tibia by flushing the medullary cavities using Hanks' balanced salt solution (GIBCO BRL, Gaithersburg, MD) and a 27-gauge needle. The cells were washed twice in Hanks' solution, and single cell suspensions were obtained by passage through a cell strainer (40 mm, Becton Dickinson, Franklin Lakes, NJ). These cells were then counted, diluted to the desired concentration, and immediately injected into the recipients peritoneal cavity. The use of temporal vein injection in newborn animals has been discontinued in our lab because of the technical difficulties associated with this procedure and the lack of assurance that the cells are indeed delivered into the circulation. This work was carried out jointly by me and Dr. Silvia Miranda.

*Transplantation Protocol.* Thirty-five 1-day-old newborn ASMKO mice were used as recipients. Before receiving the transplant, the recipients were subjected to a single total body irradiation dose of 200 cGy from a dual $^{137}Cs$ source (dose rate = 80 cGy/min). Each animal received a total of 3x $10^7$cells/g of body weight given in two doses

on sequential days. Age-matched litter-mates (normal, n=6; and ASMKO, n=6) were used as irradiation controls (i.e., irradiated, but not transplanted). The animals were maintained on a 12-hr light/dark cycle, water ad libitum, and Purina rodent chow 5001. No antibiotics or other supportive care were given to any of the animals before or after transplantation. This work also was carried out jointly with Dr. Miranda.

*Post-Transplant follow-up and Tissue Processing.* The weights of the transplanted and control animals were registered monthly by Dr. Miranda. Peripheral blood also was obtained by retinal orbit bleeding for monthly post-BMT enzyme analysis in white blood cells (WBCs) (done by me). WBCs were isolated after lysis of the red cells using a hemolytic buffer (0.1 M $NH_4Cl$, 12 mM $NaHCO_3$, 10 mM EDTA, pH 8.0). Three months after BMT, a set of 10 ASMKO mice were anesthetized with ketamine (Sigma. St. Louis. MO; 0.5 g/kg of body weight) and subjected to cardiac perfusion. An incision was made in the right atrium to allow blood to flow out, and a cannula was introduced through the left ventricle into the aorta, delivering 50 ml of warm 0.9% NaCl solution. After perfusion, tissues were collected and immediately frozen on dry ice for subsequent sectioning and enzyme/lipid analysis. Tissue samples for light microscopy were fixed and given to Dr. Mark Haskins from the Department of Pathology at the University of Pennsylvania School of Veterinary Medicine for analysis.

*Purkinje Cell Counting.* Paraffin sections of cerebella were analyzed by Dr. Silvia Miranda for the presence of Purkinje cells.

*ASM and Protein Assays.* Sphingomyelin covalently linked to the fluorescent probe BODIPY (BODIPY dodecanoyl sphingosyl phosphocholine; B12SPM) was synthesized as previously described for lissamine rhodamine sphingomyelin (LRSPM) (Dinur et al. 1992), except that BODIPY dodecanoic acid (Molecular Probes Inc., Eugene, OR) was condensed with sphingosyl phosphocholine. Cleavage of Bl2SPM by ASM releases fluorescent ceramide, which can be quantified after separation by thin layer chromatography (TLC) or organic extraction using a spectrofluorometer or fluorescent imaging instrument. WBCs or tissues were obtained as described above and homogenized in 0.2% Triton X-100 on ice using three 10-sec bursts of a Potter-Elvehjem tissue homogenizer (Thomas Scientific, Swedesboro, NJ). Total protein was determined by the method of Stein et al. (Stein et al. 1973). The standard 15-μl ASM assay mixture consisted of 10 μl of protein extract (from homogenized cells or tissues), and 2 nmol of B12SPM suspended in 0.1 M sodium acetate buffer, pH 5.2, containing 0.6% Triton X-100 and either 5 mM EDTA (for detection of the non-zinc-dependent ASM activity) or 0.1 mM $ZnCl_2$ (for detection of the zinc stimulated ASM activity) (Schissel et al. 1996).

After incubating the assay mixture at 37°C (up to 3 hrs), the samples were loaded on TLC plates (TLC LK6 D Silica gel 60, Whatman Clifton, NJ) and resolved using chloroform/methanol (95:5 v/v). After resolution, the band containing the fluorescently labeled ceramide was scraped from the TLC plates, extracted in chloroform/methanol/water (1:2:1 v/v/v) for 15 min at 55°C, and quantified in a spectrofluorometer (fluorescence spectrophotometer 204-A; Perkin-Elmer Cetus,

Norwalk, CT). The instrument's settings were excitation 505 nm and emission 530 nm. This work was carried out mainly by myself.

*Sphingomyelin Analysis*. Sphingomyelin (SPM) levels were determined from the phosphate content in each sample (Rousseau et al. 1986). Tissues (brain, liver, lung, and spleen) were homogenized in chloroform/methanol (1:2 v/v) for lipid extraction. Alkaline phospholipids hydrolysis was performed on the liver, lung, and spleen samples in 0.4 N KOH/90% methanol for 2 hr at 55°C. To avoid interference from plasmologen and other phospholipids in the brain extracts, acidic hydrolysis was carried out in these samples using 0.6 M HCI in ethanol for 30 min at 37°C before the alkaline hydrolysis. After hydrolysis, the samples were incubated with perchloric acid (70%) for 40 min at $180^0$C, followed by treatment with 0.5% ammonium molybdate and Fiske & Subbarow reducer (4 mg/ml; Sigma Chemical, St. Louis, MO) for 10 min at $100^0$C. Absorbency at 830 nm was measured in a spectrophotometer (model 1201, Milton Roy Spectronic Inc. Rochester, NY). This part was mainly done by Dr. Miranda with my help.

*Statistical Analysis*. Descriptive statistics and t test were performed using the SigmaStat statistical software (version 2.0; Jandel Corp.).

# Results

*Survival and Growth of Transplanted ASMKO Mice.* Figure 1 shows the effects of BMT on the survival of ASMKO mice that were transplanted as newborns after irradiation with 200 cGy. ASMKO "control" animals (i.e., irradiated, but not transplanted) survived up to 5.5 months, whereas wild-type irradiation control animals had a normal life-span. Of note, ASMKO animals receiving BMTs had a prolonged life-span when compared with the non-transplanted ASMKO control group, from a mean of ~5 months to 9 months.

The weight of normal, ASMKO, and transplanted ASMKO animals were measured monthly after BMT (Fig. 2). The transplanted ASMKO mice gained weight normally up to 4 months after BMT, whereas in non-transplanted ASMKO controls weight loss was observed by the third month of age. However, despite the delayed onset, by the fifth month after BMT significant weight loss was seen in the transplanted group, and, from then on, the growth of these animals followed a pattern similar to the non-transplanted ASMKO controls. Although the mean weight values were always higher in males as compared with females, no significant differences were observed between genders when the overall data were submitted to statistical analysis (ASMKO transplanted, P=0.311; ASMKO controls, P=0.2271; normal controls, P=0.3260).

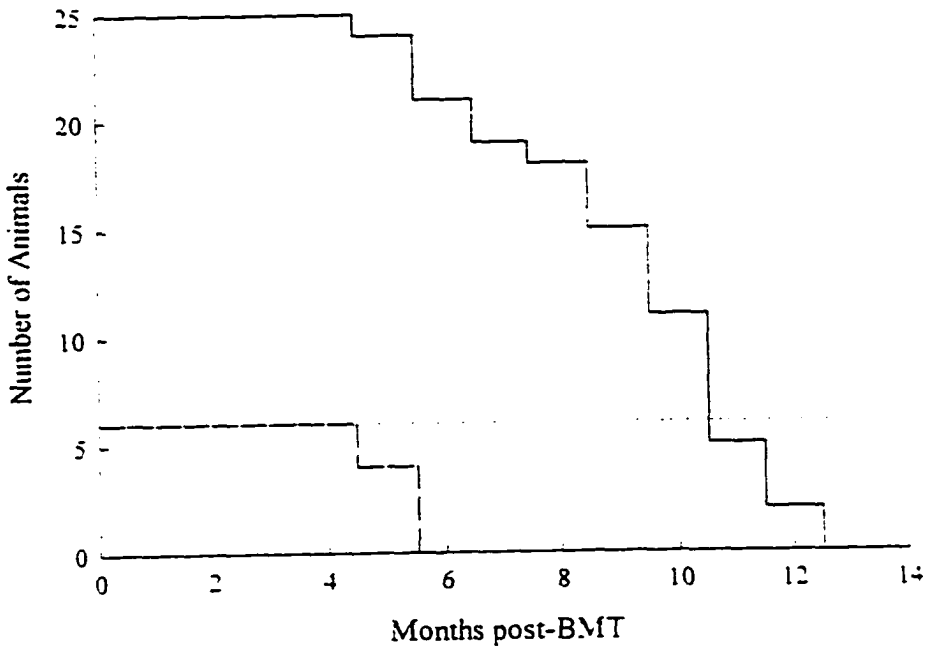


**Fig. 1. Survival rate of ASMKO transplanted and control mice after BMT.** Dotted line, normal irradiation controls; hatched line, ASMKO irradiation controls; solid line, transplanted ASMKO mice.

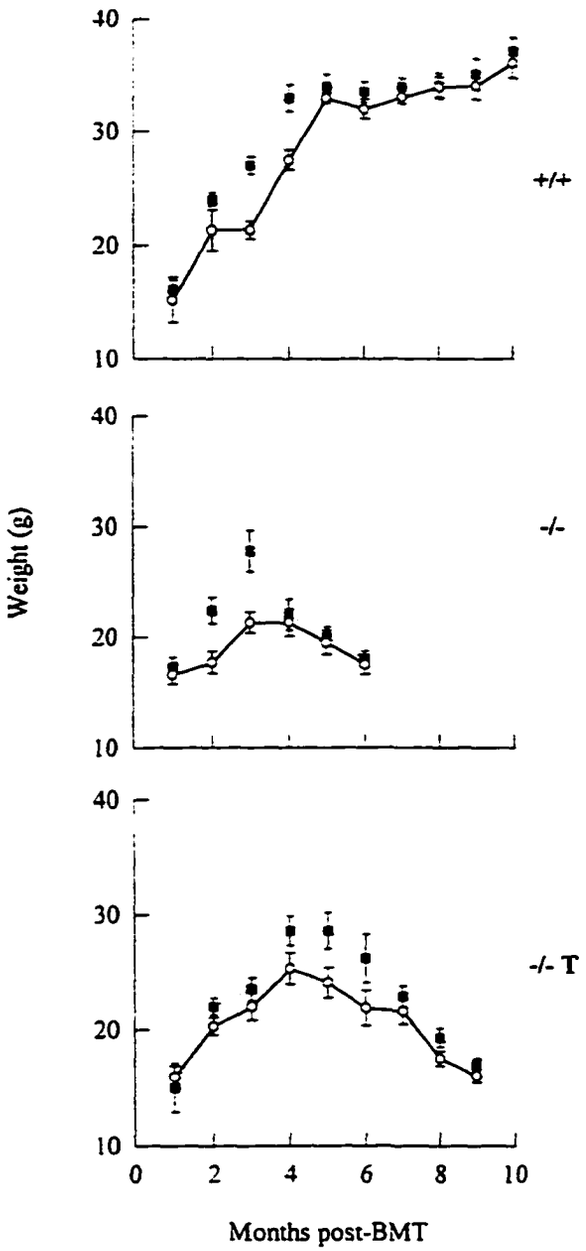


**Fig. 2. Growth profiles of transplanted ASMKO and control mice after BMT.** +/+, normal control mice (n=6); -/-, ASMKO control mice (n=6); -/- T, transplanted ASMKO mice (n=25). ■, males; ○, females. Significant differences were found among transplanted and normal control animals (ASMKO transplanted males vs. normal control males, *P=0.0065;* ASMKO transplanted females and normal control females, *P=0.0056).* However, no statistical differences were observed between ASMKO transplanted and non-transplanted mice (males, *P=0.58;* females, *P=0.3422).* Mean values are shown along with the standard error of the mean.

*Engraftment.* To monitor engraftment, WBC ASM activities were measured by me monthly after BMT. Our previous work (chapter 2) had shown that WBC ASM activities were directly correlated with the number of donor derived cells in the blood of transplanted ASMKO mice (as determined by Y-chromosome in situ hybridization), and were an accurate measure of engraftment (Miranda, Erlich et al. 1997). In the present study (Fig. 3), 97% of the transplanted animals had ASM activities that were greater than ASMKO control values (ranging from a mean of 40-60% of normal). However, the activities varied widely among individual animals, from 7% to 100% of normal. Engraftment in males (gender-matched transplants) were higher than in females (gender-mismatched), although the differences were not statistically significant (P=0.1579). When the WBC ASM activities were analyzed in individual animals surviving at various times after BMT (Fig. 1), no direct correlation was observed between activity (i.e., engraftment) and survival.

*Tissue ASM Activities and Sphingomyelin Levels.* In order to assess tissue enzyme levels and substrate clearance, 3 months after BMT a set of 10 transplanted animals was euthenized and the ASM activities and SPM contents were measured. In Figure 4, the ASM activities in several tissues are shown, whereas Figure 5 depicts the SPM content. Note that, in the transplanted group, significant ASM activities were detected in the WBCs and bone marrow, and that the two activities were correlated in individual animals. Indeed, in several animals, the enzyme activities from these sources were essentially normalized. Among other tissues however, only the liver, lung, and spleen had low levels of enzyme detected, whereas, in brain, heart, and kidney, there was essentially

no detectable ASM activity. Addition of zinc to the assay mixtures, which facilitates the detection of the secreted, zinc-activated form of ASM (Schissel, Schuchman et al. 1996), did not significantly alter the results (not shown).

Regarding SPM storage, a marked accumulation of lipid was evident in each of the four tissues analyzed from the ASMKO control mice (Fig. 5), whereas, in the 10 transplanted animals, the SPM levels were reduced from an average of 35% in the brain to 55% in the liver. Although, in some tissues, an inverse correlation was observed between WBC ASM activities and SPM levels, no general trend was found among individual animals.

*Pathology.* To evaluate the effects of BMT on the ASMKO histology, bone marrow, brain, liver, lung, and spleen were sectioned and analyzed by light microscopy (by Dr. Haskins). Figure 6 shows an example of this analysis in the bone marrow, liver, and spleen from two transplanted animals, one engrafted at 14% and the other 90%. Note that in the bone marrow and liver, both transplanted animals showed a marked improvement in storage. However, in the spleen, only the highly engrafted animal appeared normalized, whereas the animal engrafted at 14% still had evidence of substantial storage. Analysis of lung sections was similar to the spleen (not shown).

An intriguing aspect of the ASMKO phenotype is an almost complete loss of the Purkinje cell layer in the cerebellum (Horinouchi, Erlich et al. 1995). This phenotype becomes obvious by 3 months of age, when these cells are nearly absent. In the

transplanted ASMKO animals studied 3 months after BMT (by Dr. Miranda), Purkinje cells were found in the lingula, central, and culmen lobules of the cerebellum, and quantitative analysis showed an increased number of Purkinje cells in those regions (Fig. 7). In accordance with these findings, we observed that the onset of ataxia was delayed in many of the transplanted animals, and storage was reduced in the spinal cord (Figure 8) and cerebral cortex (not shown).

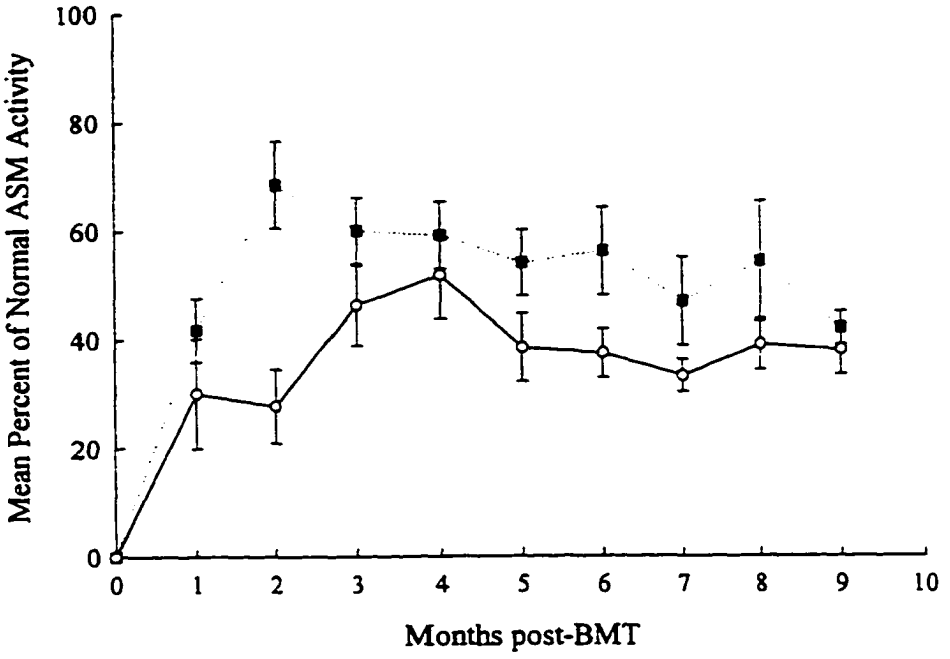


**Fig. 3. ASM activities in WBCs of transplanted ASMKO mice (n=25) after BMT. ■, males; ○, females. Mean values are shown along with the standard error of the mean.**

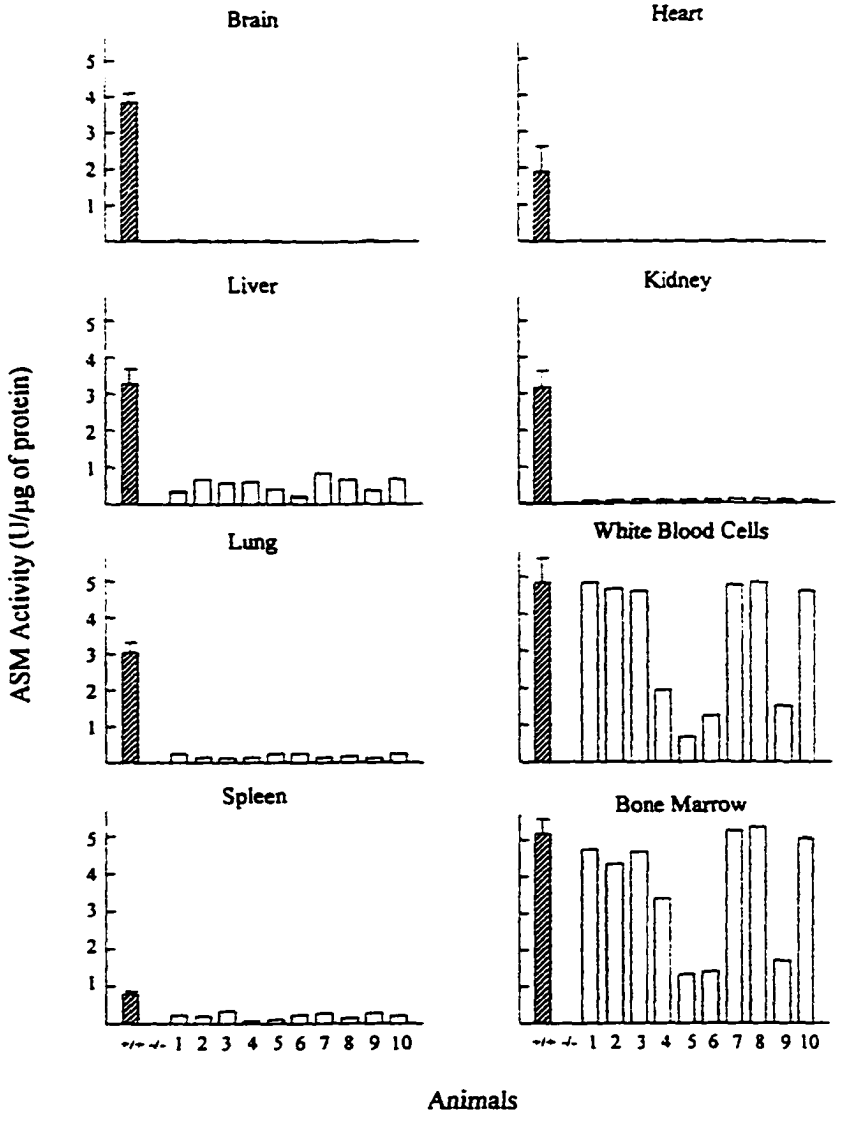


**Fig. 4. ASM activities in tissues of transplanted ASMKO and control mice 3 months after BMT.** Hatched bar represents normal irradiation controls *(+/+, n = 6)* and open bars show data from individual transplanted ASMKO mice (1-10). Note that ASMKO irradiation control animals *(-/-,* n=6) had no residual ASM activity. Animals were perfused before harvesting the tissues, and the plotted values were obtained from enzyme assays performed without the addition of zinc.

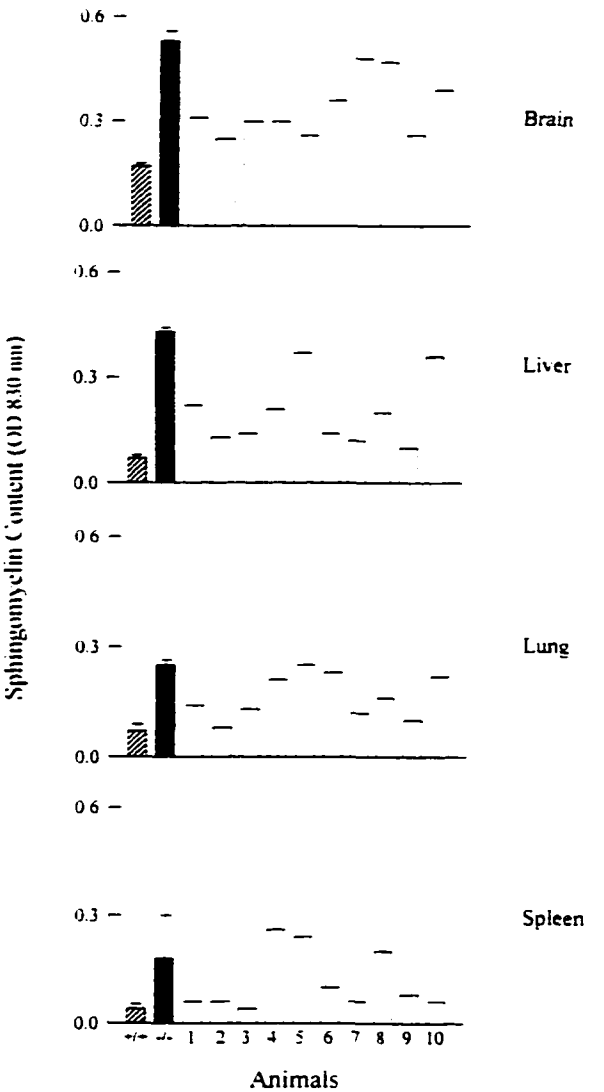


**Fig. 5. Sphingomyelin content in tissues of transplanted ASMKO and control mice 3 months after BMT.** Hatched bar represents normal irradiation controls (+/+; n=6), solid bar represents affected irradiation controls (-/-; n=6), and open bars show data from the individual, transplanted ASMKO mice (1-10).

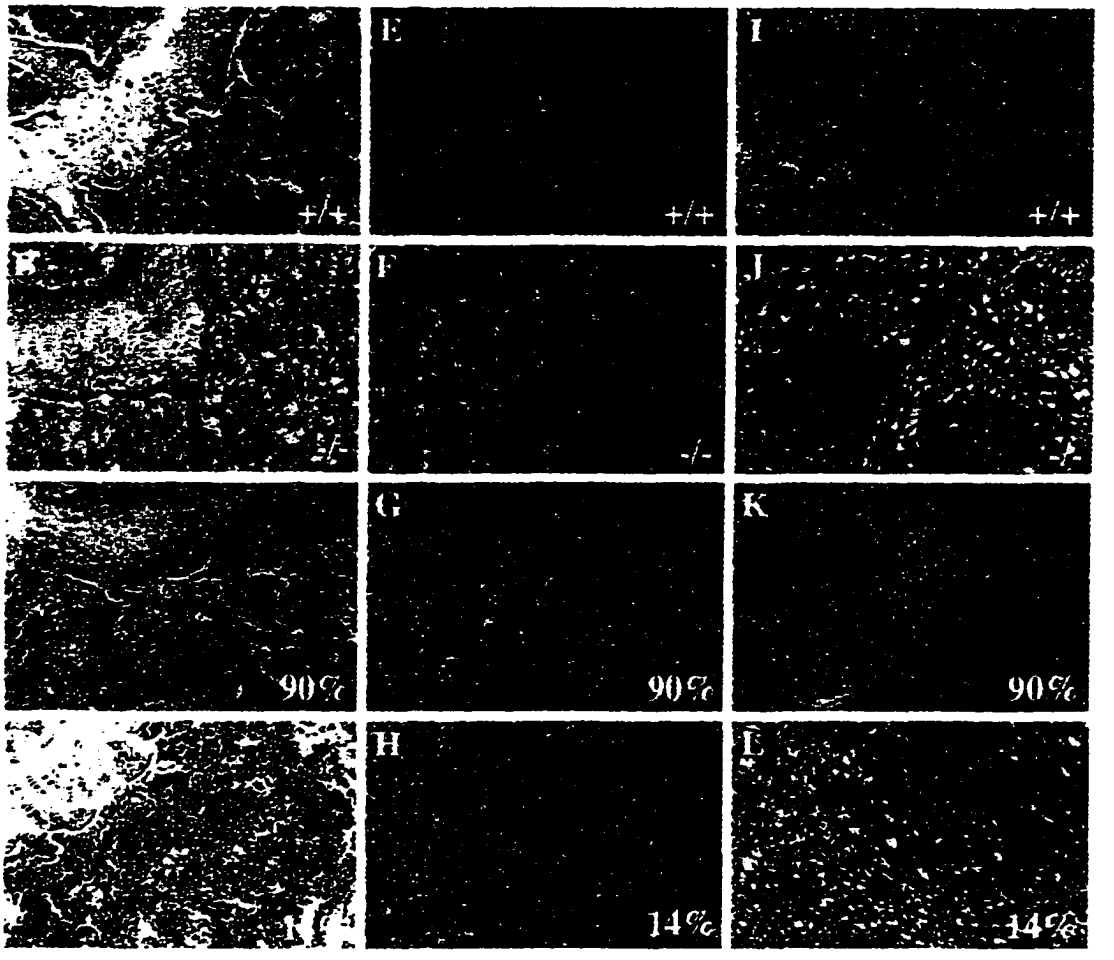


**Fig. 6. Bone marrow (panels A-D), liver (panels E-H), and spleen (panels I-L) sections from transplanted ASMKO and control mice 3 months after BMT.** +/+, normal control mice; -/-, affected control mice; 90% and 14% refer to the engraftment in two representative transplanted ASMKO animals (original magnification. x 200).

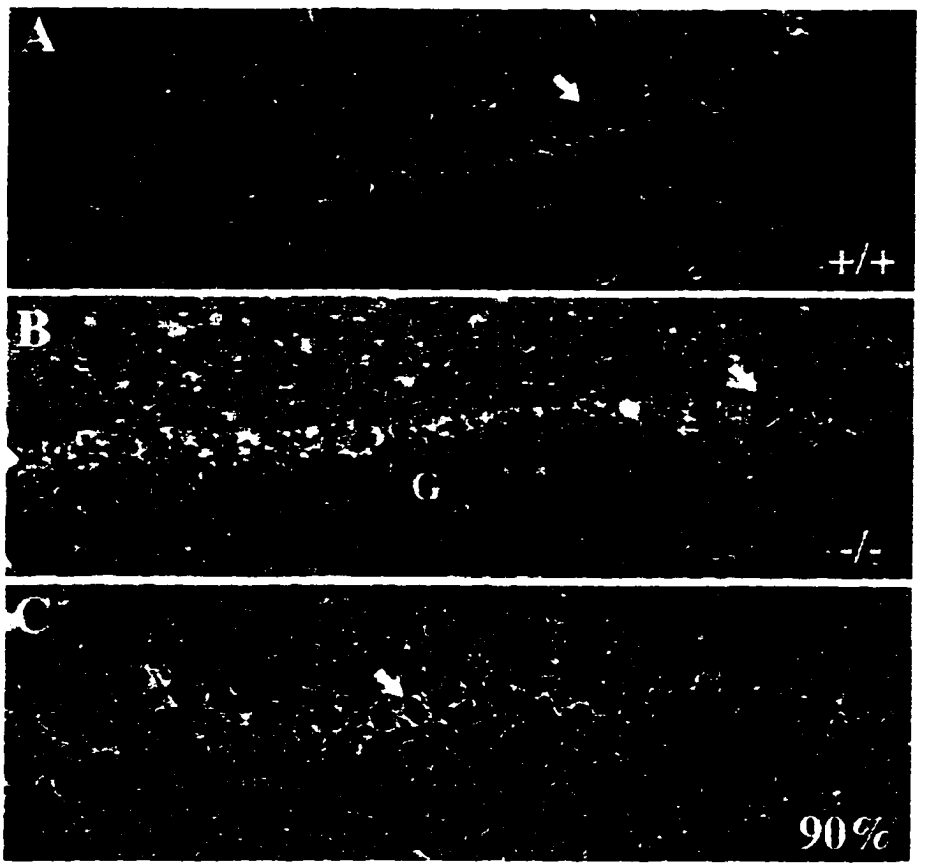


**Fig.7. Cerebellum sections of transplanted ASMKO and control mice 3 months after BMT.** (A) Normal control (+/+); (B) ASMIKO control (-/-); (C) transplanted ASMKO mouse (90% engraftment). Arrows point to Purkinje cells (large, round, and highly eosenophilic cells between the granular and molecular layers of the cerebellum). Note that normal animals have a layer of Purkinje cells throughout the cerebellum. Non-transplanted ASMKO animals have virtually no Purkinje cells, whereas transplanted animals have regions without the layer (not shown) and some regions (lingula, central, and culmen lobules) with a layer very similar to normal controls. (original magnification, x800).

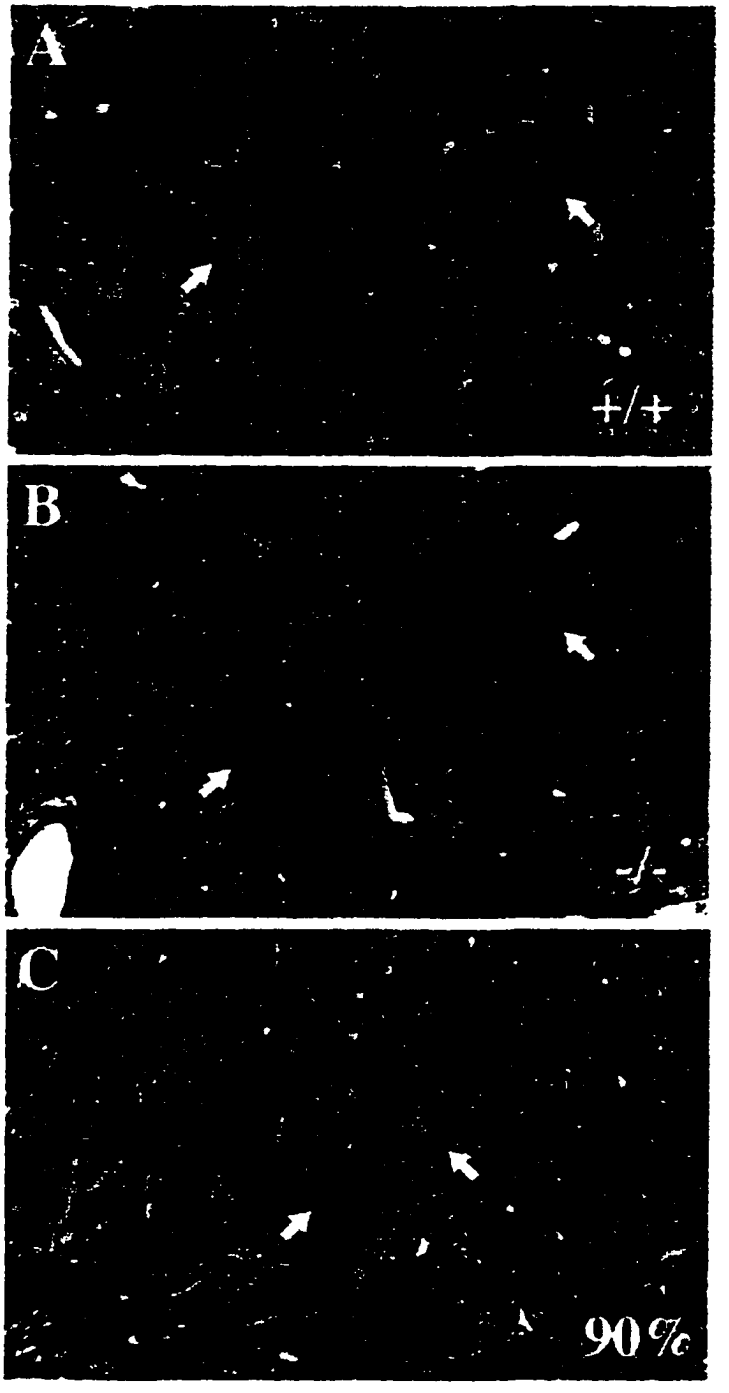


**Fig. 8. Spinal cord of transplanted ASMKO and control mice 3 months after BMT.** (A) Normal control (+/+); (B) ASMKO control (-/-); (C) transplanted ASMKO (90% engraftment). The arrows point to motor neurons Note the highly basophilic granules in the cytoplasm of ASMKO control as compared with normal animals and the fact that this material was reduced in the transplanted ASMKO animals (original magnification, x400).

# Discussion

In the present study, the ASMKO mouse model of types A and B NPD was used to evaluate the efficacy of BMT for the treatment of this disorder. Base on previous work from our laboratory (chapter 2) (Miranda, Erlich et al. 1997), recipient ASMKO newborns were preconditioned with a "sublethal" dose of 200 cGy which was effective yet safe (Miranda, Erlich et al. 1997). The results of our study revealed that high engraftment led to correction of the reticuloendothelial (bone marrow, liver, and spleen) and the lung pathology, and that even low levels of engraftment significantly improved pathology in some of these tissues. Moreover, CNS improvements also could be achieved as a result of BMT. However, despite these positive findings and an average increased life-span by ~4 months, all of the transplanted animals eventually developed ataxia and died.

These data in the animal model system support the limited information available from the human transplantation literature (Vellodi, Hobbs et al. 1987; Bayever, August et al. 1992; Bayever, Kamani et al. 1992) and suggest that BMT is a reasonable therapeutic option for Type B NPD, where the primary sites of pathology are within the reticuloendothelial system. However, with regard to the neuronopathic Type A NPD, although our results indicate that if BMT was accomplished during early infancy the onset of some neurologic symptoms may be delayed, they also suggest that complete treatment of the neurologic disease is highly unlikely by BMT alone. Indeed, even when nearly complete engraftment was achieved in the transplanted animals, only partial

changes in the CNS pathology were observed. Thus, treatment of Type A NPD patients will likely require high level over-expression and secretion of the ASM protein, intervention during fetal development, and/or a completely different therapeutic strategy.

An interesting CNS feature found in the ASMKO mice is an almost complete absence of the Purkinje cell layer (Horinouchi, Erlich et al. 1995). The loss of Purkinje cells is a well characterized phenomenon in several neurodegenerative diseases (Dick et al. 1986; Higashi et al. 1993; Khan 1993; Doughty et al. 1995; Triarhou et al. 1995; Tu et al. 1997) and, in NPD mice, occurs in an orderly and chronologic fashion, starting from the anterior vermis and progressing into the posterior vermis (Kuemmel et al. 1997). Although the mechanisms involved in the loss of this particular cell type are not totally understood, it is possible that the high metabolic rate in these cells intensifies the toxic effects of substrate accumulation as compared with other neurons (Kuemmel, Schroeder et al. 1997). This may lead to cell death in the Purkinje cells before other cell types. Interestingly, histologic analysis of the brains of transplanted ASMKO mice revealed an increased number of Purkinje cells, primarily in the lingula, central, and culmen lobules of the cerebellum. This observation is the likely explanation for the reduced ataxia observed in these animals and the increased life-span.

One implication of this finding is that the presence of Purkinje cells at 3 months after BMT is caused by normal ASM secreted from donor-derived microglia. Indeed, previous work has shown that microglial cell lines secrete ASM (Schissel, Schuchman et al. 1996). It has also been clearly demonstrates that the enzyme secreted from primary

glial cultures can be taken up by neurons (Miranda et al. 1998). However, as shown in Figure 4, almost no enzyme activity was detected in brain homogenates, consistent with our previous data showing that very few donor-derived cells crossed the blood brain barrier in the transplanted animals (Miranda, Erlich et al. 1997). Thus, the question arises as to why the Purkinje cells phenotype was as improved as it was. Although a definitive answer to this question remains unknown, perhaps the high metabolic rate of these cells, which could be targeting them for early cell death, also allows them to internalize secreted ASM more rapidly than other neurons after transplantation. Alternatively, they may simply have more receptors for ASM uptake than other cell types.

In summary, these studies provide valuable information concerning the efficacy of BMT for the treatment of NPD. However, as with many other rare metabolic disorders, the likelihood of finding HLA-compatible normal or heterozygous donors for most NPD patients is small. Thus, another viable therapeutic option for these patients may be autologous transplantation of genetically corrected hematopoietic stem & progenitor cells, alone or in combination with enzyme replacement. Toward this end, our lab has shown that ASM retroviral vectors can correct the metabolic defect in NPD cells and lead to overexpression of the enzyme in the transduced cells (Dinur, Schuchman et al. 1992; Suchi et al. 1992; Yeyati, Agmon et al. 1995). I have also developed a fluorescence-based system to isolate transduced NPD hematopoietic stem cells (see chapter 5). Studies are currently under way to evaluate this approach in the ASMKO mouse model (see chapter 4).

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# Chapter 4

## Retrovirally Mediated Gene Therapy for Niemann-Pick Disease.

# Abstract

Currently, there is no effective treatment for Types A & B Niemann-Pick Disease (NPD). Our previous work has shown the validity of using bone marrow transplantation (BMT) in the treatment of Type B NPD. However, due to the complications associated with BMT we have also investigated hemopoietic stem cell gene therapy (HSCGT) as an alternative therapeutic approach.

Previous work from our laboratory (Chapter 2) has helped us in identifying and establishing a safe yet effective protocol for BMT in ASMKO mice. Using a modification of this standard protocol, thirty-two newborn ASMKO animals were transplanted with ASMKO bone marrow transduced with an ecotropic retroviral vector containing the ASM cDNA. In about half of these animals an increased ASM enzymatic activity (up to 9-fold above normal activity) was detected in the white blood cells (WBC) at 1 month post-transplant. Animal growth patterns and enzymatic activity were monitored monthly in the transplanted animals. In addition, brain pathology and behavioral studies have been conducted at 4-5 month post transplant to determine the effect of the high enzymatic activity on the brain of transplanted animals. More comprehensive pathology studies will be initiated soon (at 6 month post-transplant) by Dr. Silvia Miranda.

# Introduction

Based on our BMT studies in the ASMKO mouse (see Chapters 2 and 3) we have determined that BMT is a good therapeutic approach for Type B Niemann-Pick disease. However, the use of allogenic BMT as standard procedure in the treatment of NPD is complicated by several problems such as the lack of fully compatible sibling donors in most families and complications due to lethal total body irradiation, immuno-suppression, infections, and graft vs. host disease (GVHD), which are often associated with this procedure. Therefore we are trying to develop and evaluate hemopoietic stem cell gene therapy (HSCGT) as an alternative to BMT (Dinur et al. 1992; Suchi et al. 1992; Yeyati et al. 1995) for the treatment of NPD. Using this approach, the two major problems of donor availability and GVHD are eliminated. Furthermore, enzymatically corrected cells (obtained by retroviral-mediated gene transfer) usually express the enzyme at higher levels than that found in normal cells. This high level of enzymatic activity might augment the moderate therapeutic effect seen in the brain of BMT treated animals thereby making HSCGT a useful therapeutic approach for the treatment of Type A NPD.

Since the discovery of the human ASM gene, our group has been active in the development and evaluation of gene therapy for NPD. One of the first goals of this research was to construct retroviral vectors which could be used to transduced cells from NPD patients, thereby correcting their metabolic defect. Three such vectors have been constructed by introducing the full-length human ASM cDNA into the retroviral vectors

NAT, pBC 140 and MFG (Dinur, Schuchman et al. 1992; Suchi, Dinur et al. 1992; Yeyati, Agmon et al. 1995). All of these vectors are based on the Moloney leukemia virus (MLV) genome. The pBC 140 is one of the classic retroviral vectors initially used for gene therapy. In addition to the ASM gene, which is under the control of the CMV promoter, it contains the neomycin resistance gene (*neo*) under the control of the viral 5' LTR (Fig 1-A). The inclusion of *neo* confers upon the transduced cells the resistance to the drug G418, and allows for their isolation before returning them to the patients. In the NAT vector, the ASM gene is coupled to a Herpes Simplex thymidine kinase (TK) promoter and cloned into the 3' LTR. Upon integration, the ASM gene is duplicated and moved to the 5' LTR region and its transcription is driven by the TK promoter (Fig 1-B). The third vector, MFG, is a very simple vector that does not contain any marker gene. However, it has been engineered to allow high level transgene expression by inserting the ASM gene into the translation initiation codon of the viral envelope protein (Fig 1-C). It has been previously shown that the MFG vector is capable of transducing mouse hemopoietic stem cells and can result in high expression of the transgene both *in vitro* and *in vivo*. Each of the retroviral DNA vectors have been converted into replication defective viral units by electroporating them into the envAM12 amphotropic packaging cell lines (Markowitz et al. 1988; Markowitz et al. 1988; Markowitz et al. 1988; Markowitz et al. 1988).

In a series of experiments conducted in our lab, it has been shown that virus containing media is capable of transducing skin fibroblasts from Types A and B NPD patients. The reconstituted enzyme levels in the corrected cell lines often exceeded that

found in normal cells (up to 16-fold higher) and the accumulated SPM levels diminished (Suchi, Dinur et al. 1992; Yeyati, Agmon et al. 1995). In all cases, the MFG vector was the most efficient and led to the highest expression of the enzyme. Furthermore, *in situ* ASM assay studies in the corrected cells have shown that the expressed enzyme in the transduced cells was correctly targeted to the lysosome (Suchi, Dinur et al. 1992). In this chapter the initial *in vivo* evaluation of HSCGT as a treatment modality for NPD is described.

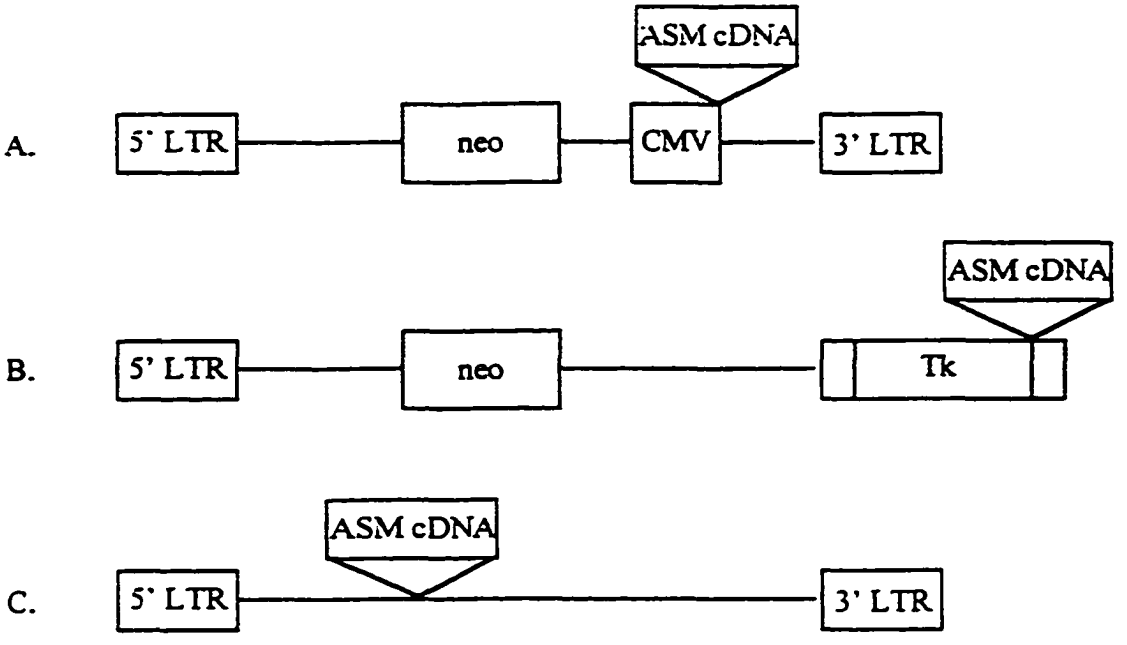


**Fig 1. ASM retroviral constructs.** A, pBC140 B, NAT C, MFG.

# Materials and Methods

*Preparation of Ecotropic Producer Cells.* To prepare ecotropic producer cells an existent amphotropic producer cell line containing the ASM /MFG viral vector was grown to ~80% confluency in a T-75 flask containing 30 ml Dulbecco's modified Eagle's media (DMEM; GibcoBRL, Gaithersburg, MD), 20% fetal calf serum (GibcoBRL, Gaithersburg, MD) and antibiotics. After 24 hr the media was harvested, concentrated to a 3 ml volume using 300K Macrospec filter tubes (Pall Filtron Corporation, East Hills, NY), combined with 7 ml of fresh media and antibiotics as well as 8 μg/ml polybrene (Sigma, St Louis, MO) and transferred to a 10 cm dish containing the gp86 ecotropic packaging cell line. The process was repeated 3 times over a 3-day period and was followed by harvesting of the transduced ecotropic producer line and dilution of the cells to a final concentration of 100 cells/10 ml. The cells were then transferred to 10 cm dishes and grown until individual colonies were present. Each colony was picked up using ring cylinders and expanded. DNA was prepared from each line and subjected to quantitative PCR (see below) and the lines exhibiting the highest copy number of the provirus were retained and used to transfect ASMKO mouse bone marrow cells.

*Mice.* The ASMKO mouse colony was established from heterozygous breeding pairs obtained by gene targeting of 129/Sv embryonic stem cells and subsequent micro-injection into C57BL/6 blastocysts (Horinouchi et al. 1995).

*Preparation of Donor Cells.* Normal male mice (6-12 weeks old) from within the colony were used as donors. Donor animals were pretreated with 5-FU (150 mg/Kg) 2 days before harvesting, were killed by cervical dislocation, and the bone marrow cells were harvested from the femura and tibia by flushing the medullary cavities using Hank's balanced salt solution (GIBCO BRL, Gaithersburg, MD) and a 27-gauge needle. The cells were washed twice in Hank's solution, and single cell suspensions were obtained by passage through a cell strainer (40 mm, Becton Dickinson, Franklin Lakes, NJ). Low-density bone marrow cells (<1.085 g/cm$^3$) were isolated by discontinuous density gradient centrifugation using Nycoprep (Nycomed Pharma AS, Oslo, Norway) (Bertoncello et al. 1987) and washed with buffered Hank's solution containing 5% heat inactivated fetal calf serum (HSA). These cells were then counted, diluted to the desired concentration, and retrovirally transduced.

*Retroviral Transduction of Bone Marrow Cells.* To achieve retroviral transduction, adult bone marrow were cultured for 48 hr in 10 ml of Dulbecco's Modified Eagle's Media containing concentrated amphotropic or ecotropic ASM/MFG retroviral vector.(see above) , 20% heat-inactivated fetal calf serum and antibiotics. Cultures were carried out in the presence of SCF (50 ng/ml), IL-3 (20 ng/ml), IL-6 (10 ng/ml) (Genzyme, Cambridge, MA) and 8 μg/ml polybrene (Bodine et al. 1990; Okada et al. 1991; Brugger et al. 1993; Koller et al. 1993). After the first 24 hr the media was changed to fresh media containing newly harvested and concentrated viral vector and fresh growth factors and polybrene.

*Transplantation Protocol.* Thirty-two 2-day-old newborn ASMKO mice were used as recipients. Before receiving the transplant, the recipients were subjected to a single total body irradiation dose of 200 cGy from a dual $^{137}Cs$ source (dose rate = 80 cGy/min). Each animal received a total of 1 x $10^6$ cells/g of body weight.. Age-matched litter-mates (normal, n=5; and ASMKO, n=5) were used as irradiation controls (i.e., irradiated, but not transplanted). The animals were maintained on a 12-hr light/dark cycle, water ad libitum, and Purina rodent chow 5001. No antibiotics or other supportive care were given to any of the animals before or after transplantation.

*Post-Transplant Follow-up and Tissue Processing.* The weights of the transplanted and control animals were registered monthly by Dr. Miranda. Peripheral blood also was obtained by retinal orbit bleeding for monthly post-BMT enzyme analysis in white blood cells (WBCs) (done by me and Dr. Miranda). WBCs were isolated after lysis of the red cells using a hemolytic buffer (0.1 M $NH_4Cl$, 12 mM $NaHCO_3$, 10 mM EDTA, pH 8.0).

*PCR Analysis.* A modification of our previously described procedure was used (Yeyati, Agmon et al. 1995). Murine and human ASM sequences were amplified using one common sense primer, 5'-TGCTGAGGATCGAGGAGACAA-3' (P1) constructed from human and murine ASM exon 3, and two species-specific antisense primers, 5'-GGGTAGAGTGACAGAAGATTGA-3' (P2) and 5'-GGCACAAGAGTAGCCAGACG -3' (P3), constructed from murine ASM intron 3 and human ASM exon 6, respectively. Primer pair P1 and P2 amplified a 211-bp genomic murine ASM product, while primer pair P1 and P3 amplified a 554-bp product from the human ASM/MFG sequence. Each

amplification reaction (~100 µl final volume) contained 200 pmol of primer P1, 100 pmol each of primers P2 and P3, 300 ng of genomic DNA, lx PCR buffer (Promega, Madison, WI), 1.5 mM $MgCl_2$, 5 U of Taq polymerase (Promega, Madison, WI) and 200 mM each of dNTPs. A standard curve was generated using DNA mixtures as described in Yeyati *et al.* (Yeyati, Agmon et al. 1995). Following amplification (30 cycles, each consisting of 1 min at 93°C, 1 min at 61°C, 1 min at 72°C), PCR products were separated by electrophoresis on 1.5% agarose gels and stained with ethidium bromide. The intensity of the bands was determined using the NIH Imager software package. By comparing the intensity of the two amplified bands, the number of transduced cells in the sorted populations could be estimated. This calculation was based on the assumption that each transduced cell contained one proviral genome. It should be noted that the murine-specific ASM band is present in all samples and serves as an internal control, and that while the ASMKO mice have no ASM activity, the murine ASM genomic sequences are still present.

*Enzyme Assays.* Fresh or cultured adult bone marrow cells were harvested, washed once with HSA, and incubated on ice for 15 min in 0.2% Triton X-100. Total protein was determined by the method of Stein *et al.* (Stein et al. 1973). The standard 15 µl ASM assay mixture consisted of 10 µl of protein source and 2 nmol of B12SPM suspended in 0.1 M sodium acetate buffer, pH 5.2 containing 0.6% Triton X-100 and 5 mM EDTA. After incubating the assay mixture at 37°C (up to 3 hr), the samples were loaded onto thin layer chromatography plates (TLC LK6 D Silica gel; Whatman, Clifton, NJ) and resolved using chloroform/methanol (95:5 v/v). After resolution, the band

containing the fluorescently-labeled ceramide (the product of B12SPM hydrolysis) was scraped from the plates, extracted in chloroform/methanol/water (1:2:1 v/v) for 15 min at 55°C, and quantified in a spectrofluorometer (fluorescence spectrophotometer 204-A, Perkin-Elmer). The instrument settings were excitation 505 nm and emission 530 nm.

# Results

*Failure of the Amphotropic ASM/MFG Retroviral Vector to Transduce Mouse Bone Marrow Cells.* Mononuclear ASMKO mouse bone marrow cells were transduced with the amphotropic ASM/MFG retroviral vector for 3 consecutive days. Although the enzyme activity increased each day of the transfection period (Fig 2-A), PCR analysis shown that the transfection rate was very low and reached measurable levels only on the third day (Fig 2-B). Therefore, the detected enzymatic activity was probably the result of enzyme uptake from the transfection media.

*Transduction of ASMKO Bone Marrow Cells with Ecotropic ASM/MFG Retroviral Vector.* Following the failure of the amphotropic retroviral vector to transduce ASMKO mouse bone marrow cells, I prepared an ecotropic version of this vector. Using this new vector and the same 3-day transfection protocol a much higher transfection rate of ASMKO mouse bone marrow cells was achieved, over 2 viral copies per cell (Figure 3). I then transplanted the cells into thirty-two 2-day old ASMKO mice at a dose of 1 x $10^6$ transduced cells/g. The recipients were pretreated with a radiation dose of 200 cGy.

*Long Term Follow-up of WBC ASM Activity in Transplanted Animals.* WBC ASM activities were measured by me (1-2 month post-transplant) and Dr. Miranda (3-5 month post-transplant) monthly after BMT. As can be seen from Figure 4 seventeen out of the thirty-four transplanted animals had ASM activities ranging widely from 11% to 955% of normal. During the 5 month follow-up period there was an increase in

the enzymatic activity in all of these animals and by the end of the fifth month post-transplant the enzyme levels that were found ranged from 73% to 2790% of normal. Of note, none of the transplanted animals that failed to show ASM enzymatic activity at 1 month post-transplant produced measurable enzyme levels thereafter.

*Survival and Growth Patterns of Transplanted ASMKO Mice.* ASMKO "control" animals (i.e., irradiated, but not transplanted) survived up to 5.5 months of age, whereas wild-type irradiation control animals had a normal life-span. None of the ASMKO animals receiving HSCGT had died at 6 month post transplant. Similarly to the results of the allogenic BMT experiments, there was a delay in the weight loss of the transplanted ASMKO compared to non-transplanted ASMKO controls. However, despite the delayed onset, by the fifth month after BMT significant weight loss was seen in the transplanted group (not shown-work done exclusively by Dr. Miranda).

*Behavioral Studies.* The effect of HSCGT on cerebellar function of transplanted mice were determined by Dr. Miranda using the roto-rod behavioral test machine. This device can be used to test the ability of mice to adjust to walking or running on a spinning rod that accelerates with time. This ability to adjust to the accelerating rod is related to the possible malfunction and death of Purkinje cells, as well as other neural defects, in the mice's brains that are a result of the progression of the disease. The performance of transplanted animals was somewhat better then untransplanted animals of the same age (4-5 month). However, there was no correlation between the level of enzymatic activity

and the mouse performance on the test (not shown-work done exclusively by Dr. Miranda).

*Pathology*. To evaluate the therapeutic effects of HSCGT on the ASMKO phenotype, the two animals that performed best on the roto-rod device test were sacrificed at 5 month post-transplant and the bone marrow, brain, liver, lung, and spleen were sectioned and sent for analysis by light microscopy (by Dr. Mark Haskins, University of Pennsylvania-School of Veterinary Medicine). In addition, Dr Victor L. Freidrich, Jr., of the Brookdale Center for Molecular Biology, Mt. Sinai School of Medicine is studying the brain sections of these mice to determine the number of conserved Purkinje cells. The results of these analyses have not yet been furnished by these investigators.

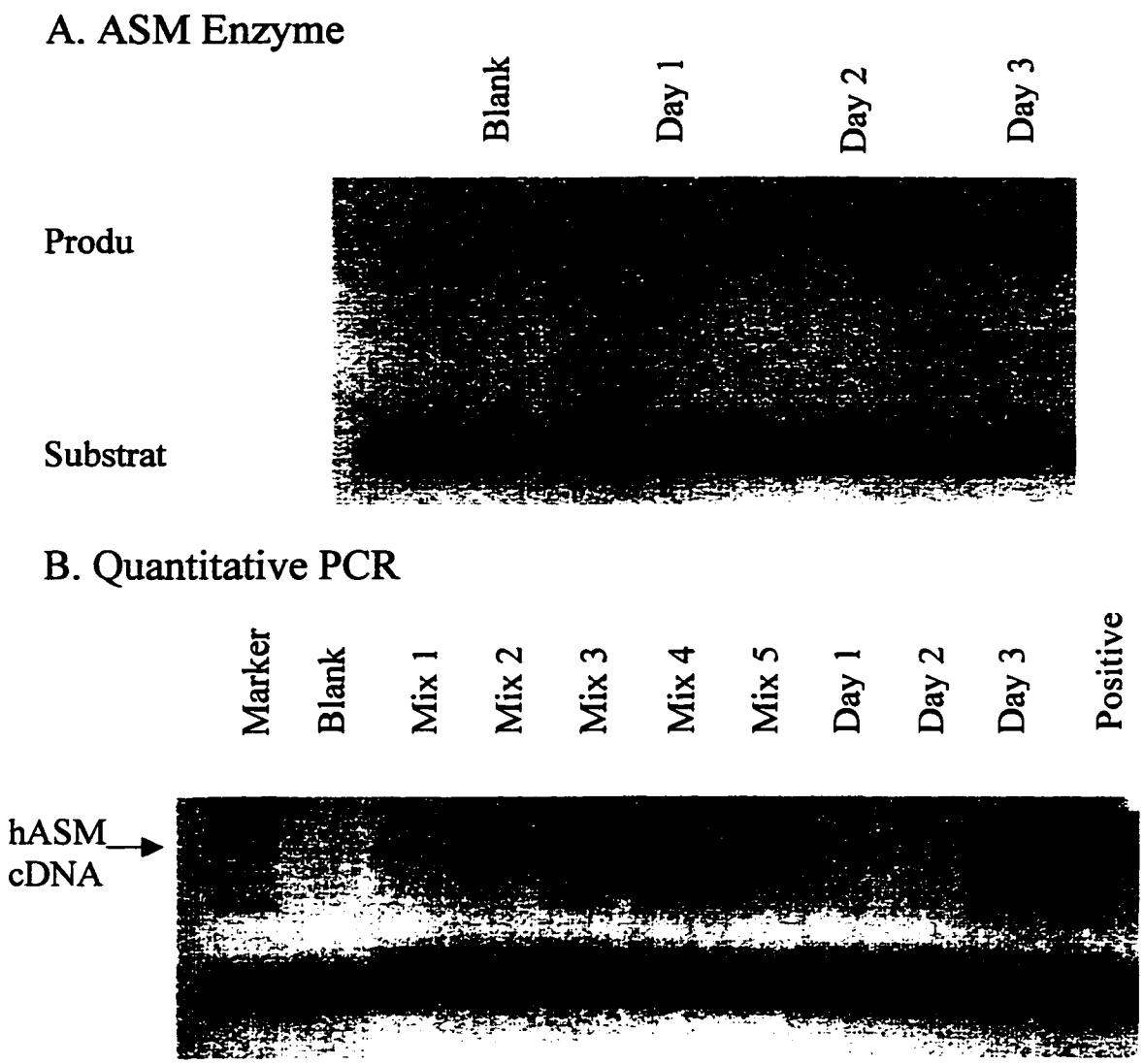


**Fig. 2. Retroviral Transduction of ASMKO bone marrow cells using the amphotropic ASM/MFG retroviral vector.** A, ASM enzyme assays and B, Quantitative PCR were used to determine the transduction rate of the bone marrow cells. Transduced cell sample were taken every day over the 3 days incubation with viral containing media and the DNA and protein were extracted and submitted to these assays. ASM/MFG and mouse genomic DNA mixtures were used in order to create a standard curve: Mix 1 = 1 ASM/MFG genome per 100 mouse genomes; Mix 2 = 1 ASM/MFG genome per 50 mouse genomes; Mix 3 = 1 ASM/MFG genome per 10 mouse genomes; Mix 4 = 1 ASM/MFG genome per 4 mouse genomes; Mix 5 = 1 ASM/MFG genome per 2 mouse genomes.

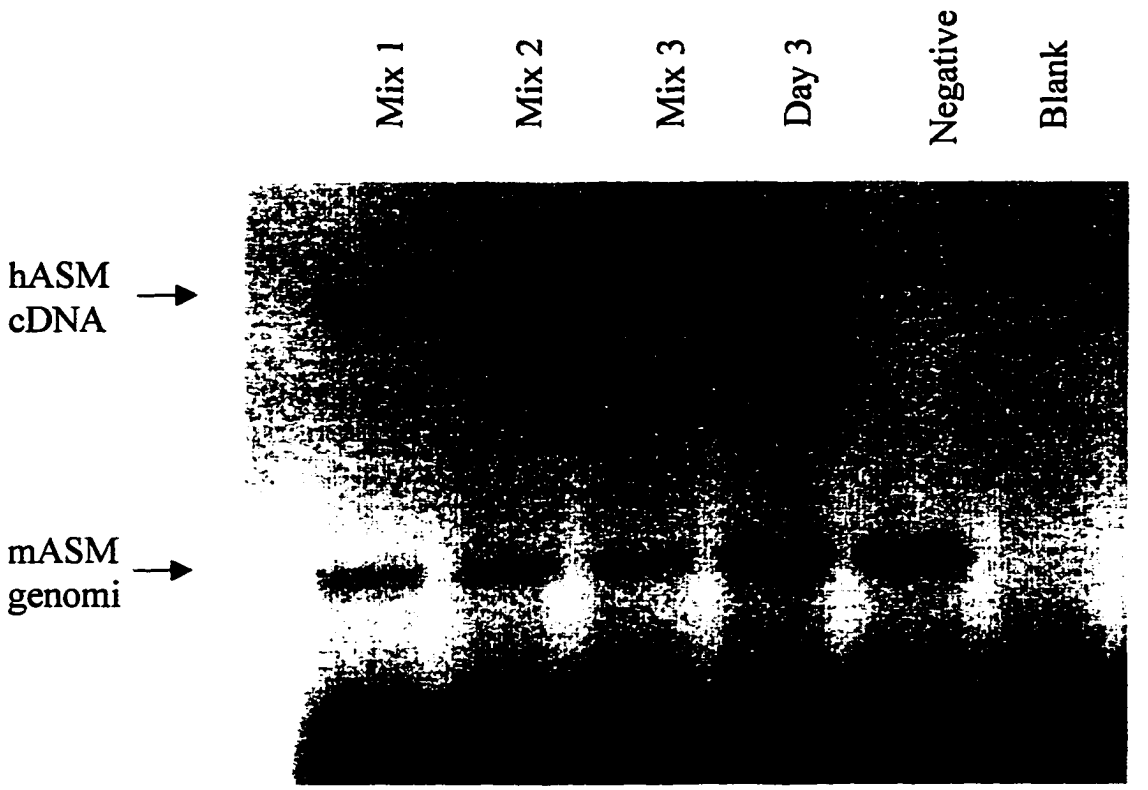


**Fig. 3. Retroviral Transduction of ASMKO bone marrow cells using the ecotropic ASM/MFG retroviral vector.** Quantitative PCR was used to determine the transduction rate of the bone marrow cells. Transduced cell sample were taken after the 3 days incubation with viral containing media and the DNA was extracted and submitted to the PCR assay. ASM/MFG and mouse genomic DNA mixtures were used in order to create a standard curve: Mix 1 = 1 ASM/MFG genome per 2 mouse genomes; Mix 2 = 1 ASM/MFG genome per 1 mouse genomes; Mix 3 = 2 ASM/MFG genome per 1 mouse genomes.

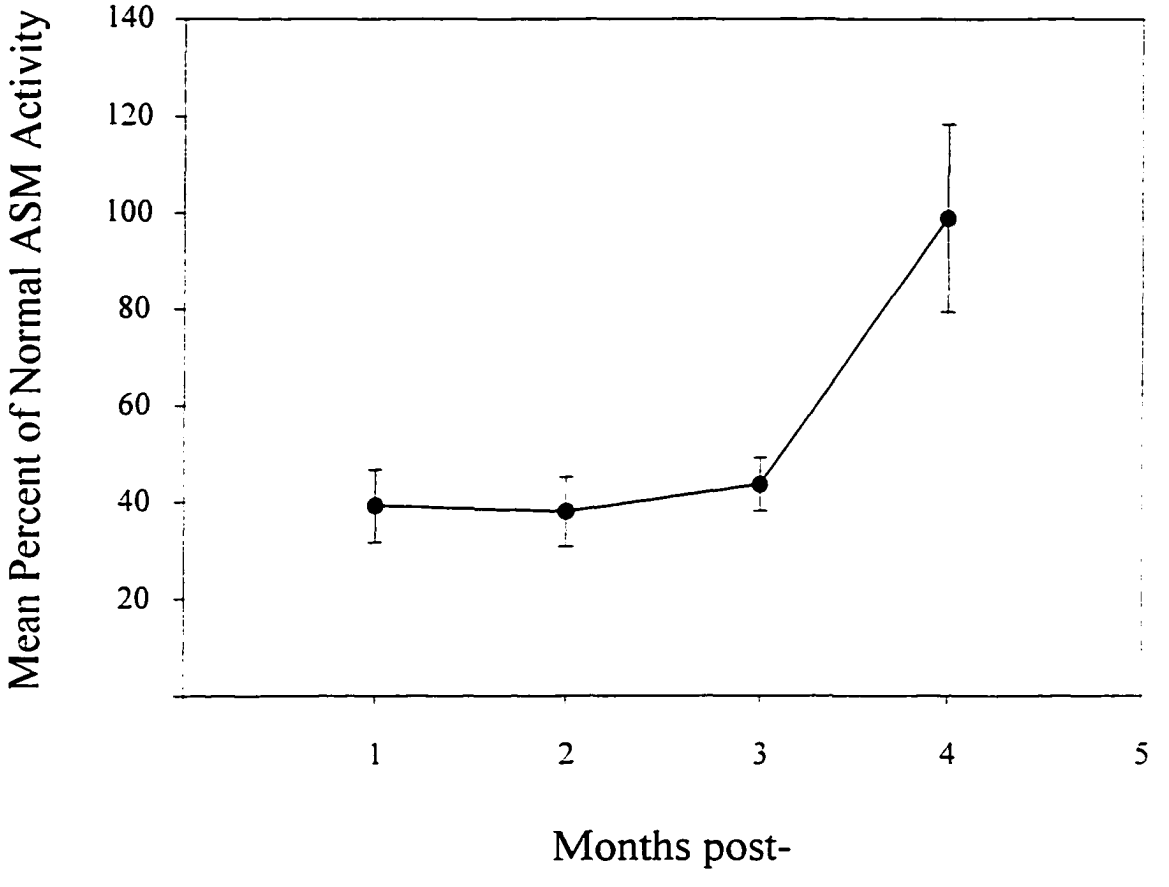


**Fig. 4. ASM activities in WBC of transplanted ASMKO mice (n=18) after BMT with retrovirally-corrected bone marrow.** Mean values are shown along with the standard error of the mean.

# Discussion

In an effort to solve some of the problems associated with allogenic BMT our lab has been active in the development of HSCGT strategies for the treatment of NPD. Previous efforts were concentrated in constructing a range of retroviral vectors and evaluating their ability in to transfect NPD fibroblasts and produce high levels of ASM in the transfected cells (Suchi, Dinur et al. 1992; Yeyati, Agmon et al. 1995). In the first stage of this study I have tested the effectiveness of the ASM/MFG vector in transfecting bone marrow cells. It was found that the amphotropic ASM/MFG vector led to very low transfection rate of bone marrow cells. Indeed no enzymatic activity could be found in the WBC of mice transplanted with these transduced cells. In contrast, bone marrow cells that were transduced with an ecotropic ASM/MFG vector presented a high degree of transfection and ASM enzymatic activity. These phenomena were also described by others (Orlic et al. 1997) and may be due to the low number of amphotropic virus receptor on the membrane of stem and progenitor cells (Orlic et al. 1996).

In addition to the *in vitro* studies we have began a series of transplantations of transduced cells into ASMKO mice, in order to evaluate the efficacy of HSCGT for the treatment of NPD. Base on previous work from our laboratory (chapter 2) recipient ASMKO newborns were preconditioned with a "sublethal" dose of 200 cGy which was effective and safe (Miranda et al. 1997). Donor animals were pretreated with 5-FU before harvesting of the bone marrow, in order to achieve high transduction rate of stem and progenitor cells (Szilvassy et al. 1989). Indeed, about half of the transplanted animals

showed increased ASM enzymatic activity (on average 4-8 fold the normal activity) even at six month post transplant. Y-chromosome in-situ hybridization studies are currently being performed by Dr. Miranda in order to determine the percent of engraftment of donor cells in the recipient bone marrow. Correlating the results of these studies with those of ASM enzymatic activity can reveal the actual level of enzyme in the transduced-engrafted cells and help us determine whether any significant "vector-shutdown" has occurred (Jolly et al. 1986; Xu et al. 1989; Duch et al. 1994).

Based on these very preliminary results, it seems that the clinical effect of HSCGT is very similar to that achieved by allogenic BMT (chapter 3). In the allogenic BMT experiments we have seen an almost complete disappearance of the pathology in organs of the reticulo-endothelial system, even when the ASM enzymatic activity was as low as 50% of normal. The results of the HSCGT experiments show that animals express at least two-fold the normal activity and therefore it is reasonable to speculate that these animals will also show a marked improvement of the disease phenotype in organs such as the liver and spleen. The effect of HSCGT on the CNS is also similar to that seen in the BMT experiment. There is a slight improvement in the mice motor capabilities and rescue of some Purkinje cells in the cerebellum. However, the mice still perform worse then their normal littermates and may have a reduced life-span. We therefore propose that BMT and HSCGT may be used to treat Type B but not Type A NPD. In order to improve HSCGT so it can be used to treat type A NPD our lab intend to test the following approaches: transplantation with an enriched population of hemopoietic stem cells selected with out unique fluorescence-based technique (see Chapter 5), injection of

purified corrected hemopoietic stem cells into the brains of ASMKO mouse newborns and fetal transplantation of transduced bone marrow. We hope that these improvements will lead to increase in the number of transduced cells populating the brain thereby augmenting the therapeutic effect in the CNS.

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# Chapter 5

# Fluorescence-Based Selection of Gene-Corrected Hemopoietic Stem and Progenitor Cells From Acid Sphingomyelinase Deficient Mice

# Abstract

A novel, fluorescence-based procedure for isolating retrovirally-transduced NPD hemopoietic stem and progenitor cells and assessing gene transfer and expression in these cell types has been developed. Lineage depleted hemopoietic cells were isolated from the bone marrow or fetal livers of acid sphingomyelinase deficient (ASMKO) mice, and retrovirally transduced with amphotropic or ecotropic vectors encoding a normal acid sphingomyelinase (ASM) cDNA. Anti-c-Kit antibodies were then used to label stem and progenitor enriched cell populations, and the Bodipy fluorescence was analyzed in each group after incubation with a Bodipy-conjugated sphingomyelin. Only cells expressing the functional ASM (i.e., transduced) could degrade the sphingomyelin, thereby reducing their Bodipy fluorescence as compared to non-transduced cells. The usefulness of this procedure for the *in vitro* assessment of gene transfer into hemopoietic stem cells was evaluated, as well as its ability to provide an enrichment of transduced stem cells *in vivo*. To demonstrate the value of this method for *in vitro* analysis, the effects of retroviral transduction using: 1) ecotropic vs. amphotropic vectors, 2) various growth factor combinations, and 3) adult bone marrow vs. fetal liver stem cells were assessed. The results of these studies confirmed the fact that ecotropic vectors were much more efficient at transducing murine stem cells than amphotropic vectors, and that among the three most commonly used growth factors (stem cell factor [SCF] and interleukins 3 and 6 [IL-3 and IL-6]), SCF had the most significant effect on the transduction of stem cells, while IL-6 had the most significant effect on progenitor cells. In addition, it was determined that fetal liver stem cells were only ~2-fold more "transducible" than stem

cells from adult bone marrow. Transplantation of Bodipy selected bone marrow cells into lethally irradiated mice revealed that the number of spleen colony forming units (CFU-S) that were positive for the retroviral vector (as determined by PCR) was 76%, as compared to 32% in animals that were transplanted with cells that were non-selected. These methods are particularly useful for evaluating hemopoietic stem cell gene transfer in animals since the marker gene utilized in the procedure (i.e., ASM) encodes a naturally occurring mammalian enzyme that has no known adverse effects, and the fluorescent compound used for selection (Bodipy sphingomyelin) is removed from the cells prior to transplantation.

# Introduction

As described in Chapter 4, transducing NPD cells with the MFG retroviral vector resulted in the highest level of enzyme production. However, since the MFG vector does not include any marker gene, and therefore does not confer upon the transduced cells any known selective advantage during G418 selection, it was impossible to isolate and / or enrich the metabolically corrected cells. To address this issue, a fluorescence based selection system has been developed in our laboratory in collaboration with Dr. Shimon Gatt of the Hebrew University (Dinur et al. 1992; Yeyati et al. 1995). In brief, a fluorescent derivative of SPM is added to the media of normal and NPD cells for a 24 hr pulse period, and then the cells are chased for an additional 48-72 hr in non-fluorescent ("cold") media. Following this pulse/chase incubation, FACS analysis and sorting of the transduced cells reveal two cell populations that can be distinguished by their residual fluorescence: a low fluorescence population of metabolically-corrected cells and a high fluorescence population of non-corrected cells. Quantitative PCR and ASM enzyme assays verified that the cells contained in the low fluorescence population expressed higher levels of ASM activity and were enriched for vector sequences.

Prior to my entering this project, this system had been worked out using NPD skin fibroblasts. However, as described in Chapter 4, the target of our gene therapy efforts for NPD effort are the hemopoietic stem cells (HSCs). Complications in adapting the method to hemopoietic cells included the low activity of ASM in normal hemopoietic cells, and the short time that these cells can be kept in culture without differentiating.

With the help of Dr. Gatt and Dr. Jan Visser of the New York Blood Center, I have been able to modify this method to the selection of metabolically corrected NPD bone marrow cells. We have also found that this method can be generally used to quantitatively determine gene transfer and expression in hemopoietic stem and progenitor cells.

This chapter demonstrates the utility of this method using hemopoietic stem and progenitor cells obtained from the bone marrow or fetal livers of ASMKO mice. The advantages of this technique as a general transduction assay method include the facts that 1) the target cells do not need to be proliferating (such as HSCs), and any gene transfer system can be analyzed after inserting ASM into the vector, 2) it is fluorescence based and highly sensitive, permitting analysis of rare cell populations, and 3) it can be easily used for long-term *in vivo* analysis since it requires only two days to complete, does not involve extensive cell manipulation, and the transplanted cells do not express a foreign protein with potentially adverse effects.

# Materials and Methods

*Cell Preparations.* To obtain adult nucleated bone marrow cells, the tibia and femurs of 12-16 week old C57BL/SV129 normal and ASMKO mice were flushed with buffered Hank's solution (10 mM Hepes, pH *7.5).* Single cell suspensions were obtained by passing the cells through a 0.4 micron mesh (Becton Dickinson Labware, Franklin Lakes, NJ). Low-density bone marrow cells (<1.085 $g/cm^3$) were isolated by discontinuous density gradient centrifugation using Nycoprep (Nycomed Pharma AS, Oslo, Norway) (Bertoncello et al. 1987), and washed with buffered Hank's solution containing 5% heat inactivated fetal calf serum (HSA). For the mixing experiments the cells were counted and normal and ASMKO cells were mixed at various ratios prior to the labeling stage. To obtain fetal liver cells, the livers of day 14.5 fetuses were isolated in buffered Hank's solution. Single cell suspensions were prepared by gently pipetting the tissue up and down through the bore of a 5 ml pipette. The cells were then washed with HSA.

*Lineage Depletion.* To obtain $Lin^-$ cells, a modification of the method of Bertoncello *et a.* (Bertoncello et al. 1991) was used. Isolated adult bone marrow and fetal liver cells were incubated for 30 min on ice with the biotinylated antibodies anti-TER-119, anti-CD45R/B220 and anti-Ly-6G (PharMingen, San Diego, CA). The concentration of each antibody was 1 $\mu g/10^6$ cells. The cells were than washed with HSA, resuspended in buffered Hank's solution, and incubated with streptavidin-coated magnetic beads (Dynabeads M-280 Streptavidin, Dynal, Lake Success, NY) at 4°C for 30 min at a 10:1

bead:cell ratio. Magnetic force was then applied for 1 min and the supernatant was collected. The cell pellet was washed with buffered Hank's solution 3 times using the same procedure, and the supernatants were combined.

*Anti-c-Kit Labeling.* Cultured or freshly collected cells were washed once with HSA and counted. The cells were than incubated with phycoerythrin-conjugated anti-CD117 (c-Kit) antibodies (PharMingen, San Diego, CA) at a concentration of 1 $\mu g/10^6$ cells for 30 min on ice. After labeling, the cells were washed once with HSA and resuspended in buffered Hank's solution. Control labeling was carried out with a rat $IgG_{2b}$, kappa isotype (PharMingen, San Diego, CA).

*Synthesis of Fluorescent Sphingomyelin.* Sphingomyelin to which the fluorescent probe Bodipy was covalently linked via a 12-carbon spacer (Bodipy dodecanoyl sphingosyl phosphocholine; Bl2SPM) was synthesized as previously described for lissamine rhodamine sphingomyelin (Dinur, Schuchman et al. 1992), except that Bodipy dodecanoic acid (Molecular Probes Inc., Eugene, OR) was condensed with sphingosyl phosphocholine. To incorporate B12SPM into liposomes, B12SPM was mixed with phosphatidyl choline (PC; Sigma, St Louis, MO) at a molar ratio of 1:4. The solvent was evaporated and the mixture was resuspended in buffered Hank's solution followed by a 1 min sonication.

*"Pulse-Chase" Labeling with B125PM.* B12SPM/PC liposomes (final concentration 0.5-1 nmol/ml) were incubated at 37°C for 4 hr with $Lin^-$ cells which had

been suspended in buffered Hank's solution. Labeling was terminated by centrifuging the cells (400g) and washing the pellets once with HSA. Fresh medium was then added and the cells were further incubated for 48 hr in standard culture media containing Iscove's Modified Dulbecco's Medium (IMDM; GibcoBRL, Gaithersburg, MD), 10% heat inactivated fetal calf serum (GibcoBRL, Gaithersburg, MD) and antibiotics, but no B12SPM/PC liposomes.

*Retroviral Transduction.* To achieve retroviral transduction, adult bone marrow and fetal liver cells were co-cultured for 48 hr with amphotropic or ecotropic retroviral producing cells containing an ASM/MFG retroviral vector (Yeyati, Agmon et al. 1995). Control, untreated cells were co-cultured with producer cells alone. Cocultures were carried out in 0.4 mm Transwell dishes (Coming Costar, Cambridge, MA) containing the packaging cells in the upper compartment. SCF (50 ng/ml), IL-3 (20 ng/ml), and IL-6 (10 ng/ml) (Genzyme, Cambridge, MA) were added to the media unless indicated otherwise.

*FACS Analysis and Expansion of Sorted Cells.* Cells were analyzed using a FACScan instrument (Becton Dickinson Immunocytometry Systems, San Jose, CA) and the WinMDI program. Cells were sorted using a FACStar flow cytometer (Becton Dickinson Immunocytometry Systems. San Jose, CA). Sorted cells were resuspended in expansion media containing IMDM, 10% heat inactivated fetal calf serum, SCF (50 ng/ml), IL-6 (10 ng/ml), IL-3 (20 ng/ml) and antibiotics, and then grown at 37°C for 10 days.

*PCR Analysis.* A modification of our previously described procedure was used (Yeyati, Agmon et al. 1995). Murine and human ASM sequences were amplified using one common sense primer, 5'-TGCTGAGGATCGAGGAGACAA-3' (P1) constructed from human and murine ASM exon 3, and two species-specific antisense primers, 5'-GGGTAGAGTGACAGAAGATTGA-3' (P2) and 5'-GGCACAAGAGTAGCCAGACG -3' (P3), constructed from murine ASM intron 3 and human ASM exon 6, respectively. Primer pair P1 and P2 amplified a 211-bp genomic murine ASM product, while primer pair P1 and P3 amplified a 554-bp product from the human ASM/MFG sequence. Each amplification reaction (~100 μl final volume) contained 200 pmol of primer P1, 100 pmol each of primers P2 and P3, 300 ng of genomic DNA, lx PCR buffer (Promega, Madison, WI), 1.5 mM $MgCl_2$, 5 U of Taq polymerase (Promega, Madison, WI) and 200 mM each of dNTPs. A standard curve was generated using DNA mixtures as described in Yeyati *et al.* (Yeyati, Agmon et al. 1995). Following amplification (30 cycles, each consisting of 1 min at 93°C, 1 min at 61°C, 1 min at 72°C), PCR products were separated by electrophoresis on 1.5% agarose gels and stained with ethidium bromide. The intensity of the bands was determined using the NIH Imager software package. By comparing the intensity of the two amplified bands, the number of transduced cells in the sorted populations could be estimated. This calculation was based on the assumption that each transduced cell contained one proviral genome. It should be noted that the murine-specific ASM band is present in all samples and serves as an internal control, and that while the ASMKO mice have no ASM activity, the murine ASM genomic sequences are still present.

*Enzyme Assays.* Fresh or cultured adult bone marrow cells were harvested, washed once with HSA, and incubated on ice for 15 mm in 0.2% Triton X-100. Total protein was determined by the method of Stein *et al.* (Stein et al. 1973). The standard 15 µl ASM assay mixture consisted of 10 µl of protein source and 2 nmol of B12SPM suspended in 0.1 M sodium acetate buffer, pH 5.2 containing 0.6% Triton X-100 and 5 mM EDTA. After incubating the assay mixture at 37°C (up to 3 hr), the samples were loaded onto thin layer chromatography plates (TLC LK6 D Silica gel; Whatman, Clifton, NJ) and resolved using chloroform/methanol (95:5 v/v). After resolution, the band containing the fluorescently-labeled ceramide (the product of B12SPM hydrolysis) was scraped from the plates, extracted in chloroform/methanol/water (1:2:1 v/v) for 15 min at 55°C, and quantified in a spectrofluorometer (fluorescence spectrophotometer 204-A, Perkin-Elmer). The instrument settings were excitation 505 nm and emission 530 nm.

*CFU-S Assays.* Adult nucleated bone marrow cells were obtained from ASMKO mice that had been pretreated with 5-FU (150 mg/Kg) 2 days before harvesting, retrovirally transduced with the ecotropic vector and "pulse-chase" labeled with B12SPM as described above, and then sorted by FACS. Cells representing the least 25% fluorescent in FL-1 ($B12^{low}$) were collected and $4 \times 10^4$ were injected into the tail veins of lethally irradiated (800 cGy) adult ASMKO mice. For comparison, the same number of non-selected, transduced cells were injected into another set of animals. After 14 days the mice were sacrificed, the spleens were removed and then fixed in a 70% solution of formalin : acetic acid : ethanol (1:1:20 v/v/v) for 3 days.

To prepare DNA from the CFU-S colonies, a modification of the method of Frank *et al.* (Frank et al. 1996) was used. The CFU-S colonies were separated and washed individually 3 times overnight in lx TE (pH 8.2) at 4°C, and then minced and incubated overnight again at 37°C in a solution containing 50 mM Tris (pH 8.2) and 200 ng/μl Proteinase K (Boehringer Mannheim, Mannheim, Germany). The microcentrifuge tubes containing the digested materials were then immersed in boiling water for 8 min, and the extracted DNA was placed on ice. For PCR, the DNA solutions were diluted 1:100 and 40 μl was used. The PCR was performed as described above except that only primers P1 and P3 were used and the number of cycles was 40. A positive colony was defined as a colony in which the hASM transgene-specific PCR product was found in at least three independent amplification reactions.

# Results

*Comparison Between B12SPM and LRSPM.* Bone Marrow cells from normal or ASMKO mice were incubated for 4 hr with either Bodipy-conjugated sphingomyelin (B12SPM) or lissamine rhodamine-conjugated sphingomyelin (LRSPM), and then grown at 37°C for 48 hr in standard culture media without substrate. The cells were then subjected to FACS analysis and the relative fluorescence of each cell population was determined (Fig. 1). Comparison of the two fluorescent substrates (B12SPM and LRSPM) has shown that both are useful in differentially labeling the ASMKO and normal cells. However, as expected, the B12SPM gave much higher fluorescence levels. In addition, these experiments showed that only the "white cell" component of the bone marrow is differentially labeled in normal versus ASMKO (Fig. 1). Therefore, we have found it useful not to include the bone marrow erythroid population in future analysis.

During these studies, I found that the presence of serum in the media during labeling inhibited substrate entry into the cells (not shown). Furthermore, I also found that the delivery of the substrate in liposomes, together with phosphatydil choline (PC), also enhanced the cell labeling (not shown). The findings were important since they have allowed us to optimize the labeling procedure so that it takes only 4 hours. The absence of serum for such a short period of time does not seem to have any deleterious effect on the hemopoietic cells.

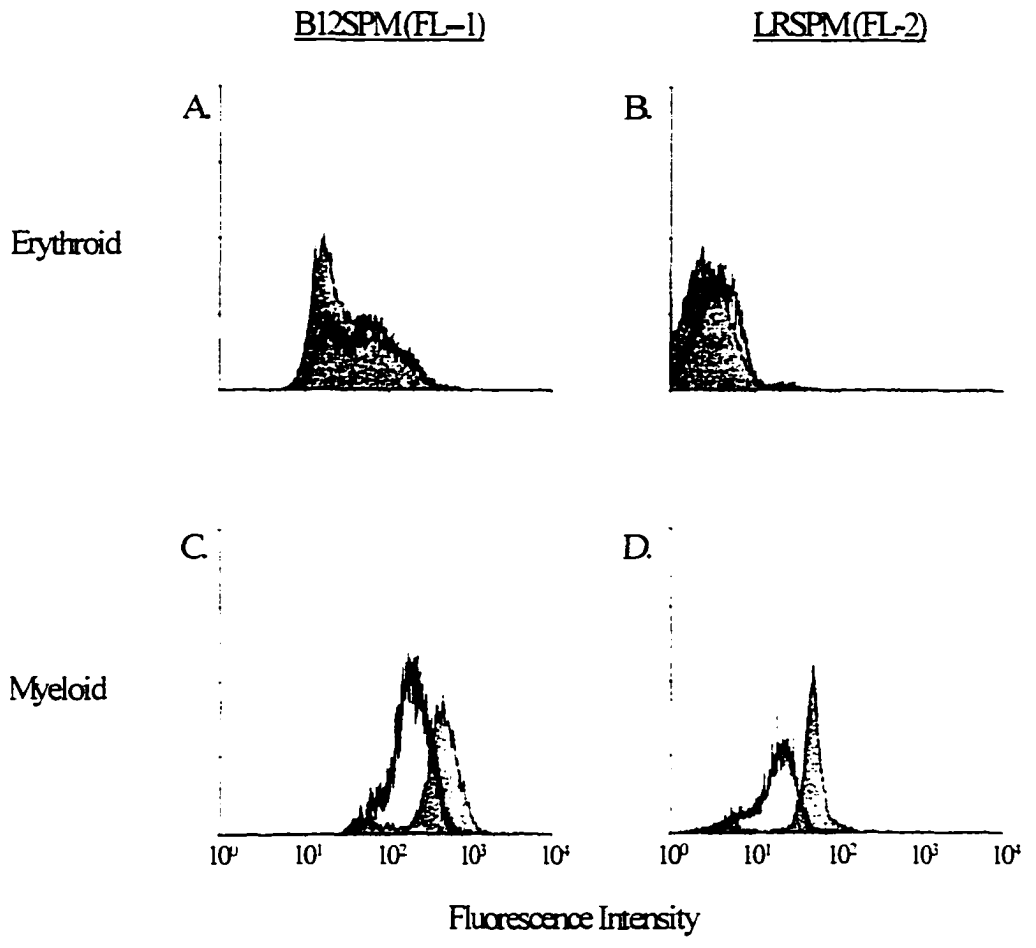


**Fig 1. Analysis of B12SPM and LRSPM labeling of normal and ASMKO bone marrow cell populations.** Panels A and C depict the red cell (erythroid) bone marrow population, while panels B and D depict the white cell (myeloid) bone marrow population. shaded area: ASMKO cells; open area: normal cells. Note that FL-1 measures B12SPM (Panels A & B) and FL-2 measures LRSPM (Panels C & D). The experiment was repeated at least three times, and the representative datum from one experiment is shown.

*"Pulse-Chase" Labeling of Normal/ASMKO Bone Marrow Cell Mixtures.* In order to demonstrate the ability of this technique to detect the retrovirally-transduced ASMKO cells in a mixed population of metabolically corrected and uncorrected cells an experiment was conducted in which normal and ASMKO cell populations were pre-mixed in different ratios. labeled with B12SPM, chased for 48 hr, and then submitted to FACS analysis. The mixing of the cells was done before the labeling stage, and 3 cell mixtures were used: 25% Normal / 75% ASMKO, 50% Normal / 50% ASMKO, and 75% Normal / 25% ASMKO. Upon FACS analysis of these cell mixtures two peaks were present: low fluorescence corresponding to the normal cells, and high fluorescence corresponding to ASMKO cells (Fig. 2). The number of the cells included in each population correlated strongly with the "known" cell number (Table 1). These experiments demonstrated the possibility of separating the two cell populations even, when the normal cells represent only 25% of the total mixture.

*Identification of Stem and Progenitor Enriched Cell Populations.* Bone marrow was harvested from normal and ASMKO adult mice and analyzed by FACS. After pre-enrichment by Nycodenz density gradient centrifugation and lineage depletion (Bertoncello, Bartelmez et al. 1987; Bertoncello, Bradley et al. 1991), two main populations of nucleated cells were identified, designated $FSC^{high}$ and $FSC^{low}$ (Fig. 3A). Labeling of these cells with antibodies against c-Kit revealed that both groups contained $c\text{-}Kit^-$ cells (Fig. 3B), and that the number of lineage depleted ($Lin^-$)/$c\text{-}Kit^-$ cells was ~2% of the total nucleated cell population. c-Kit was used as a marker since previous work had shown that the $Lin^-/FSC^{high}/c\text{-}Kit^+$ population is highly enriched for progenitor

cells, while the $Lin^-/FSC^{low}/c\text{-}Kit^-$ population is highly enriched for stem cells (Okada et al. 1991; Ikuta and Weissman 1992).

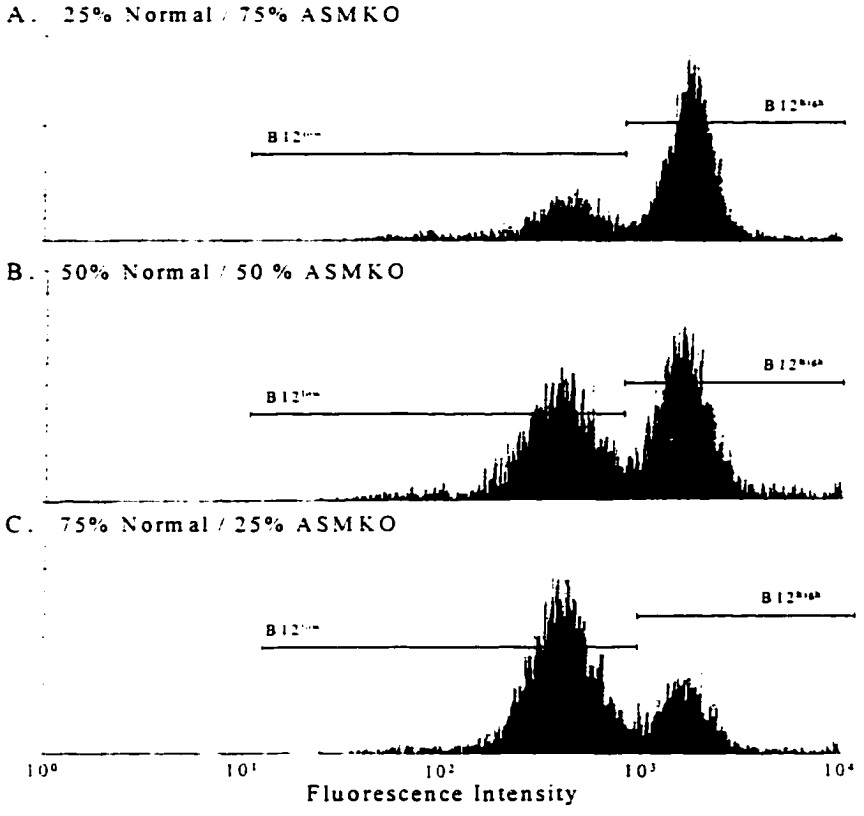


**Fig 2. FACS analysis of bone marrow cell mixtures from normal and ASMKO mice.** The cells were counted, mixed and subjected to pulse/chase labeling procedures with B12SPM. A, cell mixture 25% Normal / 75% ASMKO. B, 50% Normal / 50% ASMKO. C, 75% Normal / 25%ASMKO.

**Table 1. Separation of ASMKO/Normal Cell Mixtures by FACS**

| | Cell Mixture | | Populations Identified by FACS | |
|---|---|---|---|---|
| | Normal | ASMKO | $B12^{low}$ | $B12^{high}$ |
| A. | 25% | 75% | 25.48% | 74.52% |
| B. | 50% | 50% | 49.33% | 50.75% |
| C. | 75% | 25% | 73.80% | 26.43% |

Pulse/Chase labeling of bone marrow cell mixtures with B12SPM were carried out as described in the Materials and Methods. The cells from normal and ASMKO mice were counted and mixed at three different ratios prior to the labeling procedure. After FACS analysis of the cell mixtures two populations of low and high fluorescence (designated $B12^{low}$ and $B12^{high}$ respectively) were identified. The proportion of the cells in each of these populations is given.

*"Pulse-Chase" Labeling of Normal, ASMKO and Transduced Cells.* $Lin^-$ cells from normal or ASMKO mice were incubated for 4 hr with a Bodipy-conjugated sphingomyelin (B12SPM), and then grown at 37°C for 48 hr in standard culture media without B12SPM. Analysis of the stem and progenitor enriched populations for B12 fluorescence is shown in Figs. 4A and 4B. Comparison of the Bodipy fluorescence (FL-1) of normal and ASMKO adult bone marrow cells after labeling with Bl2SPM showed that in both the $Lin^-/FSC^{low}/c\text{-}Kit^-$ (stem cell enriched) and $Lin^-/FSC^{high}/c\text{-}Kit^-$ (progenitor cell enriched) populations. the normal cells were less fluorescent than those from ASMKO animals. These results demonstrated that stem and progenitor cells could internalize B12SPM and that both types of normal cells expressed a functional ASM.

The same experiment was then repeated on ASMKO cells that had been retrovirally transduced with an amphotropic MFG retroviral vector expressing human ASM. The results of this experiment are shown in Figs. 4C and 4D. A significant shift to lower Bodipy fluorescence was observed only in the $Lin^-/FSC^{high}/c\text{-}Kit^-$ cells (progenitor cell enriched), while only a very minor shift was observed in the $Lin^-/FSC^{low}/c\text{-}Kit^-$ (stem cell enriched) population. These results confirmed previous studies (Orlic et al. 1996; Orlic et al. 1997) showing that murine progenitor cells (but not stem cells) could be efficiently transduced by amphotropic vectors.

*PCR and Enzyme Analysis of Sorted Cells.* To further investigate this finding, retrovirally transduced $Lin^-/c\text{-}Kit^-$ ASMKO adult bone marrow cells were labeled with Bl2SPM and subjected to FACS sorting. Cells with the highest ($B12^{high}$) and lowest ($B12^{low}$) Bodipy

fluorescence were sorted from the stem and progenitor enriched groups ($Lin^-/FSC^{low}$/c-$Kit^-$ and $Lin^-/FSC^{high}$/c-$Kit^+$, respectively), grown in expansion cultures, and subjected to semi-quantitative PCR analysis and ASM activity assays (Fig. 5). Note that within the sorted $B12^{low}$ group of $Lin^-/FSC^{low}$/c-$Kit^-$ cells, a small number were transduced (1 in 230, based on the PCR assay), while in the $B12^{low}$ group of $Lin^-/FSC^{high}$/c-$Kit^-$ cells, significantly more were transduced (1 in 8). By comparison, without selection for Bodipy fluorescence, among the $Lin^-/FSC^{low}$/c-$Kit^-$ cells no transduced cells could be identified, while among the $Lin^-/FSC^{high}$/c-$Kit^+$ cells the frequency of transduced cells was ~1 in 20 (not shown). These data confirmed the fact that the stem cell enriched population from adult bone marrow was very difficult to transduce with amphotropic vectors compared to the progenitor enriched population, but also demonstrated that the Bodipy selection technique provided a significant enrichment of transduced cells. Indeed, using this method transduced cells from both groups could be identified and collected.

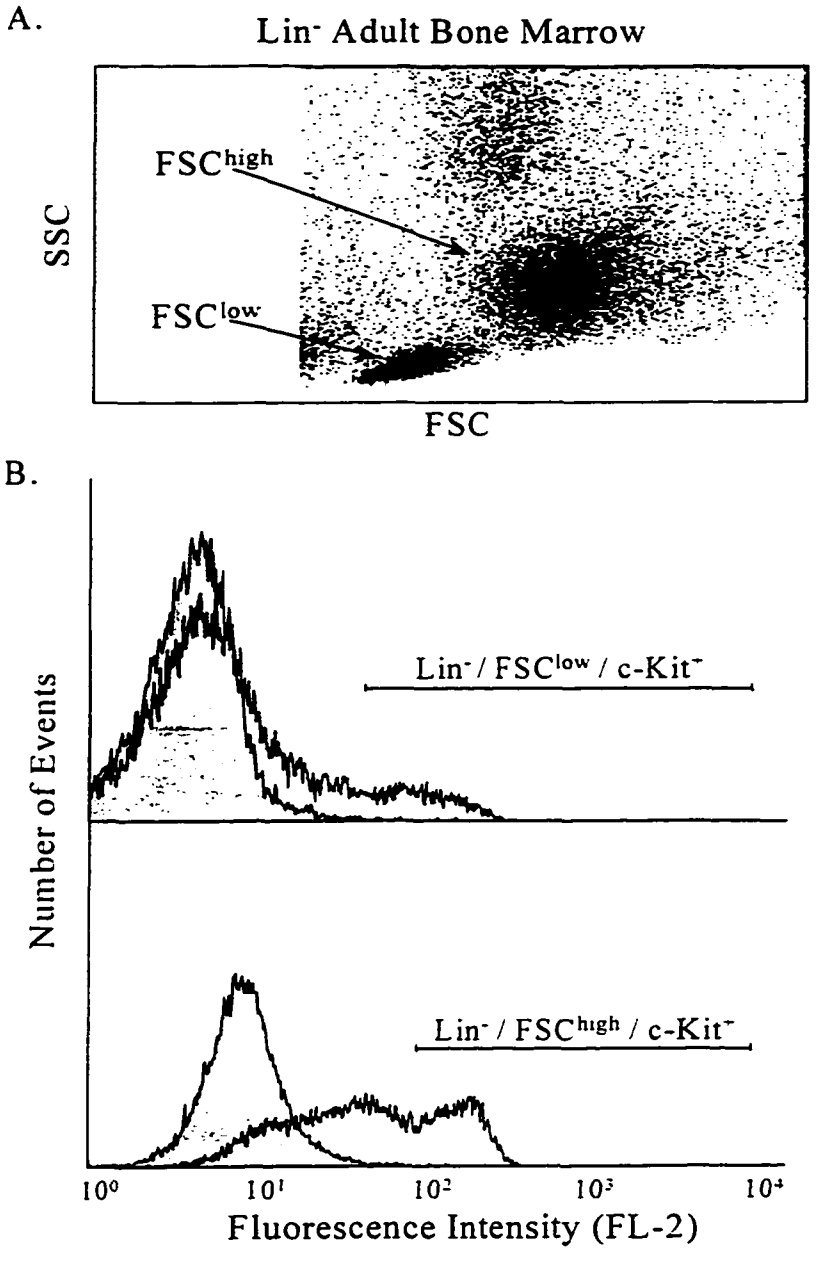


**Fig 3. Analysis of Lin$^-$ adult bone marrow cells.** The light scatter properties of the cells were measured (panel A), and two populations were identified, designated FSC$^{low}$ and FSC$^{high}$ These populations were analyzed individually for the presence of c-Kit on the cell surface using phycoerythrin (PE) conjugated anti-c-Kit antibodies (panel B). The shaded area indicates control cells, while the open area indicates cells labeled with anti-c-Kit antibodies. Note that FL-2 measures PE.

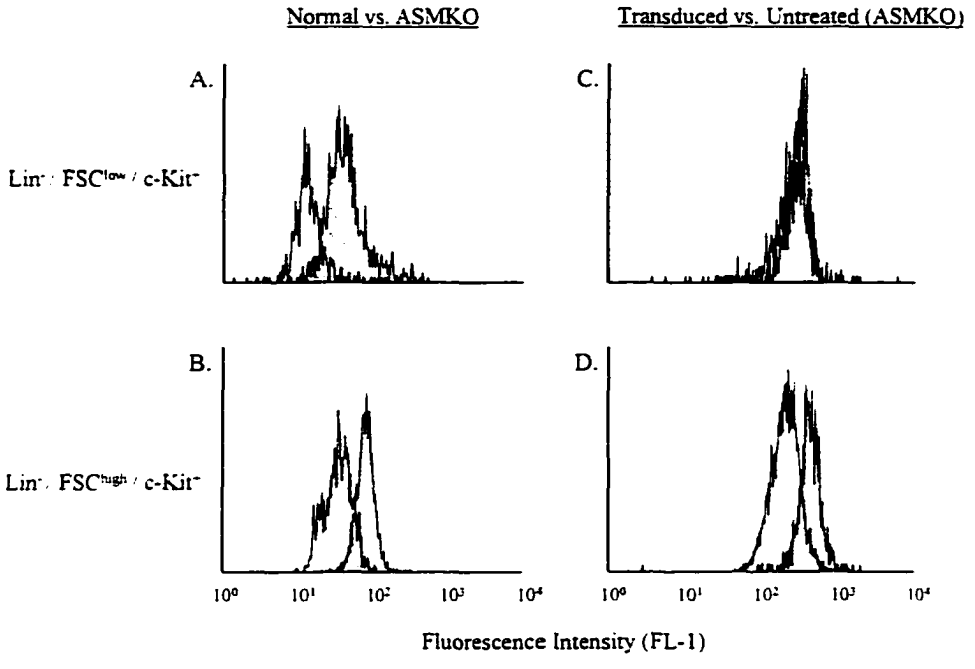


**Fig 4. Analysis of B12SPM labeling of hemopoietic stem and progenitor enriched cell populations.** Panels A and C depict the $Lin^-/FSC^{low}/c\text{-}Kit^-$ stem cell enriched population, while panels B and D depict the $Lin^-/FSC^{high}/c\text{-}Kit^+$ progenitor cell enriched population. (A & B shaded area: ASMKO cells; open area: normal cells. C & D shaded area: non-transduced ASMKO cells; open area: transduced ASMKO cells. Note that FL-1 measures B12SPM). The experiment was repeated at least three times, and the representative datum from one experiment is shown.

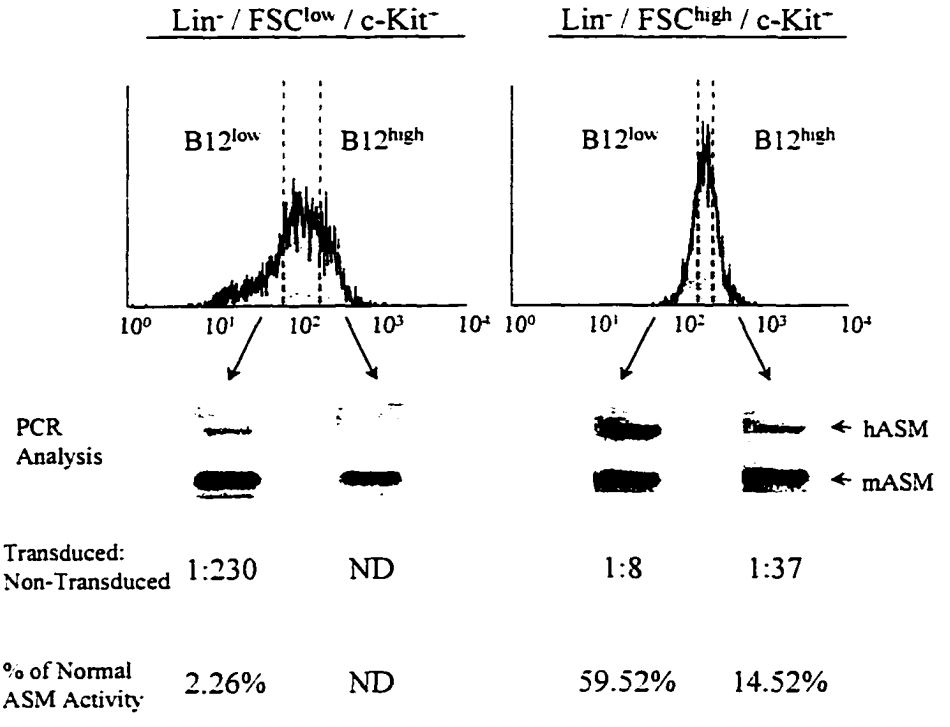


**Fig 5. Sorting and analysis of retrovirally transduced ASMKO bone marrow cells.** PCR analysis and ASM enzyme assays were conducted in triplicate on sorted $B12^{low}$ and $B12^{high}$ cells from the $Lin^-/FSC^{low}/c\text{-}Kit^-$ population (left; stem cell enriched) and $Lin^-/FSC^{high}/c\text{-}Kit^+$ population (right; progenitor cell enriched) as described in the text. ND: not detected. The experiment was repeated at least three times, and the representative datum from one experiment is shown.

*Analysis of Fetal Liver Stem and Progenitor Cells.* I next used this system to assess the transduction of fetal liver c-Kit$^+$ cells by the amphotropic vector (Fig. 6). Lin$^-$ fetal liver cells were isolated, labeled with phycoerythrin-conjugated anti-c-Kit antibodies, and analyzed on FACS. The light scatter properties of the cells were measured and, in contrast to adult bone marrow, only one population of Lin$^-$ cells was identified. This population was analyzed for the presence of the c-Kit molecule (FL-2) on the cell surface, and it was determined that the number of c-Kit$^+$ cells was ~5% of the total nucleated fetal liver cells. The Lin$^-$ ASMKO fetal liver cells were either retrovirally transduced with the ASM/MFG amphotropic vector or untreated, labeled with B12SPM, and the Bodipy fluorescence (FL- 1) of the two groups were compared. Only a small shift to lower fluorescence was observed in the retrovirally transduced population, corresponding to a low transduction efficiency of ~1 in 50 to 1 in 100 cells.

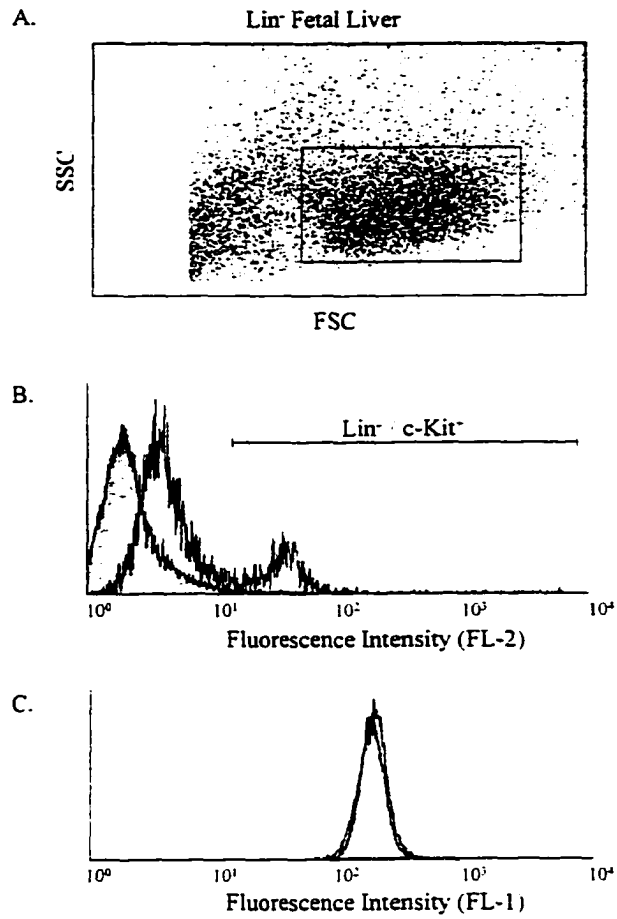


**Fig 6. Analysis of retrovirally transduced Lin⁻ fetal liver cells.** The light scatter properties of the cells were measured (panel A), and the major population was identified and gated for further analysis (shown in box). This population was then analyzed for the presence of c-Kit on the cell surface (panel B shaded area: control cells; open area: cells labeled with anti-c-Kit antibodies) Note that FL-2 measures PE. The c-Kit⁻ cells were further analyzed for B 12 fluorescence (panel C shaded area: control cells; open area: transduced cells). Note that FL-1 measures B12SPM. The experiment was repeated twice, and the representative datum from one experiment is shown.

*Comparison of the Retroviral Transduction Efficiencies Using Amphotropic vs. Ecotropic Viruses.* Since the transduction of stem cell enriched bone marrow or fetal liver cells using the amphotropic vector was low, we next sought to determine if this could be improved using an ecotropic vector. As noted above, several recent papers have reported increased transduction of murine hemopoietic stem cells using ecotropic vs. amphotropic retroviral vectors (Orlic, Girard et al. 1996; Orlic, Girard et al. 1997). As shown in Fig. 7, using the B12SPM selection technique the transduction efficiencies of $Lin^-/FSC^{low}/c\text{-}Kit^-$ stem cells with the amphotropic vs. ecotropic ASM/MFG vectors could be directly compared. Of note, the ecotropic vector led to a more than 50-fold increase in transduction over that found with the amphotropic vector.

*Effects of Individual Growth Factors on the Transduction of Murine Stem and Progenitor Cells.* To evaluate the specific contribution of growth factors to the transduction of hemopoietic cells, various combinations of SCF, IL-3 and IL-6 were used during the retroviral transduction of B12SPM labeled cells with the ecotropic vector (Fig. 8). This was followed by FACS analysis to determine the B12 fluorescence of the cells. In the $Lin^-/FSC^{low}/c\text{-}Kit^-$ population (stem cell enriched), SCF had the biggest effect on the transduction efficiency, while among the $Lin^-/FSC^{high}/c\text{-}Kit^-$ population (progenitor enriched) the largest contribution was provided by IL-6. In both groups of cells, the combination of all three growth factors led to the highest transduction efficiencies.

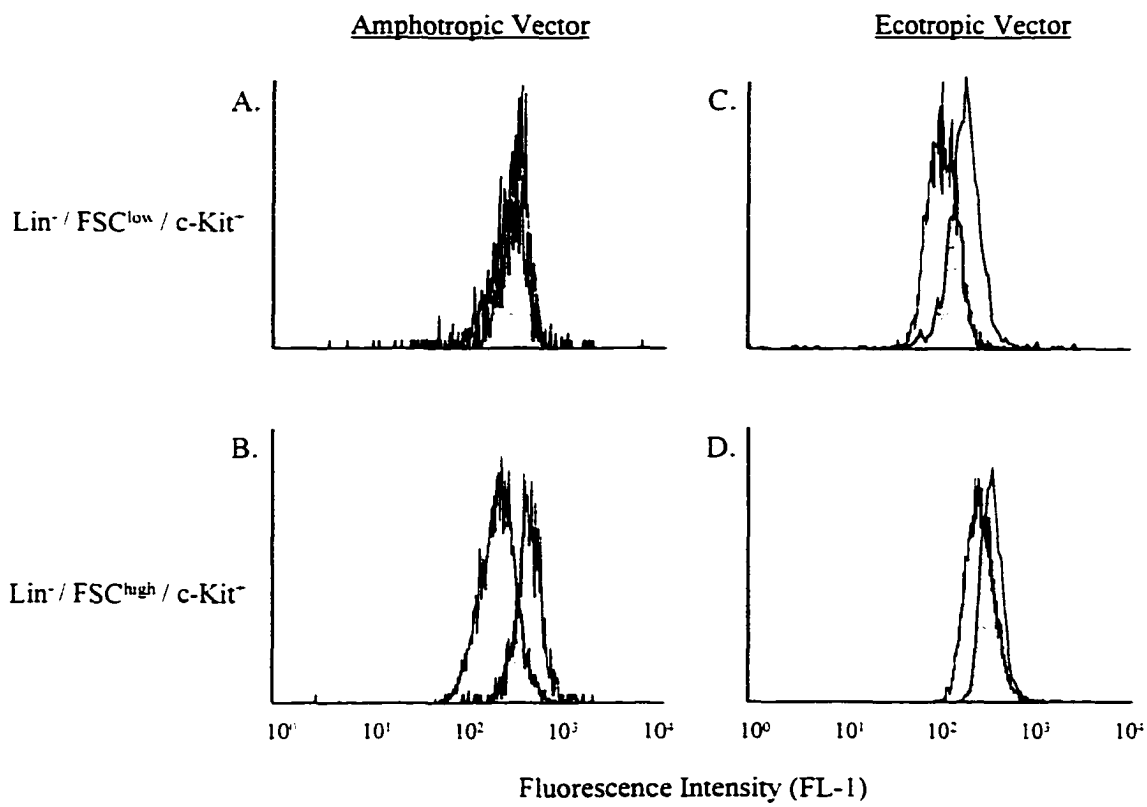


**Fig. 7. Comparison of the transduction efficiencies using amphotropic vs. ecotropic retroviral vectors.** Panels A and C depict the $Lin^-/FSC^{low}/c\text{-}Kit^-$ stem cell enriched population, while panels B and D depict the $Lin^-/FSC^{high}/c\text{-}Kit^-$ progenitor cell enriched population. The shaded areas represent non-transduced ASMKO cells, while the open areas depict transduced cells. Note that FL-1 measures B12SPM. The experiment was repeated at least three times, and the representative datum from one experiment is shown.

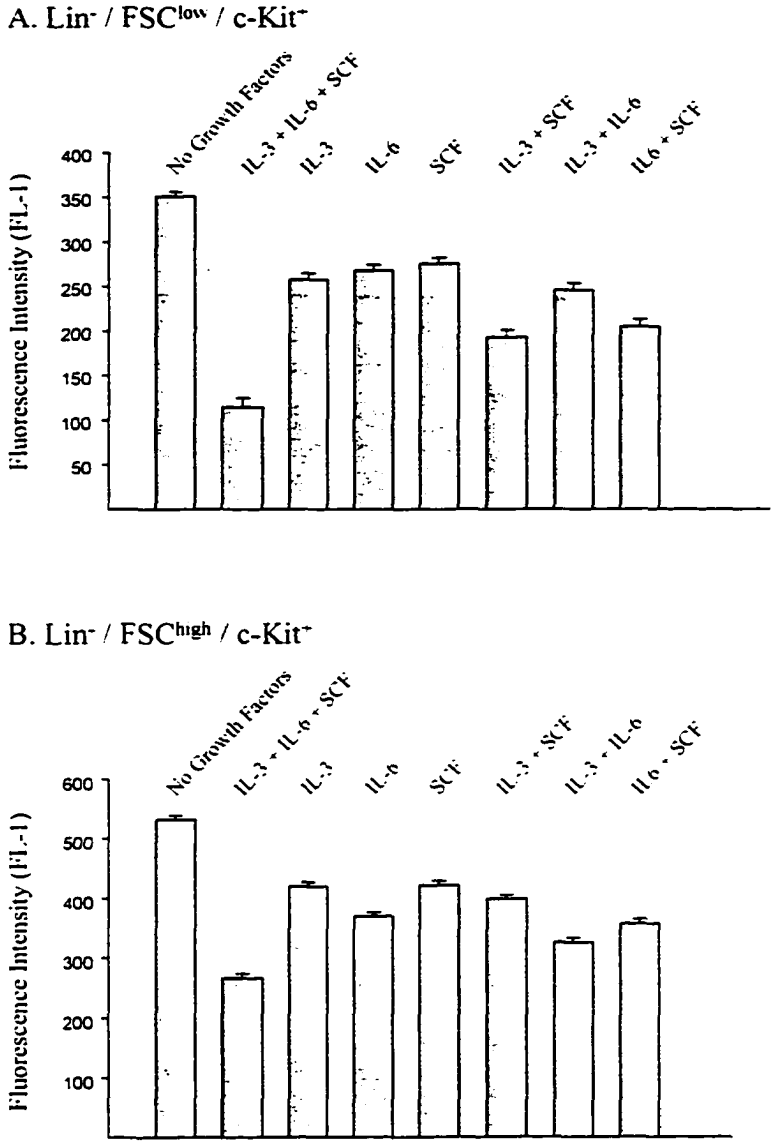


**Fig. 8. Effects of growth factors on transduction efficiencies using ecotropic vectors.** The standard retroviral transduction methods and concentrations of each growth factor are described in the Materials and Methods. FL-1 measures B12SPM fluorescence. The bars indicate +/- one standard deviation using data derived from three independent experiments.

*CFU-S Analysis of Sorted Cells.* To ensure that cells selected with these techniques were enriched for metabolically corrected hemopoietic stem cells, ASMKO bone marrow cells were transduced with the ecotropic ASM/MFG vector in the presence of SCF, 1L-3 and IL-6, $B12^{low}$ cells were collected by flow cytometry, and then transplanted into lethally irradiated adult ASMKO mice. Fourteen days later the spleens were harvested and the number of colony forming units (CFU-S) positive for the retroviral vector was determined by PCR. As shown in Table 2, in the absence of selection 32% of the CFU-S colonies were vector positive, while among animals transplanted with B12SPM-selected cells, 76% of the colonies were vector positive. Thus, these results demonstrated that a significant enrichment of transduced primitive progenitor cells can be obtained using this procedure.

**Table 2: PCR Analysis of CFU-S Colonies**

| Cell Type Injected | Total Colonies Analyzed | PCR Positive Colonies |
|---|---|---|
| Bodipy-Selected | 37 | 28 (76%) |
| Non-Selected | 28 | 9 (32%) |

CFU-S assays were carried out as described in the Materials and Methods. The number of total and PCR positive CFU-S colonies found following injection of either Bodipy-selected *(25% least fluorescent)* or non-selected transduced cells are shown. The cells selected for lower Bodipy fluorescence produced a significantly higher number of PCR positive colonies than cells that were non-selected ($\chi^2$=10.607; p=0.001).

# Discussion

As described above, a novel selection system has been previously implemented in our laboratory for the isolation of retrovirally transduced NPD cells. This fluorescence-based technique provides several important advantages over current selection schemes: 1) selection is based on metabolic correction of the diseased cell, not marker gene co-selection, 2) the transduction and selection procedures can be performed simultaneously in a short time-span of two to three days, and 3) no toxic compounds are used.

With the help of Drs. Gatt and Visser, I have been able to modify the method developed in skin fibroblasts so that it can be used for bone marrow cells. We have developed this method for two main reasons. First, we assume that the transduction of the ASM gene into NPD stem/progenitor cells does not confer any known selective advantage over non-transduced cells. Since the number of hemopoietic "niches" available for engraftment is limited (Massa et al. 1987; Quesenberry et al. 1994), it is advantageous to transplant an enriched population of transduced stem/progenitor to maximize the chance of their engraftment (versus engraftment of a non-corrected cell). Second, since the selection system is based on enzymatic expression, it is possible to select only the best enzyme expressing cells for transplantation.

In the past, lissamine rhodamine-SPM (LRSPM) was used as the fluorescent probe. However, to facilitate the use of this technique with bone marrow cells, a new fluorescent SPM derivative, Bodipy-SPM (B12SPM), has been synthesized. In my

experiments I demonstrated that while both substrates were able to label bone marrow cells, the B12SPM was much better in detecting the difference in ASM activity in normal and ASMKO cells. This can be explained by the following reasons. The B12SPM has an optimal excitation wavelength of 505 nm as compared to 560 nm for LRSPM. This wavelength is much closer to the optimal excitation wavelength of our FACStar Plus (Becton Dickenson) flow cytometer (488 nm); thus, the "specific activity" of the B12SPM probe is much higher, allowing us to detect even small differences in enzyme activity. In addition, under neutral pH the Bodipy moiety is uncharged, facilitating the entrance of the B12SPM into the cells. In contrast, the LRSPM is highly charged under the same conditions.

This selection system can be used to develop a better gene therapy protocol for NPD. The presented data clearly demonstrate that using these techniques, enriched populations of retrovirally transduced NPD hemopoietic stem and progenitor cells can be identified and collected for subsequent transplantation into patients. Such cells are currently being transplanted into ASMKO mice so that their engraftment potential and clinical usefulness can be compared to transduced hemopoietic cells which have not undergone the selection procedure. It is hypothesized that in a competitive repopulation setting, particularly in young animals that have not been lethally ablated, such selected cells may be clinically advantageous since the likelihood of engrafting a transduced, long-term repopulating stem cell will be greater due to the fluorescent enrichment procedure. Indeed, the results of the CFU-S experiment suggest that this may be the case. If successful, similar methods can be applied to $CD34^+$ cells obtained from human NPD

patients. While obtaining sufficient numbers of such $B12^{low}/CD34^{+}$ cells for human transplantation may be problematic at the present time, the development of high speed flow cytometers (Keij et al. 1991; Tanke and van der Keur 1993; Leary et al. 1994) promises to overcome this limitation.

Furthermore, we also propose that this simple analytical system can be broadly used in a variety of other gene therapy and basic research settings. The target cells do not need to be proliferating in order to take up B12SPM (Erlich and Schuchman, unpublished data), making these techniques amenable to HSCs and other quiescent cells. We have demonstrated the usefulness of this method by evaluating transduction with retroviral vectors, however other gene transfer systems can be easily studied by inserting the ASM cDNA as a marker. This would include viral or non-viral based systems. Importantly, the system relies on expression of the transferred gene as its final endpoint, and is fluorescence-based, making it highly sensitive. Also of note, the corrected cells can be isolated from the non-corrected ones using a FACS, even when they represent 1% or less of the total cell population, and the properties of the two groups can be directly compared *in vitro* or *in vivo*.

Several findings were presented in this chapter regarding the transduction of murine hemopoietic stem and progenitor cells with retroviral vectors in order to demonstrate the usefulness of these techniques. First, I confirmed the fact that amphotropic vectors could not transduce murine hemopoietic stem cells efficiently as compared to ecotropic vectors (Orlic, Girard et al. 1996; Orlic, Girard et al. 1997).

Indeed, the transduction frequency of the stem cell enriched population was increased more than 50 fold using ecotropic vs. amphotropic vectors. Of note, we also found that among the three most commonly used growth factors, SCF contributed the most to stem cell transduction, while IL-6 contributed most to progenitor cell transduction. Again, these results confirmed previous reports (Wognum et al. 1996) and demonstrated the usefulness of these techniques. Finally, we documented that c-Kit$^-$ fetal cells, which are ~2-fold more prevalent than c-Kit$^-$ bone marrow cells, were transduced with amphotropic vectors at an intermediate frequency (~1 in 100 in the Bodipy selected populations) when compared to bone marrow stem or progenitor cells.

Clearly, new vector systems, such as the Lentivirus-based retroviral vectors (Lever 1996; Poeschla et al. 1996), as well as new growth factor cocktails and packaging cell lines (Miller and Chen 1996; Schnierle et al. 1997), can be readily analyzed by these methods. Prior to the development of these and similar fluorescence-based techniques (see below), data such as these could only be obtained by transduction with vectors containing marker genes such as those encoding neomycin resistance or β-galactosidase activity, followed by labor intensive and indirect colony forming assays. Direct isolation of the transduced cells would have been impossible.

Several alternative fluorescence-based selection systems have been developed in recent years to enrich for transduced hemopoietic stem cells (Pawliuk et al. 1994; Conneally et al. 1996; Fehse et al. 1997). Among these, the use of the green fluorescence protein (GFP) has attracted considerable interest (Persons et al. 1997). However, one

important difference between our system and those which utilize GFP is that in our system the fluorescent marker (B12SPM) is not encoded by the gene transfer vector. Identification of the transduced cells relies on enzymatic expression of ASM, leading to degradation of the fluorescent molecule and a subsequent loss of fluorescence. Indeed, the target cells are only exposed to B12SPM for a short period of time, resulting in little or no toxicity. In the GFP system, the GFP gene is inserted into the vector of interest and the cells are selected for an increase in fluorescence. Stably transduced cells will continue to produce GFP and remain fluorescent, possibly leading to increased toxicity or abnormal metabolism if the transduced cells are followed for long periods of time *in vivo*.

The other methods of selecting transduced cells rely on the inclusion of a marker gene whose product is then presented on the cell surface of transduced cells (Pawliuk, Kay et al. 1994; Conneally, Bardy et al. 1996; Fehse, Uhde et al. 1997). These cells are then selected using antibodies against the cell surface marker. These methods are usually being capable of producing highly purified preparations of transduced cells. In contrast to these techniques, our system uses a derivate of a naturally occurring lipid substrate which is metabolized in most cells. Thus the chance of eliciting an immune response against the selected cells is minimized.

Thus, we believe that the selection approach described in this chapter will be of interest to a wide range of scientists, and is particularly advantageous for the development of hemopoietic stem cell gene therapy for NPD and the analysis of HSC gene transfer in general.

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# Conclusions

Type A Niemann Pick disease (NPD) is a severe, neurodegenerative storage disorder that generally leads to death before 3 years of age. Type B NPD is a milder form of the disease with little or no neurologic involvement, but progressive hepatosplenomegaly and pulmonary inflammation which generally leads to death in the second or third decades. Both diseases are allelic variants and are caused by mutations in the acid sphingomyelinase (ASM) gene, resulting in the deficient activity of the lysosomal enzyme, ASM.

Currently there is no treatment available for Niemann Pick disease. The object of my project was to use the acid sphingomyelinase knockout mouse as a model system in which new therapeutic modalities, such as bone marrow transplantation and hemopoietic stem cell gene therapy can be tested.

In Chapter 1 the ASMKO mouse was characterized on the biochemical, pathological, and clinical levels and it was found to have no residual ASM activity, and to present with a clinical and pathological phenotype that is intermediate between Types A and B NPD. Therefore we have decided that it was a suitable system in which to test BMT and HSCGT.

In Chapters 2 & 3 the efficacy of BMT for the treatment of Niemann-Pick disease was evaluated. This was done in two stages. In the first stage a large number of animals were transplanted using varying conditions (i.e., donor cell number, radiation dose). These experiments have helped us in identifying a transplantation protocol which usually

leads to high engraftment of donor stem cells in the recipients bone marrow, while at the same time minimize the deleterious side effects of BMT. In the second stage of the BMT experiments, a group of animals was transplanted using this protocol. Successfully engrafted animals were analyzed biochemically, pathologically and clinically, and it was found that although the extra-neuropathic disease phenotype can be treated successfully by BMT the therapeutic effect on the CNS phenotype is small and transient. Thus, it seems likely that BMT can be used to treat Type B but not type A NPD patients.

In Chapter 4, affected mice were also transplanted with retrovirally transduced bone marrow cells. Higher ASM activities could be achieved in these animals compared to the normal allogenic BMT results. However, preliminary results show that the effect of HSCGT is similar to that achieved by BMT.

In addition to the transplantation studies, a selection method was developed which allows us to select the metabolically corrected hemopoietic cells following retrovirus transduction of the ASM gene into the ASMKO mouse bone marrow (Chapter 5). The method utilizes an artificial, fluorescent sphingomyelin substrate that cannot be metabolized in the absence of the enzyme. By combining this labeling method with antibody selection (based on cell surface markers), we were able to select the corrected cells from various bone marrow sub-populations, including the corrected stem and progenitor cell populations. These cells can now be used for *in vivo* transplantation of ASMKO mice. In addition, this selection system has proved to be useful as an assay for the *in vitro* assessment of gene transfer into hemopoietic stem cells.

In conclusion, we have proved that BMT and HSCGT have a significant therapeutic effect on the Type B NPD phenotype. However at the present time we believe that they do not produce a large enough effect on the CNS disease to merit their use in the treatment of Type A NPD. In order to improve HSCGT so it can be used to treat type A NPD our lab intends to test the following approaches: transplantation with an enriched population of hemopoietic stem cells selected with out unique fluorescence-based technique (see Chapter 5), injection of purified corrected hemopoietic stem cells into the brains of ASMKO mouse newborns and fetal transplantation of transduced bone marrow. We hope that these improvements will lead to increase in the number of transduced cells populating the brain thereby augmenting the therapeutic effect in the CNS.

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# Introduction

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# IMAGE EVALUATION
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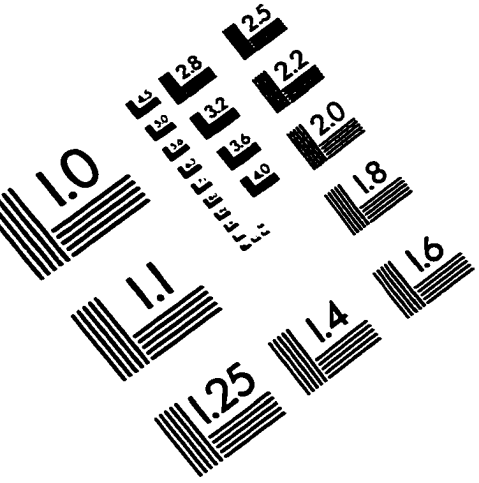


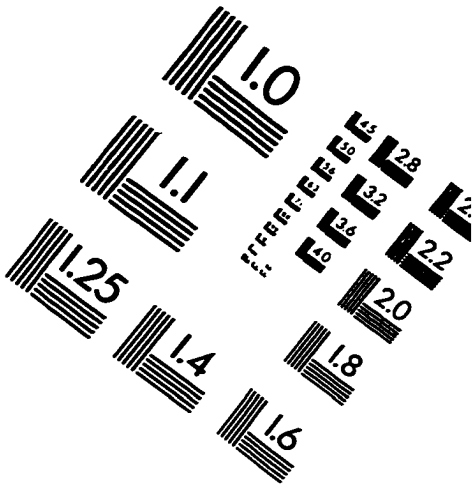


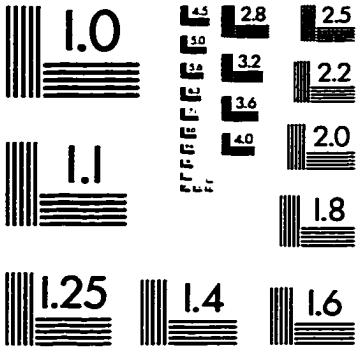


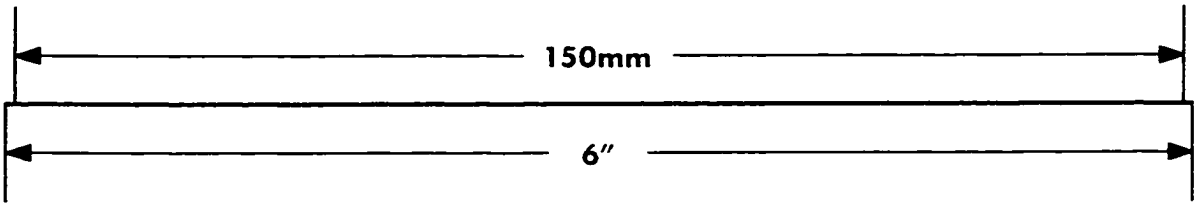


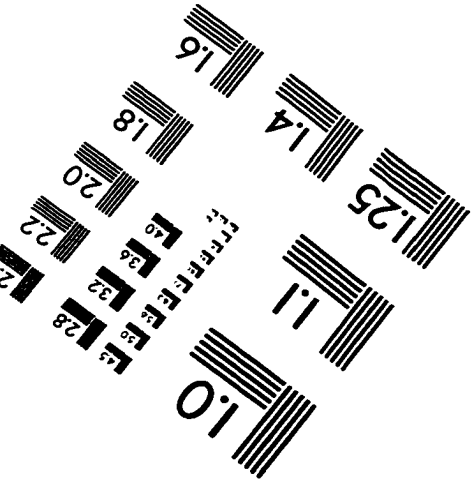


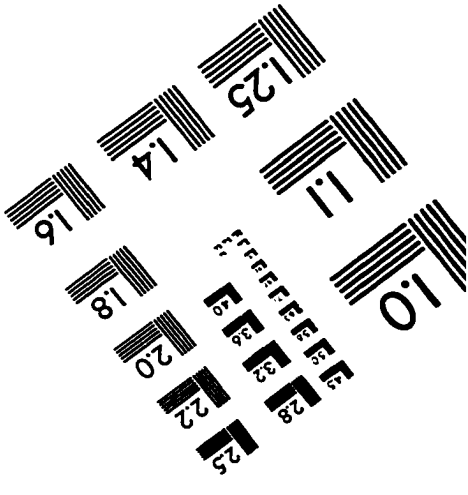


APPLIED IMAGE, Inc
1653 East Main Street
Rochester, NY 14609 USA
Phone: 716/482-0300
Fax: 716/288-5989