MUCOPOLYSACCHARIDOSIS TYPE VII

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EVALUATION OF BONE MARROW TRANSPLANTATION

AND

NON-AUTOLOGOUS SOMATIC CELL GENE THERAPY

By

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ABSTRACT

Deficiency in β-glucuronidase activity (EC 3.2.1.31) leads to the lysosomal storage disease mucopolysaccharidosis type VII not only in humans but also in a recently discovered murine mutant, the gus^{mps}/gus^{mps} mouse. Clinical and pathologic abnormalities common to the human and mouse phenotypes include shortened life span, dwarfism, dysmorphic facial features, skeletal deformities, corneal clouding, mental retardation and abnormal lysosomal storage material in the brain and peripheral organs. In the first part of this thesis, neonatal gus^{mps}/gus^{mps} mice and their normal littermates were transplanted with syngeneic normal bone marrow. Neurological function was then evaluated with two behavioral tests: the grooming test, a developmentally regulated and genetically based activity, and the Morris water maze test, which assessed spatial learning abilities. The results of these tests indicated that the behavioral deficits in the mutant mice were not restored to normal. Treated normal mice also showed significant functional deterioration, indicating the detrimental consequence of this therapy in the neonatal period. The second part of this thesis focused on a novel approach to somatic gene therapy using microcapsules. A non- autologous fibroblast cell line engineered to secrete high levels of β-glucuronidase was enclosed in perm-selective and immuno- protective microcapsules and implanted into the peritoneal cavity of gus^{mps}/gus^{mps} mice. During the 4 weeks of therapy, the biochemical and histological abnormalities of the mutant mice had significantly improved. β-Glucuronidase activity was restored to >50% of

normal in the plasma and 11.3%-65.8% in the kidney, liver and spleen. No significant activity was found in the brain. As well, the secondary elevations of other lysosomal enzymes such as β-hexosaminidase and α-galactosidase had decreased in the kidney, liver, and spleen. Urinary glycosaminoglycan content had decreased in the treated mutants indicating that the β-glucuronidase was exerting a therapeutic effect. However, after three and a half weeks of therapy, the treated mutants became severely ill and developed haemorrhagic ascites. Since normal mice treated with similar microcapsules showed no adverse effects, we hypothesized that an immune response had been generated against the foreign protein (β-glucuronidase) by the mutants, leading to the high morbidity. Thus in spite of the biochemical and histological correction observed after bone marrow transplantation and somatic cell gene therapy, the long term efficacy of these treatments needs to be further evaluated.

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Abbreviations

α	alpha
β	beta
BMT	Bone Marrow Transplantation
BSA	Bovine Serum Albumin
$CaCl_2$	Calcium chloride
cc	cubic centimetre
CHES	2-(N-Cyclohexylamino)ethanesulfonic acid
CNS	Central nervous system
DMB	1,9-dimethylmethylene blue
EDTA	Ethylenediaminetetraacetic acid disodium salt
GAG	glycosaminoglycans
HBS	HEPES-buffered saline
HCl	Hydrochloric acid
HEPES	4-(2-Hydroxyathyl)-1-piperazin-athansulfonsaure
H_2O	water
IL	Interleukin
KCl	Potassium chloride
kDa	Kilodaltons
L	litre
LB	Luria Broth
LDL	Low density lipoprotein
4-MU	4-methylumbelliferyl
mg	milligram
µL	microlitre
mL	millilitre
mM	millimolar
M	molar
M6P	Mannose 6-phosphate
M6P-R	Mannose 6-phosphate receptor
MPS	mucopolysaccharidosis
NaCl	Sodium chloride
NaOH	Sodium Hydroxide
Na_2HPO_4 $2H_2O$	di-Sodium hydrogen orthophosphate dihydrate
ng	nanograms
nm	nanometre
PBS	Phosphate buffered saline
PEG	Polyethylene glycol
PLL	poly-L-lysine

RER	Rough endoplasmic reticulum
TE	Tris EDTA
T_{CTL}	Cytotoxic T lymphocyte
T_H	Helper T lymphocyte
TNF	Tumor Necrosis Factor
Tris	Tris(hydroxymethyl)aminomethane
UDP	Uridine-diphosphate

1.0 INTRODUCTION

The rapid advancement in the technology of gene transfer methods and gene therapy are opening new dimensions in the treatment of genetic disorders. The first purpose of this introduction is to provide an overview of the different therapies used in the treatment of various genetic disorders. This will provide the basic understanding of the current approaches available. As well, it will introduce a new and rapidly emerging strategy of non-autologous somatic gene therapy using immunoisolated cells as sources of enzyme during enzyme replacement therapy. For the work of this thesis, an animal model of a lysosomal storage disease was used. It was shown that in diseases with neurological involvement, behavioural tests can provide relevant information on the efficacy of a therapy. The same murine model was used to assess the efficacy of non-autologous somatic gene therapy. Thus, the second part of this introduction gives an overview of the etiology of lysosomal storage disorders and focuses lastly on the murine model of mucopolysaccharidoses type VII.

1.1 Principles of Somatic Gene Transfer

The basic, yet broad, definition of somatic gene therapy is the use of gene transfer techniques to introduce recombinant genes into somatic cells in order to alter a disease process. Gene transfer techniques can be divided into two categories, DNA-mediated and viral-mediated gene transfer.

One of the classic methods for introducing DNA into cells is by microinjection of pure DNA into the nucleus of individual cells. More recently, researchers have shown that direct injection of DNA into tissues is also effective. Other techniques of DNA-mediated gene transfer includes electroporation, calcium phosphate precipitation, liposome fusion, receptor mediated uptake by DNA protein complexes and particle bombardment using DNA bound to metallic particles. In viral mediated gene transfer, defective viruses are used to introduce DNA into cells. Defective viruses are capable of infecting target cells (and thus transferring the packaged DNA), but lack other viral functions such as replication. Many types of viral vector systems have been developed from SV40, adenovirus, adeno-associated virus, retrovirus and herpesvirus (Mulligan, 1991; Ledley, 1992; Watt et al., 1993).

The diversity of human diseases extends further than the classical single gene Mendelian diseases and includes complex genetic diseases as well as acquired somatic diseases. The classical genetic diseases, or single gene defects include disorders such as cystic fibrosis, ADA deficiency, hemophilia, thalassemias, familial hypercholesterolemia, lysosomal storage diseases, and some cancers such as retinoblastoma and Wilm's tumour. Complex

genetic diseases (multigenic diseases) include those disease that are affected by the environment and the individual 's genetic predisposing risk factors, and include certain cancers, diabetes, and heart disease. Lastly, acquired somatic diseases are those that are brought upon by viral infection and cause diseases such as hepatitis and AIDS. Although gene therapy for the treatment of cancers and AIDS is progressing rapidly, the following discussion will focus on examples of strategies used in the treatment of classical genetic diseases.

The strategies used for gene therapy vary according to the diseases, in that the type of genetic intervention depends on the intended clinical benefit. For example, in some inherited disorders, the genetic defect alters the expression of an essential gene product that is necessary for the function of the cell in which it is synthesized. An example of such a disease where the genetic intervention would have to result in the "correction" of specific cell types is cystic fibrosis. Alternatively, the expression of a gene product in a particular cell may not be crucial for its function or survival. Rather, the gene product may be necessary for certain biological processes such as blood clotting or hormone regulation. In this case, a cell type that does not normally synthesize this gene product may be the target for gene transfer (Mulligan, 1991). There are some cases, however, where a deficiency of a gene product affects the function of the cell that normally synthesizes that product, but the target for gene transfer may be another cell type. Such is the case for lysosomal storage disorders where the enzyme can be supplied to the circulation and taken up by the affected cells through receptor-mediated endocytosis (Beutler, 1991; Neufeld, 1991). Thus in this case, the target cell for

gene transfer can be any cell type designed to secrete an adequate amount of enzyme.

To implement these strategies, the target cells can be manipulated either *in vivo* (in side the body) or *ex vivo* (in tissue culture conditions). The next section will give some examples of gene therapy using both the *in vivo* and *ex vivo* approaches. It should be noted that research in this area is extensive and involves many permutations of approaches to many diseases. Only a few will be described.

1.1.1 *In vivo* Gene Therapy

In vivo gene therapy involves the transfer of genetic material into the cells of the body. Transfer can be mediated by direct injection of DNA, liposome encapsulated DNA, particle bombardment, or by viral infection. The success of this approach depends on the receptivity of the cells to gene transfer as well as the selective targeting of the appropriate cell type. *In vivo* gene transfer into the liver, for example, can be mediated by a variety of approaches.

Liposome mediated DNA transfer involves encapsulating DNA in various lipids such as phosphatidylcholine, phosphatidylserine, cholesterol and their derivatives (Nicolau et al., 1987; Nabel et al., 1992; Stewart et al., 1992; San et al., 1993; Koike et al., 1994). The efficacy of *in vivo* gene transfer has been demonstrated by several researchers. For example, when male Wistar rats were given intravenous injections of liposome encapsulated preproinsulin I cDNA, there was a transient decrease in blood sugar levels that correlated with a transient increase in blood insulin levels (Nicolau et al., 1987). Recently, M. Baru and

his group have demonstrated that plasmids containing the Factor IX cDNA encapsulated in phosphatidylcholine liposomes can efficiently transfect the liver and spleens of mice (M. Baru, personal communication, 1994)[1]. Liposome mediated gene transfer seems to be well tolerated *in vivo*, in that it does not cause abnormal histopathology or organ toxicity (Nabel, et al., 1992; San et al., 1993). By inserting lactosylceramide (Nicolau et al., 1987) or asialoglycoprotein (Koike et al., 1994) into the liposome bilayer, the liposomes can be effectively targeted to the liver thereby increasing the potential for gene therapy on this organ.

Recombinant viruses have also been used to transfect the cells of the liver *in vivo*. When recombinant retroviruses encoding the β-galactosidase gene were infused into the portal vein of mice, a transduction frequency of 1-2% was obtained (Kay et al., 1992). Using this technique, direct gene transfer of the human α1-antitrypsin cDNA in both mice and rats resulted in stable but low α1-antitrypsin expression for six months (Kay et al., 1992; Kolodka et al., 1993). An advantage to using retroviruses for gene transfer is that the retroviral genome integrates into the host chromosomes and is therefore maintained during cell division (Watt et al., 1993). However, the use of retroviral vectors for gene delivery has its associated risks and limitations. First, there is the possibility of insertional activation of an oncogene or insertional inactivation of a tumour suppressor gene, both of which may result in vector induced malignancy (Cornetta et al., 1991). Secondly, because mitotically active cells are required for efficient retroviral infection, 70% hepatectomies were performed on the rodents

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[1]M. Baru, Octa Medical Research Institute, Bnei Brak.; 1994 meeting on Gene Therapy, Cold Spring Harbor Laboratory

prior to viral infection. Without this invasive surgery, gene expression was 30-40 fold lower (Kay et al., 1992).

Adenoviruses, on the other hand have the advantage that they can transduce non-dividing cells with high efficiencies. *In vivo* adenovirus-mediated gene delivery was used by Stratford-Perricaudet et al., 1990, to treat neonatal ornithine transcarbamylase (OTC) deficient mice (*Spf^{ash}/y*). Subsequent to intravenous injection of recombinant adenovirus vectors containing the rat OTC cDNA, the OTC activity in the liver was detected for 15 months. Other experiments of *in vivo* hepatic gene therapy using adenoviral vectors have shown that although transduction efficiency is quite high (>90%), the expression of the transduced gene declines over time (Ishibashi et al., 1993, Li et al., 1993, Smith et al., 1993, Kozarsky et al., 1994). Therapeutic levels of Factor IX was observed in mice for 4-5 weeks (Smith et al., 1993) and in dogs for 1-2 months (Kay et al., 1994). This transient peak of gene expression followed by decline to base line levels was also observed in LDL deficient mice (Ishibashi et al., 1993) and rabbits (Kozarsky et al., 1994) following *in vivo* adenoviral gene transfer of the LDLR gene.

In vivo gene delivery to specific target cells can also be accomplished by complexing ligands to gene delivery systems such as liposomes (Nicolau et al., 1987; Koike et al., 1994), adenoviruses (Curiel, 1994) and DNA-cation complexes. Wu and Wu (1988a and 1989b) have shown that a targetable DNA carrier system could be constructed by complexing galactose terminal (asialo)glycoproteins to DNA-poly L-lysine conjugates. Their hypothesis that cell surface asialoglycoproteins receptors, unique to hepatocytes, would bind to and

internalize the complexes, was confirmed by reporter gene expression in the liver 24 hours after intravenous injection (Wu and Wu, 1988b; Wu et al., 1989). Gene expression was transient but could be prolonged following partial hepatectomies. Competition assays (Wu and Wu, 1988a) and electronmicrograph and subcellular fractionation studies using radiolabelled complexes (W. Thomas, personal communication, 1994)[2] have confirmed that the DNA complexes are internalized *via* receptor mediated endocytosis. This method of *in vivo* gene delivery has been used to express, albeit transiently, albumin in Nagase analbuminemic rats (Wu et al., 1991), LDL-receptors in the Watanabe heritable hyperlipidemic rabbit and Factor IX in Sprague-Dawley rats (Ferkol et al., 1993). Mack et al., 1994 have recently shown that the addition of cationic lipids to DNA-ligand complexes enhances the transfection efficiency by tenfold.

1.1.2 *Ex Vivo* Gene Therapy

The *ex vivo* approach involves genetic manipulation of cells *in vitro*, followed by implantation of the recombinant cells into recipients. To avoid immune rejection, the patients own cells are used, or non-autologous cells are immunoisolated with inert materials prior to implantation. The strategies described for gene transfer *in vivo* can be used to transfer genes *in vitro*. Gene transfer into the haemapoetic cells provide a good example of *ex vivo* gene

[2]W. Thomas, TargeTech Inc., Meriden, Connecticut; 1994 meeting on Gene Therapy, Cold Spring Harbor Laboratory

therapy. This is especially true for diseases that can be treated by allogeneic bone marrow transplantation.

Adenosine deaminase (ADA) deficiency is one such inherited disorder that has been successfully treated with allogeneic bone marrow transplantation (BMT). Allogeneic BMT is the method of choice, since it is preferable to provide a patient with cells harbouring a normal gene rather than cells that have been genetically modified. Unfortunately, allogeneic BMT is limited by the number of HLA-matched donors (Mulligan, 1991; Anderson et al., 1990) The ultimate therapy for ADA deficiency would involve gene therapy on totipotent bone marrow cells. However, it has been difficult to obtain a suitable preparation of human stem cells for gene transfer (Williams and Orkin, 1986; Mulligan, 1991; Anderson et al., 1994). Anderson and his colleagues have hence proposed to boost the immune function of ADA deficient patients by introducing the patients own T-lymphocytes that have been transduced *in vitro* with ADA expressing retroviruses. Prior to the clinical application of this therapy, previous studies showed that genetic correction of T-lymphocytes resulted in improved immunologic function both *in vitro* and *in vivo* in both mice and monkeys (Anderson et al., 1990; Osborne et al., 1990; van Beusechem et al., 1992; Bordignon et al., 1993). Recent follow up studies on the initial clinical trial on two ADA deficient patients showed that ADA transfer into T cells resulted in prolonged T cell survival and expression

of ADA, as well as an improvement in immune function (Blaese, 1993; Blaese et al., 1993; M. Blaese, personal communication, 1994[3]).

A similar strategy has been proposed for Gaucher's disease where BMT has been shown to partially or fully reverse the disease in patients with neurological involvement (Rappeport et al., 1986; Ringdon et al., 1988; Krivit and Whitley, 1987). When bone marrow cells were transduced with retroviruses containing the glucocerebrosidase gene and implanted into irridated recipient mice, long term expression was achieved in the haemapoietic derived cell types. As well transduced cells were found in the lung, liver, CNS and spinal chord (Ohashi et al., 1992; Krall et al., 1994). Because of the difficulties in transfecting human bone marrow stem cells, the target for *ex vivo* gene transfer, like that for ADA deficiency, are the T-lymphocytes (Karlsson, et al., 1993; Robbins et al., 1994; Xu et al., 1994; R.E. Donahue[4], R. Learish[5], personal communication, 1994). A clinical protocol for the treatment of Gaucher's disease using this approach has already been approved (Anderson, 1994; Robbins et al, 1994).

Clinical improvement in patients with Gaucher's disease has also been observed following intravenous administration of glucocerebrosidase (Barton et al., 1991; Beutler,

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1992; Fallet el al., 1992; Zimran et al., 1993). However, there is a need for repeated doses and hence the need for large quantities of enzyme, both of which are very costly (Beutler, 1992; Bansal et al., 1994). Thus, for Gaucher's disease and other disorders that can be corrected by the introduction of a functional gene product into the systemic circulation, cells genetically engineered to secrete the deficient gene product can function as sources of enzyme when implanted into recipients. In a recent study, C2C12 myoblasts engineered to secrete glucocerebrosidase were implanted into the thigh muscles of C3H mice. The transduced cells secreted significant amounts of enzyme that was to be taken up by cells distant from the site of transplantation (Bansal et al., 1994; C. Liu, personal communication, 1994[6]).

Recombinant cells have also been used to deliver gene products such as α-L-iduronidase (A. Salvetti, private communication, 1994[7]), insulin (Stewart et al., 1993; Newgard, 1994), clotting factors VIII (Zatloukal et al., 1994) and IX (Dai et al., 1992; Palmer et al., 1989) and growth hormone (Chang et al., 1990; Barr and Leiden, 1991; Dhawan et al., 1991). In all these cases, autologous cells were used to avoid immune rejection. The application of this approach in humans, however, has some limitations. The isolation of a patients own cells, propagation *in vitro*, followed by genetic manipulation and characterization before implantation into that patient is labour intensive and may incur

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prohibitive costs. In 1993, Chang et. al. proposed an alternative strategy of non-autologous somatic gene therapy that would, in theory, reduce the costs of autologous *ex vivo* gene therapy. This approach involves encapsulating non-autologous recombinant cells with a biocompatible and permselective membrane. Because the cells are immunoisolated, the capsules may then be implanted into different patients that require the same gene product.

1.1.2.1 Encapsulation of cells in biocompatible polymers

The concept of implanting biological products enclosed in perm selective artificial membranes was first described by Chang in 1964. A variety of non-recombinant cell types including embryonic mesencephalon (Aebischer et al., 1988), erythrocytes (Sefton and Broughton, 1982), hepatocytes (Sun et al., 1986; Chang, 1992), PC12 cells (Aebischer et al., 1991; Winn et al., 1991; Tresco et al., 1992), parathyroid tissue (Aebischer et al., 1986), and pancreatic islet cells (Lim and Sun, 1980; O'Shea et al., 1984; Dupuy et al., 1988; Fan et al., 1990; Weber et al., 1990; Lacy et al., 1991) have since been encapsulated and maintained in vitro or implanted in animal models of human diseases.

Cells have been enclosed in both macrocapsules and microcapsules. Macroencapsulation involves filling preformed hollow fibres with a cell suspension. Macrocapsules are larger than microcapsules (1-10 mm length, 280-800 μm diameter) and are easily retrievable. However, because the capsules themselves are large and have a thick wall (100μm), nutrient diffusion can be limited and thus can compromise cell viability

(Aebischer et al., 1986; Aebischer et al., 1988; Winn et al., 1989; Aebischer et al., 1991; Emerich et al., 1992). Microcapsules in comparison are smaller (300-700 μm diameter), have a thinner wall and are spherical; characteristics that are optimal for nutrient diffusion and hence cell viability. However because of the polymers used in microencapsulation, the membrane is more mechanically fragile than that of macrocapsules (Emerich et al., 1992).

Most of the microencapsulation techniques to date involve forming droplets of a polyanion (alginate) and a cellular suspension. The cells are immobilized in a negatively charged matrix by gelling the droplets in a cationic ($CaCl_2$) bath. A permselective membrane is formed by the addition of a polycation (poly L-lysine) and a second coat of alginate. Hence, the microcapsules are formed by ionic and hydrogen bonds between the polyelectrolytes of opposite charges (Emerich et al., 1992). Once the membrane is formed, the core alginate is dissolved by a wash in sodium citrate, thus leaving room inside the microcapsules for nutrient/waste diffusion and cell proliferation. At the same time, the encapsulated cells are protected from the elements of the immune system when implanted into recipients. Hence, the use of unmatched tissue (non-autologous) is then possible by this technique.

Studies using non-recombinant microencapsulated cells have demonstrated that the cells remain viable and functional when implanted into animals. Implantation of encapsulated dopamine secreting PC12 cells was shown to improve the behavior of rats with lesions of their dopaminergic pathway (Winn et al., 1991); microencapsulated pancreatic islet cells have been used to treat diabetic rats (O'Shea et al., 1984; Fan et al., 1990; Weber et al., 1990); and

encapsulated hepatocytes have shown to increase the survival time of rats with acute hepatic failure, and to decrease bilirubin levels in hyperbilirubinemic (Gunn) rats (Sun et al., 1986; Chang, 1992).

Cells genetically modified to secrete a desired gene product provide an alternative to using cells that naturally produce that same gene product. This alleviates the problem of culturing in large amounts cell types (eg. neurons) that are not amenable to long term culture conditions. Cells that grow readily in culture such as fibroblasts and myoblasts can be used instead. Furthermore, the expression of gene products as well as the homogeneity of cell type can be controlled by using genetically modified cells.

Fibroblasts and myoblasts enclosed in alginate-poly L-lysine-alginate microcapsules have been maintained *in vitro* or implanted in animals (Al-Hendy et al., 1995; Chang et al., 1993; Liv et al., 1993; Bastedo et al., 1994a; Chang et al., 1994; Hortelano et al., 1994). Hortelano et al., 1994, demonstrated that encapsulated recombinant myoblasts are able to deliver continuous amounts of factor IX into the systemic circulation of mice for at least 4 months (Hortelano et al., 1994). Encapsulated recombinant fibroblasts have also been shown to deliver human growth hormone to the systemic circulation of rodents (Chang et al., 1993; Tai and Sun, 1993). In fact, when encapsulated mouse growth hormone secreting myoblasts were implanted into growth hormone deficient Snell mice, the mice responded physiologically and biochemically to the hormone (Al-Hendy et al., 1995). In all these cases, the recombinant cells were non-autologous and the microcapsules, when retrieved, were intact and free of inflammatory adhesions. Thus, non-autologous recombinant cells, when enclosed in

microcapsules, can survive and function *in vivo*, and are protected from the host's immune system. It seems feasible then, to use this strategy for a wide variety of diseases where the functional gene product is required in the circulation. As well, it is also conceivable that this approach can be used to treat lysosomal storage disorders as lysosomal enzymes have been shown to enter cells via receptor-mediated endocytosis.

1.2 Lysosomal Storage Diseases

1.2.1 Background

Hydrolytic enzymes within lysosomes are responsible for the degradation of a variety of macromolecules. These include bacteria, hormones and transport proteins such as low density lipoproteins (LDL), which are taken in by cells by phagocytosis or by receptor mediated endocytosis. Lysosomes are also responsible for the turnover of extracelluar matrix components as well as intracellular components such as mitochondria and cell membrane.

Like secretory proteins and plasma membrane proteins, lysosomal enzymes are synthesized on membrane bound polyribosomes in the rough endoplasmic reticulum (RER). The nascent protein is translocated into the lumen of the RER where the amino-terminal signal peptide that initiated its entry is cleaved. The protein is glycosylated with a preformed oligosaccharide chain containing three glucose, nine mannose and two *N*-acetylglucosamine residues. This is followed by further processing of the oligosaccharide chain and results in the excision of one mannose and three glucose residues. The lysosomal bound enzymes then move to the Golgi apparatus where selected mannose residues are phosphorylated through the concerted actions of UDP-α-*N*-acetylglucosamine: glycoprotein-*N*-acetylglucosamine-1-phosphotransferase (EC 2.7.8.17) and *N*-acetylglucosamine-1-phosphodiester α-*N*-acetylglucosaminidase (EC 3.1.4.45). The mannose 6-phosphate (M6P) residues on the enzymes bind with high affinity to mannose 6-phosphate receptors (M6P-R's) located in

clathrin coated pits in the Golgi. The coated pits containing the receptor-ligand complexes then buds off from the Golgi membrane to form the prelysosomal compartment. This fuses with other vesicles such as endosomes and autophagic vacuoles to form larger secondary lysosomes. It is in the acidified compartment of the lysosomes where the hydrolases become activated. A small percentage of the lysosomal enzymes fail to bind to the M6P-R's in the Golgi and are secreted along with other secretory proteins (Jourdian et al., 1984; Kornfeld, 1987; Alberts et al., 1989).

In the classic lysosomal storage diseases, deficiencies in specific lysosomal enzymes result in undegraded macromolecules, such as complex carbohydrates and lipids, to accumulate in the lysosomes. The accumulation of substrates is progressive and in effect, over time, perturbs normal cellular function. This concept was first developed by Hers in 1965 (Hers, 1965). With the widening knowledge of cell physiology and lysosomal function, the term lysosomal storage diseases then expanded to include metabolic lesions of enzyme transport. Taken individually, lysosomal storage diseases are rare. Collectively, however, they occur at a rate of approximately 5 per 10 000 births (Watts and Gibbs, 1986). Each disorder is classified into groups on the basis of enzyme deficiency (glycosidases, lipid-degrading hydrolases, and sulphatases), the primary storage product and/or metabolic lesion in transport. Within each disorder there is also considerable heterogeneity with respect to the genetics, clinical phenotype and tissue pathology (Watts and Gibbs, 1986; Neufeld and Meunzer, 1989).

The main groups of lysosomal storage disorders, individual diseases within these main groups, and the metabolic lesions are listed in Table 1. It has been proposed that this list comprises only 50 % of the potential lysosomal storage diseases as deficiencies in many other existing lysosomal enzymes have not been discovered (Reuser, 1984). The purpose of the next section is to provide an overview of the biochemistry and clinical course of the mucopolysaccharidoses, focusing lastly on MPS VII.

Table 1. Lysosomal Storage Diseases. Summary of the main groups of lysosomal storage disorders, the associated enzyme deficiencies, major storage product(s) and the organ(s) mainly affected. Data for this table was compiled from Kornfeld and Sly, 1985; Meunzer, 1986; Watts and Gibbs, 1986 and Neufeld and Meunzer, 1989.

Table 1. Lysosomal storage diseases.

Systematic Name	Eponymous Name	Enzyme Deficiency	Storage Product(s)	Organ(s) Mainly Affected
Sphingolipidoses				
G_{M1} gangliosidosis		β-D-galactoside galactohydrolase (G_{M1}-β-galactosidase; EC 3.2.1.23)	G_{M1} gangliosides, *N*-acetyl glucosaminyl oligosaccharides	CNS, skeleton, viscera
G_{M2} gangliosidosis (Type I)	Tay-Sachs disease	β-*N*-acetylhexosaminidase A (hexosaminidase A; EC 3.2.1.52)	G_{M2} gangliosides G_{A2} gangliosides	CNS
G_{M2} gangliosidosis (Type II)	Sandhoff disease	β-*N*-acetylhexosaminidase A and B (hexosaminidases A and B; EC 3.2.1.52)	G_{M2} ganglioside, G_{A2} ganglioside, globoside, *N*-acetyl-glucosaminyl oligosaccharides	CNS, skeleton, viscera
α-Galactosyl-lactosyl ceramidosis	Fabry disease	Ceramide trihexosidase (α-galactosidase A; EC 3.2.1.22)	α-Galactosyl-lactosyl ceramide	Kidney, brain, blood vessels of skin
Lactosylceramidosis	Ceramide lactoside lipidosis	Ceramide-lactoside-β-galactosidase (EC 3.2.1.23)	Lactosylceramide	CNS, skeleton, viscera
Mucosulphatidosis	Multiple suphatase deficiency	Arylsulphatases A, B and C (EC 3.1.6.8) and steriod sulphatase EC 3.1.6.2)	Cerebroside sulphate, Sulphated glycosaminoglycans, Cholesteryl sulphate	CNS, liver, spleen

Sulphatidosis	Metachromatic leukodystrophy (MLD)	Arylsulphatase A (cerebroside sulphate; EC 3.1.6.8)	Cerebroside sulphate	CNS
Galactocerebrosidosis	Krabbe disease	Galactosylceramidase (EC 3.2.1.46)	Galactocerebroside (galactosylceramide)	CNS
Glucocerebrosidosis	Gaucher disease	Glucosylceramidase (β-glucocerebrosidase; EC 3.2.1.45)	Glucocerebroside (glucosylceramide)	Brain, bone marrow, liver, spleen
Sphingomyelinosis	Niemann-Pick disease	Sphingomyelin phosphodiesterase (sphingomyelinase; EC 3.1.4.12)	Sphingomyelin	Brain, bone marrow, liver, spleen
Ceramidosis	Farber disease	Acylsphingosine deacylase (ceramidase; EC 3.5.1.23)	Ceramide	subcutaneous nodules, joints, larynx

Lipidoses

-	Wolman's disease	Acid lipase (EC 3.1.1.3)	Triglycerides, cholesterylesters	liver, bone marrow, kidney, spleen, adrenal
Cholesteryl ester stroage disease	-	Acid lipase (EC 3.1.1.3)	Triglycerides, cholesterylesters	liver, spleen, heart

Table 1. Lysosomal Storage Diseases (continued)

Systematic Name	Eponymous Name	Enzyme Deficiency	Storage Product(s)	Organ(s) Mainly Affected
Glycoproteinosis				
Fucosidosis	-	α-Fucosidase (EC 3.2.1.51)	Fragments of glycoproteins and glycolipids	CNS
Mannosidosis	-	α-mannosidase (EC 3.2.1.24)	Fragments of glycoproteins	CNS, skeleton, liver, spleen
Aspartylglucosaminu-ria	-	1-Aspartamido-β-*N*-acetyl-glucosamine amidohydrolase (amidase; EC 3.2.2.11)	Fragments of glycoproteins, aspartyl-2-deoxy-2-acetamido glucosylamine	CNS, connective tissue, bone marrow
Mucolipidosis I	Sialidosis	Neuraminidase (EC 3.2.1.18)	Fragments of glycoproteins	CNS, Skeleton, liver, spleen
Disorders of enzyme localization				
Mucolipidosis II	I-cell disease	UDP-α-*N*-acetylglucosamine: glycoprotein-*N*-acetylglucosamine-1-phosphotransferase	Mucopolysaccharides, lipids, glycoproteins	CNS, connective tissue, skeleton, heart

Mucolipidosis III	Pseudo-Hurler polydystrophy	UDP-α-*N*-acetylglucosamine: glycoprotein-*N*-acetylglucosamine-1-phosphotransferase	Mucopolysaccharides, lipids, glycoproteins	joint, connective tissues
Disorders of lysosomal efflux				
Cystinosis	-	proposed cystine efflux mediator	Cystine	kidney
-	Salla's disease	proposed sialic acid mediator	Sialic acid	CNS
Mucopolysaccharidoses				
MPS IH	Hurler	α-L-iduronidase (EC 3.2.1.76)	Dermatan sulphate Heparan sulphate	CNS, Skeleton, viscera
MPS IS	Scheie	α-L-iduronidase (EC 3.2.1.76)	Dermatan sulphate Heparan sulphate	Skeleton, viscera (relatively mild compared to MPS I-H
MPS IH/S	Hurler/Scheie	α-L-iduronidase (EC 3.2.1.76)	Dermatan sulphate Heparan sulphate	Phenotype intermediate between MPS I-H and I-S
MPS II	Hunter	α-L-idurono-2-sulphate sulphatase	Dermatan sulphate Heparan sulphate	CNS, skeleton, viscera
MPS IIIA	Sanfilippo A	Heparan sulphaminidase	Heparan sulphate	CNS

Table 1. Lysosomal Storage Diseases (continued)

Systematic Name	Eponymous Name	Enzyme Deficiency	Storage Product(s)	Organ(s) Mainly Affected
MPS IIIB	Sanfilippo B	α-D-*N*-acetylglucosaminidase (EC 3.2.1.50)	Heparan sulphat	CNS
MPS IIIC	Sanfilippo C	Acetyl CoA: α-glucosaminidase-*N*-acetyltransferase (EC 2.3.1.3)	Heparan sulphate	CNS
MPS IIID	Sanfilippo D	α-*N*-acetylglucosaminide-6-sulphate sulphatase (EC 3.1.6.7)	Heparan sulphate	CNS
MPS IVA	Morquio A	*N*-acetylgalactosamine-6-sulphate sulphatase (chondroitin sulphate sulphohydrolase; EC 3.1.6.4)	Keratan sulphate Chondroitin sulphate	Skeleton
MPS IVB	Morquio B	β-D-galactoside galactohydrolase (β-galactosidase; EC 3.2.1.23)	Keratan sulphate Chondroitin sulphate	Skeleton
MPS VI	Maroteaux-Lamy	*N*-acetylgalactosamine-4-sulphate sulphatase (arylsulphatase B; EC 3.1.6.1)	Dermatan sulphate Chondroitin 6-sulphate	Skeleton
MPS VII	Sly	β-glucuronidase (EC 3.2.1.31)	Dermatan sulphate Heparan sulphate Chondroitin 6-sulphate	CNS, skeleton, viscera

1.2.2 Mucopolysaccharidoses

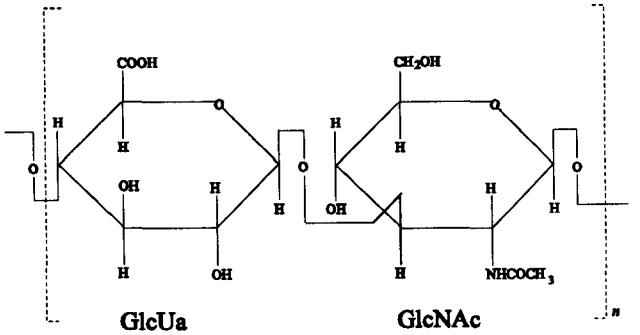
Deficiencies in specific lysosomal exoglycosidases or sulphatases result in the accumulation of glycosaminoglycans (mucopolysaccharides) in lysosomes as well as its excessive excretion in the urine. This enzymopathy is termed mucopolysaccharidoses (MPS). The clinical manifestations are heterogeneous between and within each disorder, and may result in neurological, visceral and/or skeletal involvement. The mucopolysaccharidoses, like other lysosomal storage diseases, are progressive disorders. They were first systematically classified by McKusick (1965) on the basis of clinical features, genetics and biochemistry of the excreted mucopolysaccharides. It was later modified to its present form as specific enzyme defects were elucidated (Table 1). The rest of this section will describe the chemistry of the glycosaminoglycans and its degradation. As well specific metabolic lesions in the different mucopolysacharidosis will be discussed.

1.2.3 Chemistry of Glycosaminoglycans and the Metabolic Lesions in Mucopolysaccharidoses

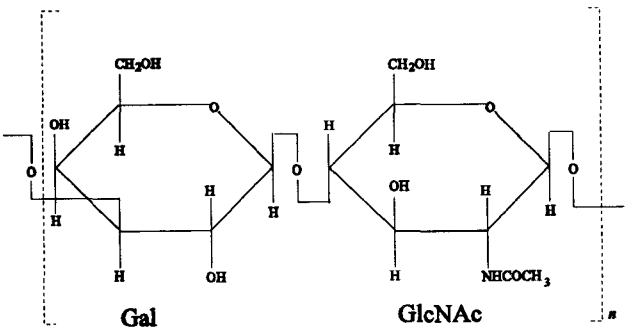
Glycosaminoglycans (GAG) are long unbranched polysaccharide chains. The long carbohydrate chain is composed of repeating disaccharide units of which one is always an amino sugar (*N*-acetylglucosamine or *N*-acetyl galactosamine) and the other is a hexuronic acid, except in the case of keratan sulphate where the second sugar is galactose (Figure 1).

The glycosaminoglycans are highly negatively charged because of the sulphate and carboxyl groups. They are distinguished by their sugar residues, number and location of sulphate groups and the type of linkage between these residues and include hyaluronic acid, chondroitin 4- and 6- sulphate, dermatan sulphate, heparin and heparan sulphate, and keratan sulphate. With the exception of hyaluronic acid, all of the glycosaminoglycans are attached to a protein core to form proteoglycans. Proteoglycans are distinguished from glycoproteins such that the latter contains relatively short branched oligosaccharide chains that comprises about 1-60% of the carbohydrates by weight. Proteoglycans, on the other hand, contain large numbers of long unbranched glycosaminoglycan chains (about 1 for every 20 amino acids) and can contain as much as 95% carbohydrates by weight. Because of the high density of negative charges glycosaminoglycan chains are strongly hydrophilic and occupy a huge volume relative to their mass (Alberts et. al., 1989).

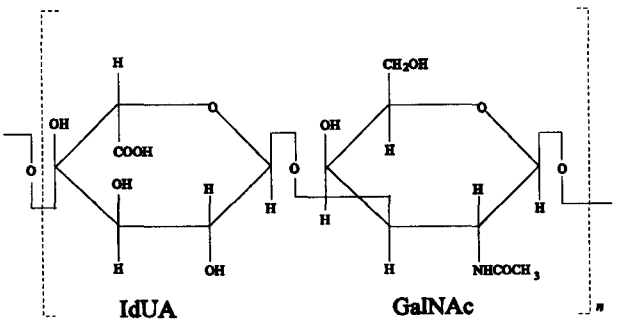
Figure 1. The main repeating disaccharide units of glycosaminoglycans: 1) hyaluronic acid; 2) keratan sulphate; 3) dermatan sulphate; 4) heparin and heparan sulphate; 5) chondroitin 4-sulphate; 6) chondroitin 6-sulphate (redrawn from Watts and Gibbs, 1986 and Alberts et. al., 1989). The sulphate groups on the sugar residues are not shown except for the chondroitin sulphates. Heparin has the same repeating units as heparan sulphate, except that in the former the majority of the D-glucuronic acid residues are replaced by L-iduronic acid residues. Abbreviations are GlcUA, D-glucuronic acid; IdUA, L-iduronic acid; GlcNAc, *N*-acetylglucosamine; GalNAc, *N*-acetyl galactosamine; Gal, D-galactose; S, sulphate.



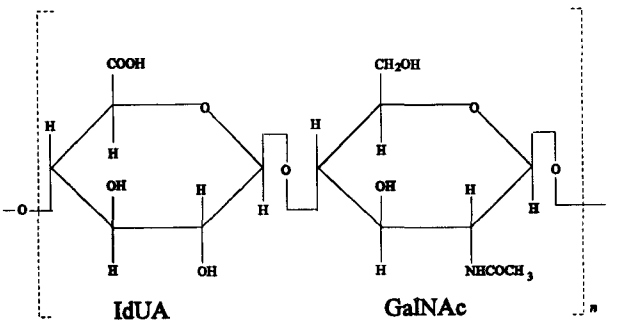
1. Hyaluronic Acid



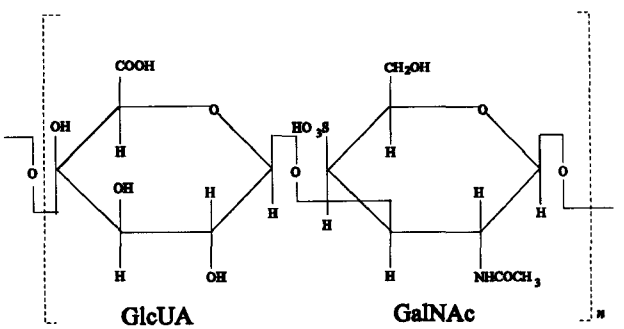
2. Keratan sulphate



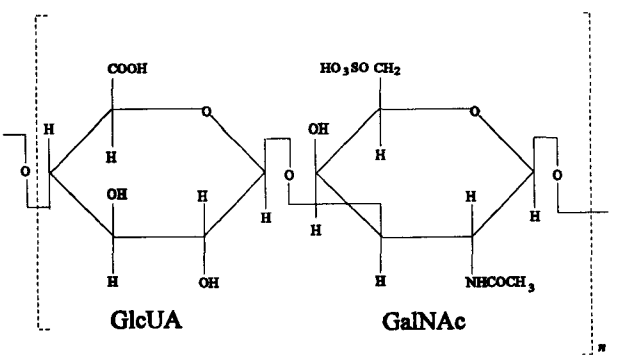
3. Dermatan sulphate



4. Heparan sulphate



5. Chondroitin 4-sulphate



6. Chondroitin 6-sulphate

Hyaluronic acid (hyaluronate) is a linear unbranched molecule that consists of repeating nonsulphated disaccharide units (Figure 1). It is found in all tissues and fluids such as connective, skin, cartlage, vitreous body, aorta, synovial and joint fluid. As well, it occurs in large amounts around proliferating and migrating cells (Brimacomb and Webber, 1964; Alberts, et al., 1989). Although the GlcUA(β1$\rightarrow$3)GlcNAc[8] linkages are susceptible to cleavage by the endoglycosidase hyaluronidase, there are no known mucopolysaccharidoses that occur as a result of abnormal or deficient hyaluronidase activity (Watts and Gibbs, 1986).

The most abundant of the glycosaminoglycans are the chondroitin 4- and 6- sulphates (chondroitin A and C respectively). They are the main normal urinary glycosaminoglycans and occur throughout the connective tissues, cartilage, as well as in skin, bone, arteries and cornea (Watts and Gibbs, 1986; Alberts et. al., 1989). Although hyaluronidase can attack the GlcUA(β1$\rightarrow$3)GalNAc glycosidic linkage of the chondroitin sulphates (Figure 1), it does not hydrolyze the linkages at regular intervals (Brimacomb and Webber, 1964; Watts and Gibbs, 1986). Degradation of chondroitin 4- and 6-sulphate requires the concerted action of the exoglycosidases β-glucuronidase and β-*N*-acetylhexosaminidase (Figure 2). Desulphatation of the 4- and 6- sulphates usually precedes hydrolysis of the glycosidic bond (Watts and Gibbs, 1986).

Dermatan sulphate (chondroitin sulphate B) is typically found in subcutaneous tissue, blood, vessel walls, heart and lung parenchyma. The major repeating disaccharide units of

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[8]Abbreviations are as follows: GlcUA, D-glucuronic acid; IdUA, L-iduronic acid; GlcNAc, *N*-acetylglucosamine; GalNAc, *N*-acetylgalactosamine; Gal, D-galactose.

dermatan sulphate are α-L-iduronic acid and α-*N*-D-acetylgalactosamine (Figure 1). However, 10-20% of the α-L-iduronate residues are replaced with D-glucuronic acid. The degradation of dermatan sulphate requires the sequential action of five lysosomal enzymes: α-L-iduronate 2-sulphate sulphatase, α-L-iduronidase, *N*-acetylgalactosamine 4-sulphate sulphatase, β-*N*-acetylhexosaminidase and β-glucuronidase (Figure 3).

Heparan sulphate and heparin are closely related glycosaminoglycans that contain α-L-iduronic acid and glucosamine residues. Herparin is stored in mast cells and found in the tissue fluids of the lung and liver. Herparan sulphate proteoglycans are found in the lung, arteries, basal laminae, and are wide spread components of cell membranes. Compared to heparin, heparan sulphate has more D-glucuronic acid residues than α-L-iduronic acid residues. As well, the majority of glucosamine residues are sulphated, a smaller number of glucosamine residues are either acetylated or unsubstituded. As shown in Figure 4, the action of seven lysosomal enzymes are required to sequentially degrade heparan sulphate.

Keratan sulphate is found in cartilage, cornea and in the intervertebral discs. It is unique among the glycosaminoglycans, in that the characteristic disaccharide units contain D-galactose instead of a hexuronic acid (Figure 1). As can be seen from Figure 5, degratation of keratan sulphate requires the sequential action of *N*-acetylgalactosamine 6-sulphate sulphatase, β-galactosidase, *N*-acetylglucosamine 6-sulphate sulphatase and β-*N*-acetylhexosaminidase.

Figure 2. Diagramatic representation of the degradation of chondroitin 4- and 6-sulphate (adapted from Watts and Gibbs, 1986). The degree of sulphatation and the sequence of carbohydrate residues are purely schematic. Deficiencies in any of the specific enzymes involved in chondroitin sulphate degradation results in MPS IVA, MPS VI or MPS VII. Note however, that a deficiency in β-D-*N*-acetylhexosaminidase results in Sandhoff's disease. Abbreviations are the same as in Figure 1.

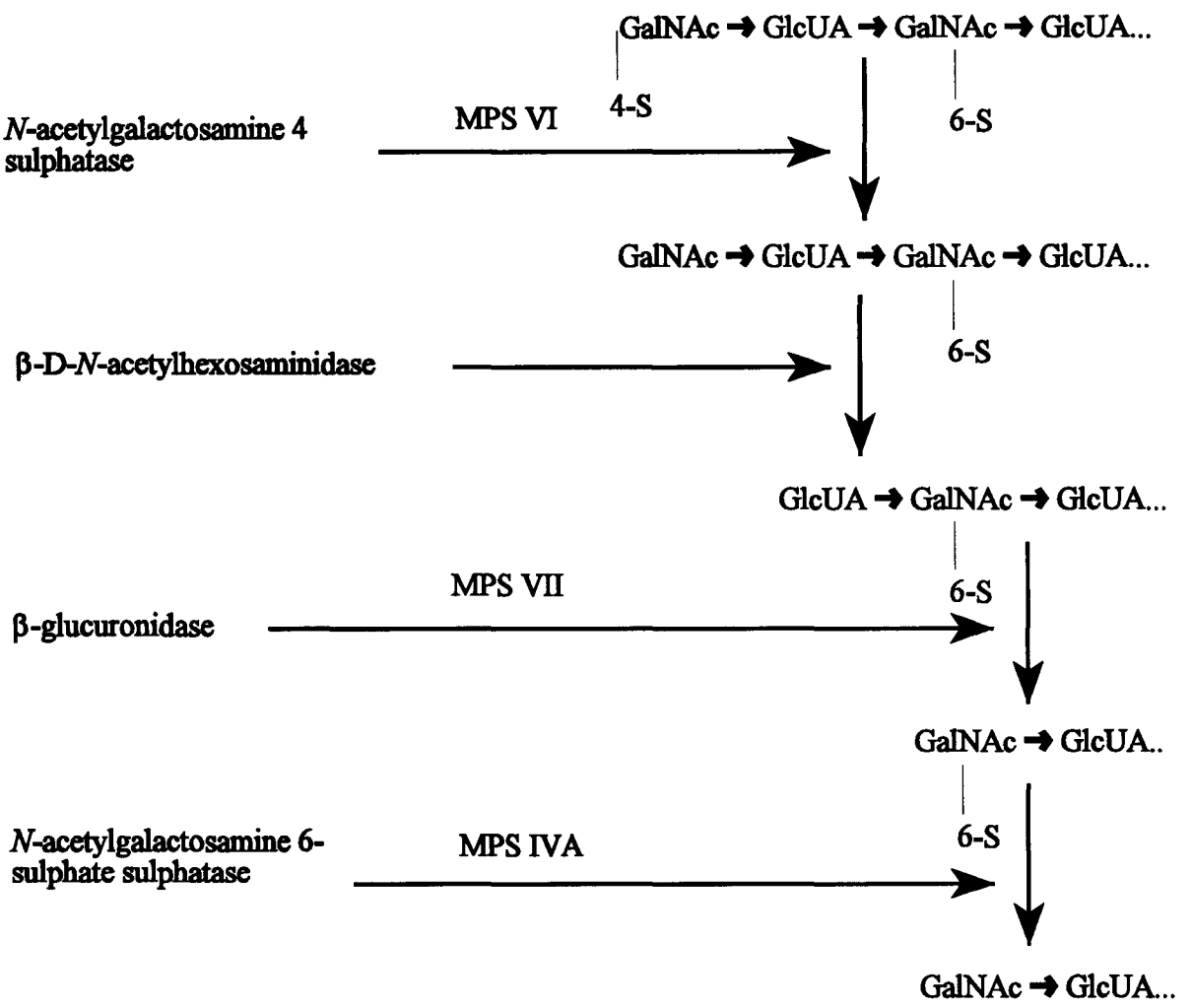

GalNAc → GlcUA → GalNAc → GlcUA...
4-S
6-S
MPS VI
N-acetylgalactosamine 4 sulphatase
GalNAc → GlcUA → GalNAc → GlcUA...
6-S
β-D-N-acetylhexosaminidase
GlcUA → GalNAc → GlcUA...
6-S
MPS VII
β-glucuronidase
GalNAc → GlcUA..
6-S
MPS IVA
N-acetylgalactosamine 6-sulphate sulphatase
GalNAc → GlcUA...

Figure 3. Diagrammatic representation of the degradation of dermatan sulphate (redrawn from Watts and Gibbs, 1986). The degree of sulphatation and the sequence of carbohydrate residues are purely schematic. Deficiencies in any of the specific enzymes involved in dermatan sulphate degradation results in MPS I, MPS II, MPS VI, or MPS VII. Note however, that a deficiency in β-D-*N*-acetylhexosamine results in Sandhoff's disease. Abbreviations are the same as in Figure 1.

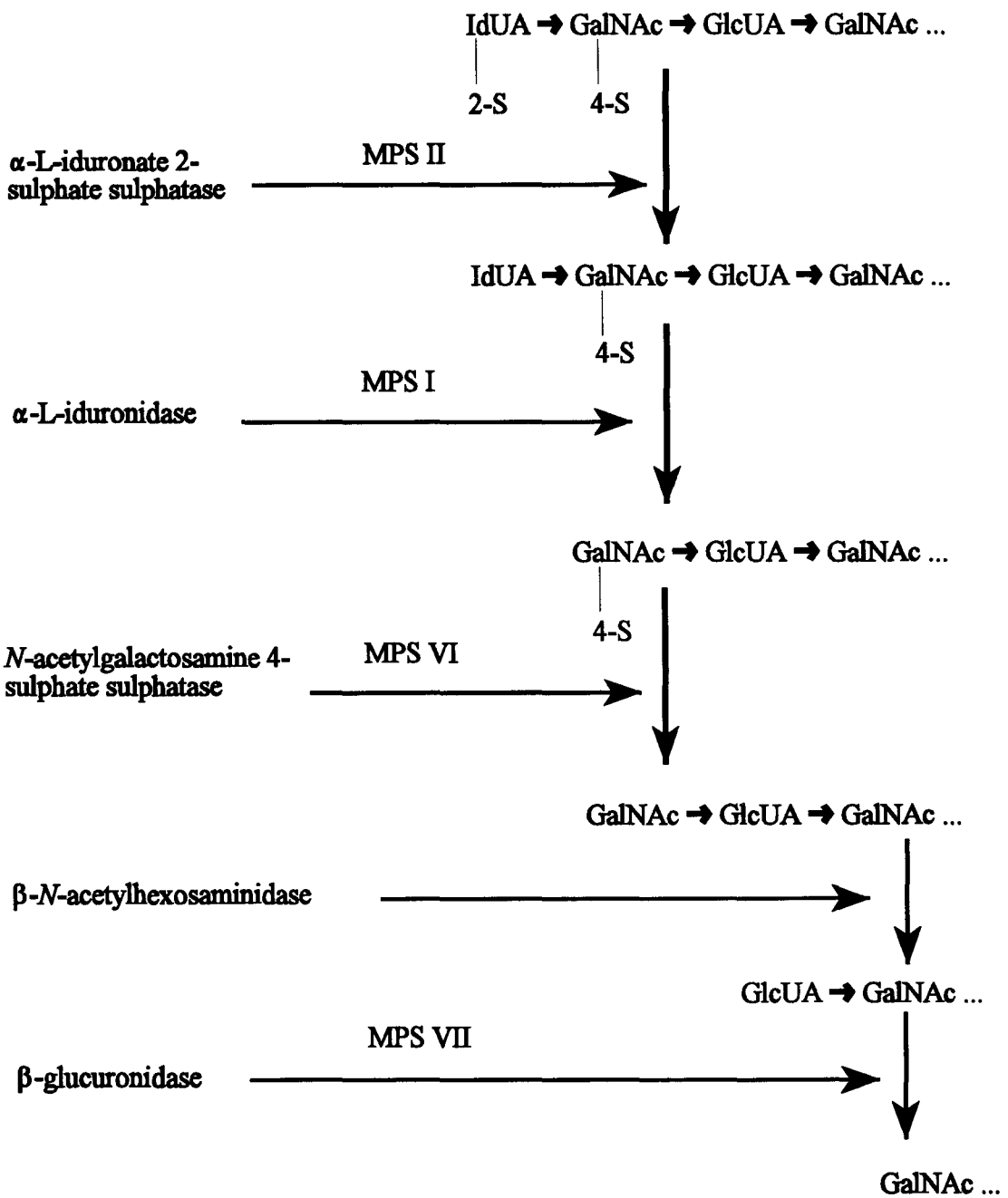

IdUA ➜ GalNAc ➜ GlcUA ➜ GalNAc ...
2-S
4-S
α-L-iduronate 2-sulphate sulphatase
MPS II
IdUA ➜ GalNAc ➜ GlcUA ➜ GalNAc ...
4-S
MPS I
α-L-iduronidase
GalNAc ➜ GlcUA ➜ GalNAc ...
4-S
N-acetylgalactosamine 4-sulphate sulphatase
MPS VI
GalNAc ➜ GlcUA ➜ GalNAc ...
β-N-acetylhexosaminidase
GlcUA ➜ GalNAc ...
MPS VII
β-glucuronidase
GalNAc ...

Figure 4. Diagrammatic representation of the degradation of heparan sulphate (redrawn from Watts and Gibbs, 1986). The degree of sulphatation and sequence of carbohydrate residues are purely schematic. Deficiencies in any of the enzymes involved in the degradation of heparan sulphate results in MPS I, MPS II, MPS IIIA-D, or MPS VII. Abbreviations are the same as in Figure 1.

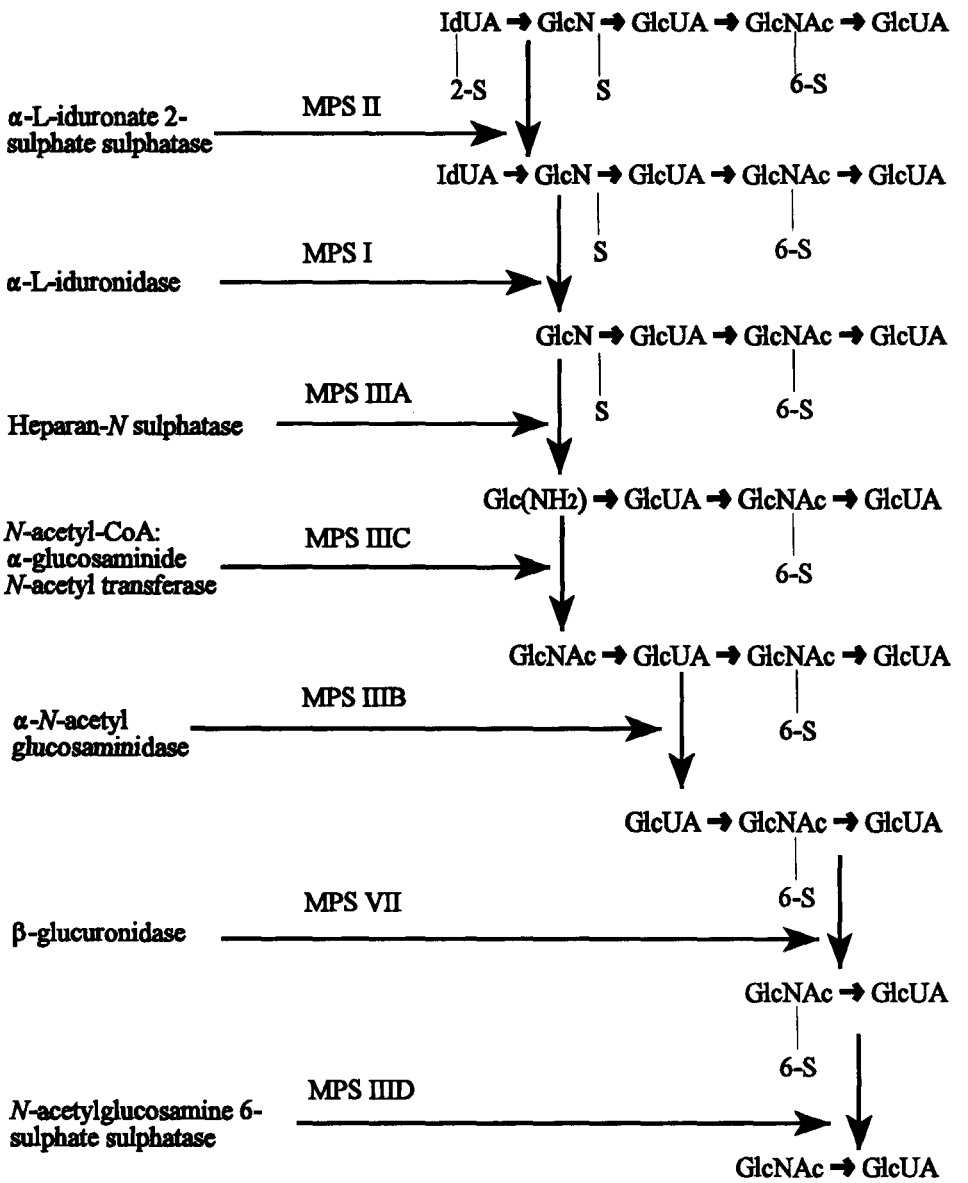
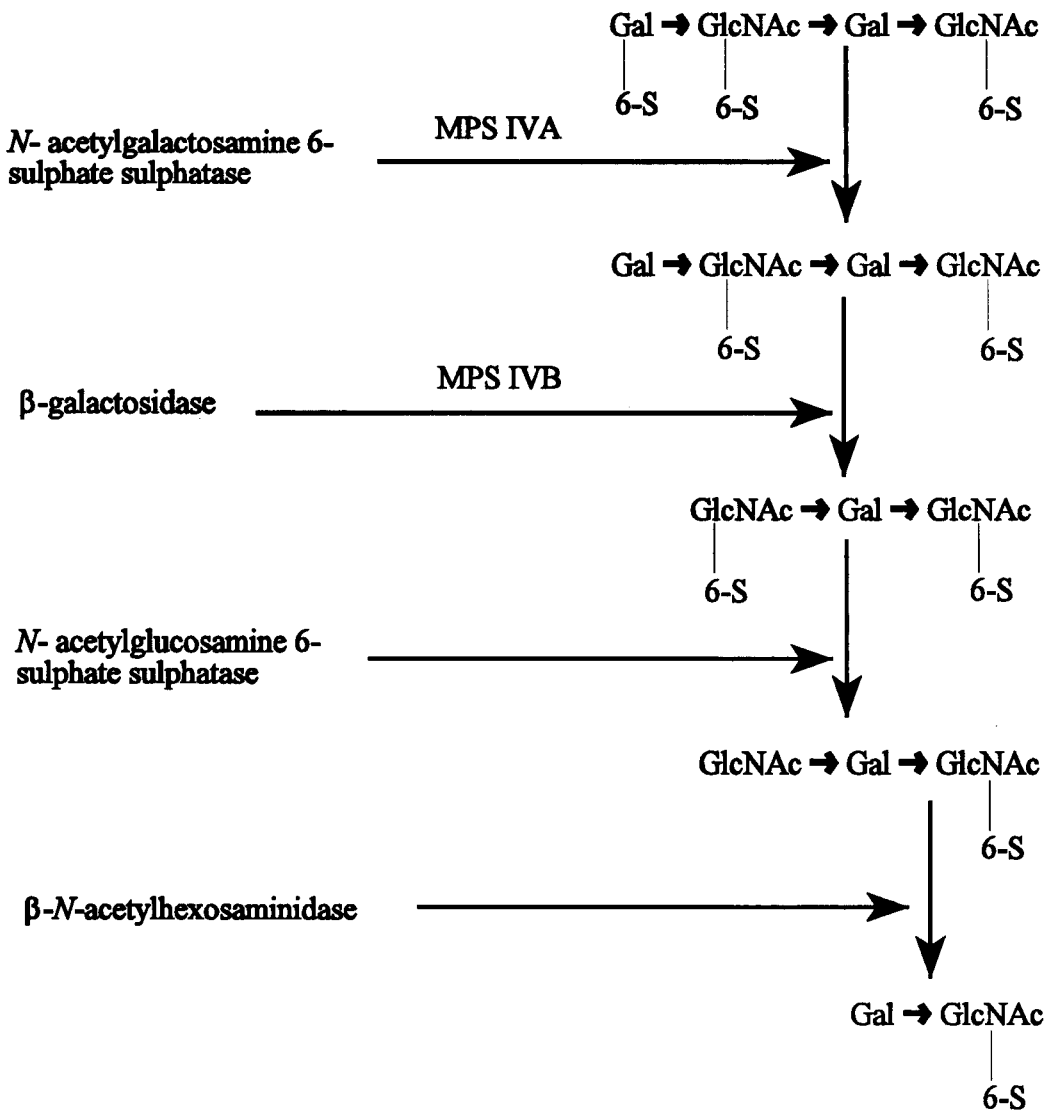

IdUA → GlcN → GlcUA → GlcNAc → GlcUA
2-S
S
6-S
α-L-iduronate 2-sulphate sulphatase
MPS II
IdUA → GlcN → GlcUA → GlcNAc → GlcUA
S
6-S
α-L-iduronidase
MPS I
GlcN → GlcUA → GlcNAc → GlcUA
S
6-S
Heparan-N sulphatase
MPS IIIA
Glc(NH2) → GlcUA → GlcNAc → GlcUA
6-S
N-acetyl-CoA: α-glucosaminide N-acetyl transferase
MPS IIIC
GlcNAc → GlcUA → GlcNAc → GlcUA
6-S
α-N-acetyl glucosaminidase
MPS IIIB
GlcUA → GlcNAc → GlcUA
6-S
β-glucuronidase
MPS VII
GlcNAc → GlcUA
6-S
N-acetylglucosamine 6-sulphate sulphatase
MPS IIID
GlcNAc → GlcUA

Figure 5. Diagrammatic representation of keratan sulphate and its degradation (redrawn from Watts and Gibbs, 1986). The degree of sulphatation and the sequence of the carhbohydrate residues are purely schematic. Deficiencies in any of the enzymes involved in the degradation of keratan sulphate results in MPS IVA or MPS IVB. Abbreviations are the same as in Figure 1.


Gal → GlcNAc → Gal → GlcNAc
6-S
6-S
6-S
N- acetylgalactosamine 6-sulphate sulphatase
MPS IVA
Gal → GlcNAc → Gal → GlcNAc
6-S
6-S
β-galactosidase
MPS IVB
GlcNAc → Gal → GlcNAc
6-S
6-S
N- acetylglucosamine 6-sulphate sulphatase
GlcNAc → Gal → GlcNAc
6-S
β-N-acetylhexosaminidase
Gal → GlcNAc
6-S

Deficiency in either a lysosomal exoglycosidase or sulphatase leads to a catabolic block in glycosaminoglycan degradation. As a result, polysaccharide chains of varying length accumulate in the lysosomes of tissues and are excreted in the urine. In Figures 2-5, the degradation step that is blocked due to abnormal or deficient enzyme activity is indicated with the corresponding mucopolysaccharidosis. The abnormally high excretion of glycosaminoglycans also serves as a diagnostic tool in determining a mucopolysaccharide disorder (McKusic, 1965; Muenzer, 1986).

1.2.3.1 MPS I (Hurler, Scheie, and Hurler/Scheie diseases)

There is great heterogeneity among patients with respect to the clinical presentation of MPS I (Table 1). Hurler's syndrome (MPS IH) is the most severe clinically among the α-iduronidase deficiencies. Clinical symptoms are progressive and include coarse facial features, hepatosplenomegaly, dystosis multiplex, short stature, mental retardation and hydrocephaly. The severe tissue involvement usually leads to premature death by 10 years of age (Meunzer, 1986; Watts and Gibbs, 1986). The clinical manifestations of Scheie's syndrome (MPS IS) is relatively mild compared to Hurler's (diagnosis is usually made between 10 to 20 years of age). Although there is mild skeletal and visceral involvement, neurological involvement is absent as reflected by normal intelligence. Patients with Hurler-Scheie (MPS H/S) syndrome, on the other hand, have intermediate clinical phenotypes. This is characterized by more progressive (but not as severe as Hurler's) skeletal and visceral involvement, and a moderate degree of mental handicap. Patients in all 3 groups of MPS I excrete excessive amounts of

dermatan and heparan sulphate (Meunzer, 1987; Watts and Gibbs, 1986). It has been suggested that such observed clinical heterogeneity is a result of multiple allelic mutations at the α-iduronidase gene.

1.2.3.2 MPS II (Hunter's disease)

There are two types of Hunter's syndrome, severe (MPS IIA) and mild (MPS IIB), both with α-iduronate sulphatase deficiency. All of the clinical manifestations observed in patients with Hurler's is seen in patients with severe Hunter's. Unlike Hurler's, Hunter's syndrome follows a slower rate of progression. Patients with severe Hunter's are usually diagnosed between the ages of 2 and 4 years, whereas those afflicted with the mild form may not be diagnosed until their late teens or early adult life. Patients with MPS IIA have clinical symptoms that resemble Scheie (MPS IS), and may survive into the fourth to sixth decades of life (Watts and Gibbs, 1986; Meunzer, 1987).

1.2.3.3 MPS III (Sanfilippo disease)

Deficiencies in four different lysosomal enzymes involved in the degradation of heparin and heparan sulphate result in four genetically different but phenotypically similar mucopolysaccharidosis (Table 1, Figure 4). These are collectively known as Sanfilippo's disease (MPS III). The enzymes deficient in MPS III are heparan *N*-sulphatase (Type A), α-*N*-acetylglucosaminidase (Type B), *N*-acetyl CoA: α-glucosaminide *N*-acetyltransferase (Type

C) and *N*-acetylglucosamine 6-sulphate sulphatase (Type D). Although heparan sulphate is excreted in excessive amounts and accumulates in all organs, visceral and skeletal involvement is mild. Rather, the CNS is severely affected in patients of the different Sanfilippo subtypes. Insomnia, hyperkinesis, destructiveness, convulsions and retardation manifest starting at 3 to 6 years of age(Watts and Gibbs, 1986; Muenzer, 1987).

1.2.3.4 MPS IV (Morquio syndrome)

Two types of Morquio syndrome's are recognized; Type A occurs as a result of deficient *N*-acetylgalactosamine 6-sulphate sulphatase activity and Type B because of deficent β-galactosidase activity. MPS IVA is characterized by severe skeletal deformities such as spondyloepiphyseal dysplasia, genu valgus, short stature, odontoid hypoplasia, sternal protrusion, and spinal chord compression. Other symptoms such as corneal clouding and hepatosplenomegaly are mild. The skeletal dysplasia observed in MPS IVB is milder than that seen in MPS IVA. However, both diseases result in excessive lysosomal accumulation and excretion of keratan sulphate and to a much lesser degree, chondroitin 6-sulphate (Watts and Gibbs, 1986; Muenzer, 1987).

1.2.3.5 MPS VI (Maroteaux-Lamy syndrome)

The clinical heterogeneity observed in Maroteaux-Lamy syndrome is similar to that seen in patients with MPS I (Hurler's). In both Type A and B forms, the metabolic lesion is

a deficiency in *N*-acetylgalactosamine 4-sulphate sulphatase activity, and results in the excretion of large amounts of dermatan sulphate. The severe form, like Hurler's, is associated with facial dysmorphism, hepatosplenomegaly, corneal clouding, short stature and dystosis multiplex. Patients with MPS VI have normal intelligence despite indications of hydrocephalus, odontoid hypoplasia, and pachymeningitis (Watts and Gibbs, 1986; Muenzer 1987).

1.2.3.6 MPS VII (Sly's disease)

MPS VII is an autosomal recessive disorder characterized by a deficiency in the lysosomal enzyme β-glucuronidase. β-glucuronidase has been purified from many sources including mouse, rat and human placenta. The mature lysosomal enzyme is a tetrameric glycoprotein composed of 4 identical subunits and has a combined molecular weight between 280 to 300 kDa (Lin et al., 1975; Tomino and Paigen, 1975; Lusis and Paigen, 1978; Mills et al., 1978;Oshima et al., 1987; Powell et al., 1988) The human β-glucuronidase gene is located in the *Gus* gene complex on chromosome 7. The *Gus* gene complex consists of the structural gene (*Gus-s*), as well as the regulatory genes involved in androgen induction (*Gus-r*), development (*Gus-t*) and systemic regulation of translation yield (*Gus-u*).

This section will describe in more detail the etiology of the disease in humans and in the murine MPS VII model. Lastly, a discussion on the various therapies performed on the MPS VII mice will provide the necessary background for the next section entitled "Rationale and Goals for this Thesis".

1.2.3.6.1 MPS VII - Elucidation of a new Mucopolysaccharidosis

In 1973, William Sly and his collegues reported a previously undescribed mucopolysaccharidosis in a young child, C.R. Clinical manifestations included coarse facial features, shortened stature, hepatosplenomegaly, granulocytic inclusions in the leukocytes and multiple skeletal abnormalities of the skull, spine, ribs, and long and short tubular bones. Initial enzyme studies indicated an absence of the lysososmal hydrolase β-glucuronidase from skin fibroblasts (Sly et al., 1973).

Prior to the discovery of C.R., Elizabeth Neufeld and her coworkers conducted a series of experiments on the fibroblasts of different MPS patients. First they showed that cells from patients with Hurler's and Hunter's diseases accumulated large amouts of radioactively labelled sulphated mucopolysaccharides and that the defect in the diseases was an inability to degrade the substrates (Frantoni et al., 1968a) Next, they showed that genetically different types of MPS cells, when co-cultivated, could correct each other. Each cell secreted an enzyme that was taken up and utilized by the other cell that lacked that particular enzyme. This complementation was observed by a decrease in the intracellular sulphated mucopolysaccharides (Frantoni et al., 1968b; Frantoni et al., 1969; Neufeld and Cantz, 1971). Later the same group showed that the accumulation of sulphated mucopolysaccharides in C.R.'s fibroblasts could be corrected by co-cultivation with every other known MPS cell type. This provided evidence for a novel MPS disease (Sly, 1993).

To date fewer than 50 cases of MPS VII have been reported, all with a wide range of clinical severity. Due to the vast clinical heterogeneity, Sewell et al., 1982 proposed to

subdivide patients with MPS VII into 3 groups: 1) those that had a severe and early fatal course, sometimes presenting as hydrops fetalis at birth as described by Beaudet et al., 1975, Nelson et al., 1982, Wilson et al., 1982 and Irani et al., 1983; 2) patients that present at adolescence with very mild symptoms, as described by Beaudet et al., 1975, Glitzelmann et al., 1978, Chapman et al., 1989; and 3) patients with intermediate clinical manifestations similar to the original patient of Sly et al., 1973 and those reported by Gehler et al., 1974, Pfeiffer et al., 1977, Sewell et al., 1982, Sheets Lee et al., 1985, Bernsen et al., 1987, Pizzutillo et al., 1989 and Wallace et al., 1990. Such clinical variability as well as the rarity of the disease makes the initiation and the evaluation of the efficacy of different therapies difficult. Hopes to develop a therapy increased in 1989 when a murine model of MPS VII was first described (Birkenmeier et al., 1989).

1.2.6.2 Murine MPS VII

In 1989, Edward Birkenmeier and his collegues described a mutant mouse model (gus^{mps}/gus^{mps}) with less than 1% of normal β-glucuronidase activity and a clinical presentation similar to human MPS VII (Birkenmeier et al., 1989; Vogler et al, 1990) Initial genetic studies showed that the mutation is inherited as an autosomal recessive disorder. It mapped to the β-glucuronidase gene complex on the distel end of chromosome 5 (Birkenmeier et al., 1989). Further genetic analysis using high resolution PAGE to detect DNA restriction fragments 20-400 base pairs in length followed by DNA sequence analysis, revealed a single base pair deletion in the β-glucuronidase gene. The single deletion of a

guanine nucleotide within exon 10 created a framshift mutation which resulted in a premature translation stop codon at codon 497 (Sands and Birkenmeier, 1993).

The clinical, pathologic, and biochemical manifestations of the murine MPS VII is similar to humans with the severe variant of the same disorder. This includes shortened life span, dwarfism, dysmorphic facial features, skeletal deformities, corneal clouding and abnormal accumulation of glycosaminoglycans in the brain, peripherial organs and macrophages (Sly et al., 1973; Birkenmeier et al, 1989; Neufeld and Meunzer, 1989; Sewell et al., 1989; Vogler et al., 1990). One year after its description, Kyle et al., 1990, showed that the gus^{mps}/gus^{mps} mice were an authentic model of MPS VII. When a human β-glucuronidase transgene was expressed in transgenic mice homozygous for the gus^{mps} mutation, the clinical and biochemical phenotype of the mutants were corrected (Kyle et al., 1990). Because the genetics and the clinical course of murine MPS VII have been extensively characterized, and because the gus^{mps} mutation occurs in a syngeneic background, the MPS VII mice serve as an excellent model to study the efficacies of different therapies and to further study the pathobiology of mucopolysaccharidosis.

Recently, the neurological function of the MPS VII mice was evaluated. Because of deficient β-glucuronidase activity, there is an accumulation of glycosaminoglycans in the CNS of mutant mice (Vogler et al., 1990). This is consistent with the mental retardation observed in most MPS patients (Beaudet et al., 1975; Pfeiffer et al., 1977; Sewell et al., 1982; Chapman et al., 1989; Neufeld and Muenzer, 1989; Pizzutillo et al., 1989; Wallace et. al., 1990). Two behavioural tests were used to analyze the neurological involvement of the

disease in the mutant mice. The first test was to monitor the amount of time the mice spent in grooming, an activity that is genetically based and developmentally regulated (Fentress and Stilwell, 1973; Berridge et al.,1987). The complex and stereotypic sets of movements innate to rodent grooming are thought to be controlled by peripheral and central control pathways (Berridge and Fentress, 1986; Berridge and Fentress, 1987a). These include central neural structures such as the corpus striatum (Dunnet and Iversen, 1982; Berridge and Fentress, 1987b) and the caudal brain stem (Kolb and Whishaw, 1981). When the amount of time spent in grooming was examined, it was found that body grooming in the MPS VII mice was depressed to 1 to 5% of the normal controls (Chang et al., 1993a). Because there is a wide spread occurrence of cytoplasmic vacuolations in the neurons, glia and mesenchymal cells of the brain (Vogler et al., 1990), the observed depression in grooming activity could be attributed to their CNS pathology.

Further neurological deficits in MPS VII mice was demonstrated through the Morris water maze test. Here the mice were placed in a water pool and monitored for the amount of time needed to locate a slightly submerged and stationary platform. The mice were given 4 trials on each day of testing. In the course of 6 days it appeared that the mutants, although able to learn the location of the platform, required 20 to 50 seconds more time than their normal litter mates to find the platform (Chang et al., 1993a). By withdrawing the platform from the pool or by changing its location, one can further test for different cognitive and spatial abilities (see Morris, 1984 for review). In each of these different tests, the mutant mice consistently performed poorly when compared to their normal littermates (Chang et al.,

1993a). The ability to process sensory imput from the surroundings, and the ability for spatial mapping in rodents, is mediated through the CNS, primarily the neocortex and the hippocampus (Kolb et al., 1993; Morris et al., 1982; Sutherland et al., 1983; Whishaw et al., 1987; Whishaw and Tomie, 1987). It is likely then that the extensive accumulation of lysosomal storage material present in these regions (Vogler et al., 1990) contributed to the mutants poor performance. The monitoring of grooming activities and the Morris water maze tests provides a quantitative means for assessing neurological function, as well as an additional parameter for evaluating the efficacy of a therapy.

1.2.6.3 Treatment of MPS VII

Following the initial characterization of the gus^{mps}/gus^{mps} mice (Birkenmeier et. al., 1989, Vogler et al., 1990) various therapies have been initiated in hopes to reverse the MPS VII phenotype.

Edward Birkenmeier and his collegues transplanted syngeneic normal bone marrow into irridated adult MPS VII mice. As a result of the transplant, significant levels of β-glucuronidase was detected in the brain (at 1000 rad), kidney, liver, lung, spleen and thymus. Despite a marked reduction in storage material in the peripheral organs, correction of storage in the CNS and skeleton was minimal (Birkenmeier et al., 1991). Due to the progressive nature of the disease, Sands et al., 1993, hypothesized that bone marrow transplantation during early life, when lysosomal storage is minimal, may be more effective. Neonatal bone marrow transplantation, although more effective at reducing storage in all organs including

the brain, also resulted in radiation dependent damage to the brain. A variation from this approach involved retroviral-mediated transfer of the β-glucuronidase gene into mutant haematopoietic stem cells, followed by transplantation into irridated MPS VII mice. Reduction in storage was evident only in the liver and spleen, albeit there was low β-glucuronidase activity in these organs (Wolfe et al., 1992b).

In an attempt to correct the CNS defect in the MPS VII mice, Wolfe et al. (1992a) designed a herpes simplex virus vector with the β-glucuronidase gene downstream of the LAT (latency-associated-transcript) promoter. The mutant mice were infected with the recombinant virus by corneal innoculation and followed for 18 weeks. β-Glucuronidase positive cells were detected in the CNS in 11 out of 13 mice, but the number of cells infected were too few to alter the mutant CNS phenotype. Recently, John Wolfe and his coworkers (J. Wolfe, personal communication, 1994)[9] transplanted recombinant β-glucuronidase secreting fibroblasts into the brains of adult MPS VII mice and found that there was a reversal in storage pathology in the neurons and glia near the site of the grafts. However, neither of these strategies dealt with the progression of the disease at the organ level.

An alternative approach to the treatment of the MPS VII mice was attempted by Philippe Moullier and his group in 1993. Syngeneic primary skin fibroblasts were transduced with retroviral vectors containing the β-glucuronidase gene. The modified fibroblasts were then embedded in a mixture of growth factors, rat collagen I and polytetra-fluroethylen fibres,

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[9]J. Wolfe, University of Pennsylvania, School of Veterinary Medicine; 1994 meeting on Gene Therapy, Cold Spring Harbor Laboratory

to form a "neo-organ" that was subsequently implanted into the peritoneal cavity. The amount of β-glucuronidase activity detected in the various organs such as the liver, spleen, lung and kidney was between 0.5 and 6% of normal. Despite the low amount of enzyme activity (relative to that obtained by bone marrow transplantation (Birkenmeier et al., 1991; Sands et al., 1993)), there was a dramatic reduction of storage material in the liver and spleen (Moullier et al., 1993). Recently, the same group showed that β-glucuronidase secreted from the neo-organs are taken up by peripherial organs when implanted into dogs (P. Moullier, personal communication)[10]. Both Moullier's (1993) and Wolfe's (1992b) work showed that low amounts of β-glucuronidase activity can have a marked effect at reducing storage lesions in the liver and spleen.

The most successful therapy reported to date involved intravenous injection of purified β-glucuronidase into newborn pups. As little as one hour after injection, β-glucuronidase activity in bone, liver, spleen, kidney and other tissues was greater than or equal to normal values. β-glucuronidase activity in the CNS was 31% of normal, an amount exceeding that observed in other therapies (Vogler et al., 1993). Furthermore, enzyme replacemant therapy during the first six months of life totally reversed mutant pathology in the brain and various organs (personal communication, Mark S. Sands, 1994)[11]. However, like other forms of enzyme replacement therapy, the treatment needs to be continuous.

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[10]P. Moullier, Laboratoire Retrovirus and Transfert Génétique, Institut Pasteur, Paris, France; 1994 meeting on Gene Therapy, Cold Spring Harbor Laboratory

[11]M.S. Sands, University of Pennsylvania, School of Veterinary Medicine.

1.3 Rationale and Goals for this Thesis

The parameters used to evaluate the efficacy of the various therapies performed on the MPS VII mice are as follows: clinical observation of features, gait and lifespan; enzyme activity in organs; measurement of urinary glycosaminoglycans; and histopathology. In humans afflicted with MPS VII, progressive mental retardation has been observed in the intermediate to severe cases (Gehler et al., 1974; Beaudet et al., 1975; Pfeiffer et al., 1977; Sewell et al., 1982; Sheets Lee et al., 1985; Bernsen et al., 1987; Pizzutillo et al., 1989; Wallace et al., 1990). To date, there has been no measurement as to how the therapies affect, if at all, neurological function. As described earlier, monitoring both the grooming activities and the Morris water maze test has been used to show that the MPS VII mice have neurological deficits as a consequence of their CNS pathology. We had the opportunity to assess the neurological function of mutant mice treated with bone marrow transplantation (Sands et al., 1993). Hence, chapter 3 includes an article published in the Journal of Clinical Investigation, 1994, which describes in detail the results and conclusions obtained from the behavioural studies.

Syngeneic bone marrow transplantation in neonatal mice was shown to be effective in reversing the pathology of the disease (Sands et al., 1993). However in humans, the procedure carries high morbidity and mortality (Wiznitzer et al., 1984). Although gene therapy on the haemopoetic stem cells eliminates the problem of finding appropriate donors, it also has its shortcomings. Firstly, human haemopoetic stem cells have proven difficult to

isolate and maintain in culture (Mulligan, 1991; Anderson, 1994). Thus corrected peripheral lymphocytes would have to be administrated at regular intervals as in the ADA clinical trial (Blaese, 1993). Secondly, the procedure of isolating the patients own cells, manipulating them *in vitro*, selecting for the appropriate recombinants and then reimplanting back into the patient, is labor intensive and may incur prohibitive expenses when done on a routine basis. This problem of cost and labour also extends to Moullier's work where primary syngeneic fibroblasts were used to avoid immune rejection (Moullier et al., 1993).

The use of microcapsules containing non-autologous cells engineered to secrete a desired protein of interest was reviewed earlier. We proposed to use this technology to treat the MPS VII mice. Chapter 4 section discusses the strategy and the results of such an experiment.

2.0 MATERIALS AND METHODS

Animals

Male mutant mice (*gus*^{mps}/*gus*^{mps}) and their normal littermates (+/+ and +/*gus*^{mps}) obtained from the mutant strain B6.C-H-2*bml*/ByBir-*gus*^{mps}/+, were supplied by the Jackson Laboratory. Mice were maintained on 4.5% Purina Rat Chow and housed on a 12:12 light:dark cycle.

Grooming Tests

Each mouse was tested twice each day, once under baseline conditions with no external stimulus and once under stimulated conditions with the mouse lightly moistened by a water mister. The mouse was videotaped at close up for 10 minutes under each condition while enclosed within a cylindrical wall 10 cm in diameter and 15 cm high. The videotapes were reviewed at 1/5 the actual speed and the total time spent in face grooming, body grooming, and in other movements were determined. Actions which contributed to face grooming was any movement of the forelimbs around the face and head such as: flailing of the forelimbs below the face; licking of the forepaws; overhand, parallel or single strokes of the forelimbs overtop of the head, side of snout or face; and shimmying (Fentress, 1972 and Fentress and Stilwell, 1973). Any grooming that did not include the face and head were

categorized as body grooming. This consisted of scratching the body with the hind legs and licking the abdomen, genitals and flanks. Other movements were activities that did not contribute to grooming such as digging, rearing, locomotion and standing still (Fentress, 1972). Only the time spent in face and body grooming were analyzed in detail.

Morris Water Maze

The procedure for this test was modified from R. Morris (Morris, 1984). A pool 182 cm in diameter was filled 30 cm deep with water at 21°C. Four cardinal points around the circumference of the pool were designated as North, South, East, and West, thus dividing the pool into four quadrants (SW, NW, NE, SE). A transparent circular plexiglass platform 10 cm in diameter was placed 40 cm from the wall; its top surface 1 cm below the surface of the water. On all trials, mice were released into the water facing the wall of the pool. Four trials were made for each mouse on each day, and each cardinal point used once as the starting point. The sequence of start positions was randomly selected for the day but all mice followed the same order.

The behavioural testing was conducted on 13 consecutive days. The first day was to habituate the animals to the water by letting them swim in the pool, without the platform, for 30 seconds per trial. On days 2 to 6, the platform was located in the SW quadrant and the time needed to reach the platform was recorded. If the mouse found the platform within 120 seconds, it was allowed to remain on the platform for 30 seconds. If it did not find the platform during the allocated time, it was guided by the experimenter to the platform and

allowed to remain there for 30 seconds. On day 7, the platform was removed and the amount of time each mouse spent in any one quadrant was recorded. On days 8 to 11, the quadrant was placed back in the SW quadrant and the procedures of days 2 to 6 were repeated. On days 12 and 13, the platform was moved to the NE quadrant and again the procedures of the previous days repeated. No less than 90 seconds and no more than 180 seconds elapsed between two trials during which the mouse was dried with a towel and placed under a heating lamp. Each trial was recorded with an overhead camera. No attempt was made to obstruct the mice from the view of the room. All the cues in the room were held constant throughout the testing (e.g. experimenter, work bench, rack of cages, T.V. monitor, light fixtures, etc.).

Cell lines

Unless stated differently, cells were maintained on 100 mm tissue culture dishes and incubated at 37°C in a 5% CO_2 water jacketed incubator. The basic culture media consisted of Dulbecco's modified minimal essential growth medium (Gibco) supplemented with 10% fetal bovine serum, 100 µg/mL penicillin, 100 µg streptomycin, and 2 mM L-glutamine. The 2A-50 fibroblasts were obtained from Dr. W. Sly. 0.1 g/L sodium pyruvate, 2.2 g/L sodium bicarbonate and 3.2 µM methotrexate were added to the medium.

Preparation of Competent DH5α Cells

A single colony of *E. coli* DH5α cells was inoculated into 50 mL LB medium and grown overnight at 37°C. Four mL of this culture was inoculated into 400 mL LB medium

and grown to mid-log phase (OD_{600} of 0.3 to 0.5). The culture was then aliquoted into 50 mL prechilled sterile polypropylene tubes and left on ice for 10 min. The cells were then centrifuged at 3000 rpm for 10 min, the supernatant decanted and the remaining cell pellet resuspended in 10 mL ice cold $CaCl_2$. This step was repeated again, this time leaving the cells in $CaCl_2$ and on ice for 1 hour. Cells were then rapidly frozen in liquid nitrogen and stored at -70°C.

Heat Shock Transformation of DH5α Cells

Competent cells were rapidly thawed and dispensed into 100 µL aliquots in sterile eppendorf tubes. Plasmid DNA (2-10 ng) was gently mixed into the cell suspension and left on ice for 10 min. The cells were then placed in a 42°C water bath for 90 seconds. One mL of LB was added and the cells allowed to incubate for 1 h at 37°C. Aliquots of this transformation culture was then plated on LB-ampicillin agar plates and incubated overnight at 37°C.

Large Scale Preparation of Plasmid DNA

Bacteria were grown overnight in 5 mL LB medium containing 50 µg/mL ampicillin at 37°C. 250 mL of LB containing 50 µg/mL ampicillin was inoculated with 100 µL of the overnight culture. This was grown at 37°C overnight. The cell suspension was then transferred to 2-250 mL polypropylene centrifuge bottles and the cells collected by centrifugation in a Sorvall GSA roter at 4100 *g* for 20 min at 4°C. After decanting the

supernatant, the pellet of cells was resuspended in 5 mL solution I, by vortexing, and transferred to 50 mL round bottom centrifuge tubes. Ten mL of freshly made solution II was gently mixed in with a pipet and the solution allowed to stand 10 min on ice. To precipitate high molecular weight nucleic acids, SDS-protein complexes and other cellular debris, 7.5 mL solution III was added and the suspension mixed very hard. The flocculent white precipitate that formed was separated from the solution by centrifugation in a Sorval SS-34 roter at 3000 *g* for 15 minutes at 4°C. Next, the supernatant containing plasmid DNA was transferred to a fresh tube, 13.5 mL isopropanol added to precipitate the DNA, and the tube placed on ice for 10 min. The DNA was then centrifuged in a Sorval SS-34 roter at 12000 *g* for 30 min to obtain a pellet that was then resuspended in 3 mL TE.

To precipitate high molecular weight RNA, 3 mL ice cold 5 M LiCl was added to the DNA solution. This was mixed well and centrifuged at 12000 *g* for 15 min at 4°C. The supernatant was transferred to a fresh 15 mL sorvall tube, 7 mL isopropanol added to precipitate the DNA, and the mixture centrifuged at 12000 *g* at 4°C. The supernatant was then aspirated and left to dry. It was then dissolved in 300 µL TE + 20 µg/mL RNase A, transferred to an eppendorf tube and incubated at room temperature for 30 min. An equal volume of 13% PEG in 1.6 M NaCl was then added, the tubes vortexed for 5 min and centrifuged for 10 min at 4°C. The supernatant was aspirated and the pellet dissolved in 400 µL TE. The DNA was then extracted with an equal volume of phenol and chloroform. Following this, 20 µL of 10 M ammonium acetate and 2 volumes (800 µL) of absolute ethanol were added to precipitate the DNA. After incubation at room temperature for 10

min, the solution was centrifuged for 10 min. The pellet that was collected was washed once with 200 µL 70% ethanol and vacuum dried. DNA was stored at -20°C in TE.

Encapsulation of Cells

Cells were encapsulated on ice and under sterile conditions. Approximately 2 x 10^6 trypsinized cells were washed once in 0.9% NaCl and resuspended in 5 mL filtered Potassium-alginate. This mixture was drawn through a 10 cc syringe with a 27 gauge blunt needle and placed in a Razar A-99 syringe pump. The size of the capsules was regulated by maintaining the extrusion rate at 39.3 cc/h and air flow along the needle at 3 L/min. Capsules were extruded into 35 mL 1.1% $CaCl_2$, collected in a 50 mL conical polypropylene tube and then subjected to the following washes: 0.55% $CaCl_2$; 0.28% $CaCl_2$; 0.1% CHES, 3 min; 1.1% $CaCl_2$; PLL, 6 min; 0.1% CHES; 1.1% $CaCl_2$; 0.9% NaCl; 0.03% potassium alginate, 4 min; 0.9% NaCl; 0.055 M sodium citrate, 6 min; 2 x 0.9% NaCl; 2 x cell culture media. The capsules were then maintained in regular tissue culture conditions.

Secretion Analysis

All samples were taken in triplicate. Cells or intact capsules were washed twice in sterile PBS before being replaced with the appropriate pre-equilibrated media. This was considered as t = 0 h. Thereafter at regular intervals (1, 2, 4 h or 2, 4, 6 h), an aliquot of media was removed for appropriate enzyme analysis.

Implantation

Capsules were washed 5 to 6 times in 0.9% cold NaCl. This was followed by final wash in TC Hanks solution (Difco), where the capsules remained on ice until needed. Each mouse was anaesthetized with a combination of isofluorane (1-chloro 2,2,2-trifluorethyl difluoromethyl ether) (Anaquest), oxygen (0.3 L/min) and nitrogen oxide (1-1.5 L/min). A catheter tube with a 16 gauge needle in the cannula was used to pierce the abdominal wall and peritoneum. This canula was used to direct capsules from a 10 cc syringe into the peritoneum.

Blood Collection

Mice were anaesthetized with a combination of isofluorane (1-chloro 2,2,2-trifluorethyl difluoromethyl ether) (Anaquest), oxygen (0.3 L/min) and nitrogen oxide (1-1.5 L/min). One to two hundred microlitres of blood was collected retro-orbitally using 1.2 mm heparinized capillary tubes (Chase Instruments). Samples were spun at 16000 *g* for 15 min at 4°C and the supernatant (plasma) collected.

DMB Assay for Glycosaminoglycans

The protocol was originally taken from Whitley, 1989 and modified by Mouillier, 1993. Briefly 600 µL of 20 µM 1,9-dimethylmethylene blue (DMB) (Aldrich) was added to 2 µL of urine. After 20 min incubation, absorbance at 540 nm was read. Amount of GAG present was determined against a standard prepared from chondroitin sulphate C (Sigma).

Creatinin Assay

This protocol was modified by Mouillier, 1993, so it could be assayed in 96-multiwell plates. Briefly, 10 µL of a tenfold diluted urine sample was mixed with 50 µL 0.2 M NaOH and 50 µL saturated picric acid (Sigma). After 20 min, absorbance at 490 nm was read and compared to a creatinin standard (Sigma).

Lysosomal Enzyme Assays

Dissected tissues were either stored at -70°C or immediately homogenized. Homogenates were sonicated on ice for 20 seconds and then centrifuged at 16000 x *g* for 30 minutes. Supernatants were assayed fluorometrically for lysosomal enzyme activities by using 4-methylumbelliferyl (4-MU) substrate derivatives (Sigma) (Glaser and Sly, 1973).

a) β-Glucuronidase: One hundred microlitres of 10 mM 4-MU-β-D-glucuronide in 0.1 M sodium acetate buffer (pH 4.5), supplemented with and 1 mg/ml BSA, was added to 10 µL homogenate and incubated at 37 C. After one hour, 1 mL of glycine-carbonate buffer (pH 10.5) was added to stop the reaction.

b) β-Hexosaminidase: One hundred microliters of 5 mM 4-MU-N-acetyl-β-D-glucosaminide in 12 mM sodium citrate-20 mM sodium phosphate buffer (pH 4.4), supplemented with and 1 mg/ml BSA, was added to 10 µL homogenate and incubated for one hour at 37 C. Two millilitres of 0.1 M 2-amino-2-methyl-1-propanol (pH 10.0) was added to stop the reaction.

c) α-Galactosidase: One hundred microliters of 4 mM 4-MU-α-D-galactoside in 0.04 mM citrate phosphate buffer (pH 4.8), supplemented with 1 mg/ml BSA, was added to 10 µL

homogenate and incubated at 37 C. After one hour 2 mL of 0.1 M 2-amino-2-methyl-1-propanol (pH 10.0) was added to stop the reaction.

An AMINCO fluorometer was used to read the excitation at 365 nm and peak emission at 455 nm.

Protein Determination

Protein concentration was determined according to the method of Lowry et al., 1973. In brief, 1 mL of reagent C was added to a sample of homogenate and allowed to stand at room temperature. After 10 minutes 100 μL of Folin-Phenol reagent was added, the solution vortexed and allowed to stand for 30 minutes. Absorbance was read at 750 nm and milligrams of protein was determined against a BSA standard.

Processing and Embedding of Tissue

Dissected tissues were immediately cut into 1-1.5 mm cubes and fixed in 2% gluteraldehyde (pH 7.4) for 1 h at 4°C. This was followed with a wash in 0.2 M sodium cacodylate buffer and left overnight at 4°C. The next morning, tissues were washed twice for 5 min each in 0.2 M sodium cacodylate buffer and once in 1% osmium tetroxide for 1 h at 4°C. The tissues were then dehydrated with stepwise washes of 5 and 10 min each of 50%, 70%, 95%, 100% ethanol and 100% propylene oxide. Tissues were then infiltrated with 50:50, 25:75, propylene oxide:firm Spurr Resin for 30 min each. This was followed with

two 1 h incubations with 100% firm Spurr Resin. Tissues were embedded in mold blocks with fresh 100% Spurr Resin and allowed to polymerize overnight at 65°C.

Sectioning and Staining

Tissue blocks were trimmed and 1 μm sections were cut on a Reichert OmU2 microtome. A standard glass cutting knife was used. Sections were placed in warm Toluidine Blue for 1-3 min, rinsed 2 times with distilled water, and incubated in warm water for 1-3 min. The sections were then placed on slides and gently heated until all water had evaporated. Permount was placed over the sections followed by a coverslip.

Data Analysis

All statistical analyses was performed with the BMDP™ Statistical Software on the VAX. The design for Analysis of variance (ANOVA) with repeated measures, or one way and two way ANOVA's were of either 2 groupings and 2 within factors, or 2 groupings and 1 within factor. Orthogonal components were requested and analyzed.

RESULTS

This section is divided into two chapters, each containing a manuscript that has already been published (Chapter 3) or in the process of being submitted (Chapter 4) for publication. Each paper is preceded by a brief summary of my specific contributions to the paper.

3.0

Bastedo, L., Sands, M.S., Lambert, D.T., Pisa, M.A., Birkenmeier, E., Chang, P.L. **Behavioral Consequences of Bone Marrow Transplantation in the Treatment of Murine Mucopolysaccharidosis Type VII.** J. Clin. Invest. 94:1180-1186, 1994.

My Specific Contributions to Bastedo et al., 1994.

Dr. M.S. Sands at the Jackson laboratory transplanted neonatal MPS VII mice and their normal littermates with syngeneic normal bone marrow. After receiving 24 mice from M.S. Sands I performed the grooming and the Morris water maze tests. Three weeks after the behavioural tests the mice were sacrificed, the organs retrieved, and tissues analyzed for lysosomal enzyme activities. Prior to this, D. Lambert had performed the same behavioural tests on another group of 23 mice. I analyzed his results, and upon advice from Dr. M. Pisa, I pooled the results from my work and D. Lambert's. Only the behavioural data were pooled. Once all the data were gathered, I performed statistical analysis using the BMDP statistical software package.

Bastedo, L., Sands, M.S., Lambert, D.T., Pisa, M.A., Birkenmeier, E., Chang, P.L. **Behavioral Consequences of Bone Marrow Transplantation in the Treatment of Murine Mucopolysaccharidosis Type VII.** J. Clin. Invest. 94:1180-1186, 1994.

Behavioral Consequences of Bone Marrow Transplantation in the Treatment of Murine Mucopolysaccharidosis Type VII

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Abstract

The gus^{mps}/gus^{mps} mouse is a model of the human lysosomal storage disease mucopolysaccharidosis type VII caused by deficient β-glucuronidase activity. Bone marrow transplantation has been shown to correct some of their biochemical and pathological abnormalities but its efficacy in correcting their neurological functional deficits is unknown. We transplanted the neonatal gus^{mps}/gus^{mps} mice and their normal controls and evaluated their central nervous system function with two behavioral tests: the grooming test, a developmentally regulated and genetically based activity, and a Morris water maze test which assessed spatial learning abilities. The two transplanted groups groomed less than the normals, were unable to remember the location of an invisible platform from day to day, and were severely impaired at developing strategies to locate the platform in unfamiliar locations. The performance of both normal and mutant transplanted groups was clearly inferior to the untreated normals and, in some instances, close to or worse than the untreated mutants, even though the enzyme abnormalities of the mutants have been partially corrected. Hence, the behavioral deficits in the mutant mice were not restored to normal while similarly treated normal mice showed significant functional deterioration, indicating the detrimental consequence of this therapy in the neonatal period. (*J. Clin. Invest.* 1994. 94:1180–1186.) Key words: Sly syndrome • lysosomes • central nervous system diseases • behavioral sciences • β-glucuronidase

Introduction

Deficiency in β-glucuronidase activity (EC 3.2.1.31) leads to the lysosomal storage disease mucopolysaccharidosis type VII (MPS VII,[1] Sly disease) not only in humans (1) but also in a recently discovered murine mutant, the gus^{mps}/gus^{mps} mouse (2). Clinical and pathologic abnormalities common to the human and the mouse phenotypes include shortened life span, dwarfism, dysmorphic facial features, skeletal deformities, corneal clouding and abnormal lysosomal storage material in the brain, peripheral organs and macrophages (1–7). Although only the more severely affected human patients demonstrate mental retardation, the mutant mice have clearly defined neurological deficits which result in cognitive, memory, and central nervous system (CNS)–mediated functional deficiencies (8). The mutation of the gus^{mps}/gus^{mps} mouse is due to a single base pair deletion in the β-glucuronidase gene, resulting in a premature stop codon within the open reading frame and a complete absence of β-glucuronidase messenger RNA, thus accounting for the total enzyme deficiency (9).

Because the gus^{mps}/gus^{mps} mice have been extensively characterized in their genetics, biochemistry, pathology, and behavioral abnormalities, they provide an excellent model system to study the pathobiology of lysosomal storage diseases and to evaluate the efficacy of different therapies. Various therapeutic strategies such as bone marrow transplantation (BMT)[1] with either syngeneic normal donors (10, 11) or retrovirally transfected mutant stem cells (12), infection with viral vectors (13), and somatic cell therapy with genetically modified skin fibroblasts (14) have been performed on these gus^{mps}/gus^{mps} mice. These treatments resulted in variable histochemical, biochemical, and pathological improvement of the peripheral organs, while the restoration of β-glucuronidase activity in the CNS has been minimal. It is not clear if any improvement in CNS function has resulted from these therapeutic interventions.

The accumulation of storage material in the CNS of the mutant mice is consistent with the mental retardation observed in severely affected MPS VII patients. We have recently demonstrated that the generalized pathology and neurological involvement of the disease in the mutant gus^{mps}/gus^{mps} mice result in CNS functional deficits that can be measured with behavioral tests (8). One behavioral test is to monitor the amount of time spent in grooming, an activity that follows a complex and stereotypical set of movements (15, 16) characteristic of the species, strains, and developmental stages of the animals (17). It was suggested that the widespread occurrence of cytoplasmic vacuolation in neurons, glia, and mesenchymal cells in the brains of gus^{mps}/gus^{mps} mice (7) contributed to levels of grooming activity that are significantly lower than normal (8). Another behavioral test, the Morris water maze (18), measures the ability of rodents for spatial mapping, reference memory, and discrimination reversal. The pathological presentation of the gus^{mps}/gus^{mps} mice includes lysosomal distention in the hippocampus and the neocortex (7), structures recognized as critical for spatial learning (19–23). This further supports a specific neurological basis for the deficits in cognition and memory demonstrated by the mutant mice during the water maze tests (8).

Of all the experimental therapeutic interventions performed on the mutant mice, only BMT has been tried in humans (24).

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1. *Abbreviations used in this paper:* BMT, bone marrow transplantation; MPS VII, mucopolysaccharidosis type VII.

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Many patients with various lysosomal storage diseases have been treated worldwide with variable degrees of success (25). Because of the inherent difficulties in human studies and the phenotypic variability of lysosomal storage diseases, it has been difficult to evaluate the efficacy of BMT, particularly in lysosomal storage disorders which involve neurodegeneration. Since the neurological deficits of the gus^{mps}/gus^{mps} mice can be demonstrated with behavioral tests, we propose to evaluate the efficacy of BMT in the prevention of neurological deficits in the mutant mice.

Methods

Animals. Male mutants (gus^{mps}/gus^{mps}) and their normal littermates (+/+ and $+/gus^{mps}$) obtained from the mutant strain B6.C-H-2*bml*/ByBir-gus^{mps}/+, were supplied at the Jackson Laboratory (2). The 47 mice were divided into 4 groups. 11 normal (+/?) and 9 mutant littermates were treated with BMT performed on day 1 after birth. In brief, the mice received 2 Gy and then were transplanted with bone marrow cells from normal (+/+) syngeneic females obtained from the B6.C-H-2*bml*/ByJ strain (11). 17 normal and 10 mutants did not receive any treatment. Mice were maintained on 4.5% Purina Rat Chow (Ralston Purina Co., St. Louis, MO) and housed on a 12:12 light/dark cycle. Behavioral testing was always conducted during the animal colony's light phase.

Assessment of engraftment. Peripheral blood smears were prepared and stained with Wright's stain as previously described (2). Donor-derived granulocytes were differentiated from those of the host by the absence of dense granules in the cytoplasm.

Grooming tests. Each mouse received two test trials on the same day, under baseline conditions with no external stimulus and stimulated conditions with the mouse lightly moistened by a water mister. The mouse was videotaped close up for 10 min under each condition while enclosed within a cylindrical wall 10 cm in diameter and 15 cm high. The videotapes were reviewed at one-fifth the actual speed and the total time spent in face grooming, in body grooming, and in other movements was determined. Actions that qualified as face grooming included any movement of the forelimbs around the face and head such as: flailing of the forelimbs below the face; licking of the forepaws; overhand, parallel, or single strokes of the forelimbs overtop of the head, side of snout, or face; and shimmying (16, 17). Any grooming that did not include the face and head was categorized as body grooming. This consisted of scratching the body with the hind legs and licking the abdomen, genitals, and flanks. Other movements were activities that did not contribute to grooming, such as digging, rearing, locomotion, and standing still (17). Only the time spent in face and body grooming were analyzed in detail.

Morris water maze. The procedure for this test was modified from R. Morris (18). A pool 182 cm in diameter was filled 30 cm deep with water at 21°C. Four cardinal points around the circumference of the pool were designated as North, South, East, and West, thus dividing the pool into four quadrants (SW, NW, NE, SE). A transparent circular plexiglass platform 10 cm in diameter was placed 40 cm from the wall, its top surface 1 cm below the surface of the water. On all trials, mice were released into the water facing the wall of the pool. Four trials were made for each mouse on each day, using each cardinal point once as the starting point. The sequence of start positions was randomly selected for the day, but all mice followed the same order.

The behavioral testing was conducted on 13 consecutive days. On the first day, the animals were habituated to the water by letting them swim in the pool, without the platform, for 30 s per trial. On days 2–6, the platform was located in the SW quadrant and the time each mouse needed to reach the platform was recorded. If the mouse found the platform within 120 s, it was allowed to remain on the platform for 30 s. If it did not find the platform during the allocated time, it was guided by the experimenter to the platform and allowed to remain there for 30 s. On day 7, the platform was removed and the amount of time each mouse spent in any one quadrant was recorded. On days 8 to 11, the quadrant was placed back in the SW quadrant and the procedures of days 2 to 6 were repeated. On days 12 and 13, the platform was moved to the NE quadrant, and again the procedures of the previous days were repeated. No less than 90 s and no more than 180 s elapsed between two trials, during which the mouse was dried with a towel and placed under a heating lamp. Each trial was recorded with an overhead camera. No attempt was made to obstruct the mice from the view of the room. All the cues in the room were held constant throughout the testing (e.g., experimenter, work bench, rack of cages, TV monitor, light fixtures, etc.).

Lysosomal enzyme assays. Dissected tissues were either stored at −70°C or immediately homogenized in 20 mM Tris·HCl, pH 7.5/140 mM NaCl/10 mM β-mercaptoethanol/0.25% Saponin homogenization buffer. Homogenates were sonicated on ice for 20 s and then centrifuged at 16,000 *g* for 30 min. Supernatants were assayed fluorometrically for lysosomal enzyme activities by using 4-methylumbelliferyl (4-MU) substrates (26). β-Glucuronidase, β-hexosaminidase, and α-galactosidase activities were assayed using the substrates 4-MU-β-glucuronide, 4-MU-*N*-acetyl-β-D-glucosaminide, and 4-MU-α-D-galactoside (Sigma Chemical Co., St. Louis, MO), respectively. Proteins were determined according to the method of Lowry (27).

Data analysis. All the behavioral tests and biochemical analyses were performed double-blind. The codes were broken when all test data had been collected. All statistical analysis was performed with the BMDP™ Statistical Software on the VAX. The designs for analysis of variance (ANOVA) with repeated measures, or one-way and two-way ANOVAs, were of either two groupings and two within factors, or two groupings and one within factor. Orthogonal components were included for analysis.

Results

The animals were divided into four groups based on their genotypes (normal or mutant) and whether they received neonatal BMT (treated or untreated). The time spent in different grooming activities under various conditions and their abilities to locate an invisible platform in the Morris water maze were assessed.

Grooming

Under baseline conditions, body grooming in the 14-wk-old untreated mutant mice was depressed to 10.5% of that of their normal littermates. In mutants treated with BMT, body grooming was depressed to 33% of normal. However, ANOVA indicated that there was a significant difference only between the normal and mutant genotypes ($F_{1,37} = 12.64$, $P < 0.01$) but no difference between animals as a result of the BMT treatment. Follow-up tests (Tukey) indicated that only the untreated mutants were significantly different from the normal controls ($P < 0.05$). At 19 wk of age, the amount of time spent in body grooming was again only significantly different between animals with different genotypes ($F_{1,33} = 6.70$, $P < 0.02$). No significant difference attributable to the BMT was observed between the experimental groups. Therefore, BMT was not associated with improved grooming activities in the mutants under baseline conditions at either 14 or 19 wk of age.

When the mice were stimulated with a mist of water sprayed on their bodies, the amount of time spent in body or face grooming increased (Fig. 1). The increase in body grooming was observed in all animals at both 14 and 19 wk of age and no significant difference was observed among the four groups (normal and mutant: treated and untreated). From ANOVA of face grooming, however, significant main effects of genotype ($F_{1,52}$

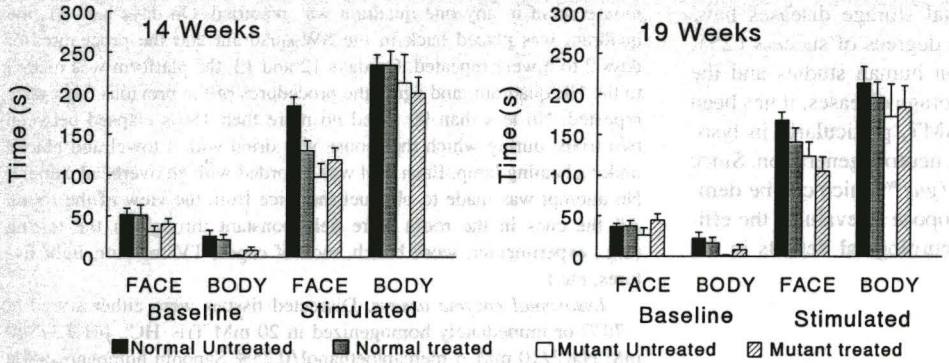

Figure 1. Grooming behavior of the normal and mutant mice after bone marrow transplantation. Each animal at both 14 and 19 wk of age was monitored for the amount of time spent in face and body grooming under normal conditions (baseline), as well as after stimulated with a spray of water mist (stimulated). Data are the mean±SEM.

= 12.03, $P < 0.002$) and genotype × treatment interaction ($F_{1,52} = 7.16$, $P < 0.01$) were found for mice at 14 wk of age. This indicated that the amount of time spent in face grooming was significantly different between animals of different genotypes and that BMT has exerted some effect. Follow-up tests (Tukey) clarified that compared with the normal untreated controls, face grooming was reduced in all three remaining groups (95% level of confidence in the treated normals and treated mutants; 99% level of confidence in the untreated mutants), but none of these three groups differed significantly from each other. Hence, BMT was associated with a lower level of performance when normal mice were treated, while no improved performance was obtained when the mutants were treated. At 19 wk of age, however, only significant main effects of genotype ($F_{1,42} = 7.24$, $P < 0.02$) were found. This time, face grooming was reduced only in treated mutants and their untreated controls (95% level of confidence). The normal treated animals may have recovered from the BMT sufficiently to perform such CNS-mediated reflex activities at similar levels as the normal untreated controls.

Morris water maze test

Acquisition. From days 2 to 6, the mice were tested for their ability to learn the location of the platform placed in the SW quadrant. The normal controls showed a gradual decline in the time needed per trial to find the platform. Although the treated mice (normal and mutant) and untreated mutants also showed a decrease in search time over these days, they were much slower than the normal controls (Fig. 2).

ANOVA for repeated measures showed significant main effects of treatment ($F_{1,43} = 11.62$, $P < 0.002$), genotype ($F_{1,43} = 10.14$, $P < 0.003$), trial ($F_{3,129} = 10.23$, $P < 0.0001$), and day ($F_{4,172} = 10.29$, $P < 0.0001$). This indicates that the performance of the mice was affected by all four parameters: the BMT, the genotype, the sequence of the four trials during the same day, and the sequence of the days from day 2 to day 6. In addition, there were significant interactions between genotype × treatment ($F_{1,43} = 7.83$, $P < 0.01$), trial × treatment ($F_{3,129} = 3.30$, $P < 0.05$), and day × treatment ($F_{4,172} = 2.90$, $P < 0.05$), indicating that the rates of improvement between the two genotypes throughout the four trials of each day and the 5 d during the acquisition phase were different as a result of the BMT. The results were further analyzed with posteriori comparisons (Tukey) as summarized at the top of Fig. 2. It can be seen that the BMT therapy had no effect on improving the performance of the mutant mice, and even retarded the learning performance of normal mice.

Transfer. On day 7, the platform was removed from the SW quadrant and the mice were monitored for the amount of time spent in each quadrant. All four groups were observed to spend more time swimming in the quadrant where the platform was originally located (SW) and less time in the other three quadrants (Fig. 3). When tested with ANOVA for repeated measures, the difference among quadrants was highly significant ($F_{1,129} = 35.54$, $P < 0.0001$). Moreover, a significant interaction was observed between the main effects of quadrant × treatment ($F_{3,129} = 4.63$, $P < 0.02$), indicating that the relative amount of time spent in the four quadrants differed as a result of the BMT. When each quadrant was analyzed separately,

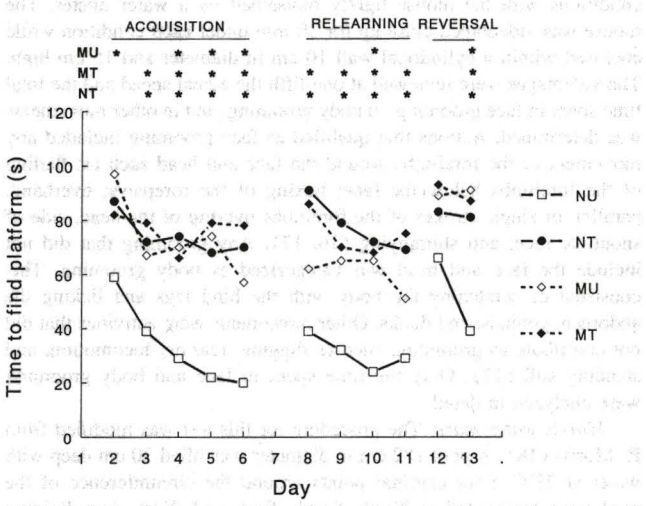

Figure 2. Morris water maze test on normal and gus^{mps}/gus^{mps} mice after bone marrow transplantation. Each animal was released into a water pool from each of the four cardinal points once per day. The time taken to find and land on an invisible platform placed in the pool was recorded and averaged over the four trials as latency per day. Days 2 to 6 comprised the acquisition phase, when the platform was placed in the same location throughout. On day 7, the platform was removed to measure the transfer phase (Fig. 3). Days 8 to 11 comprised the relearning phase, when the platform was replaced to the original position. Days 12 and 13 comprised the reversal phase when the platform was moved to the opposite quadrant. Stars indicate days in which the group differed from the control untreated normal group at the 95% level of confidence. Data are the mean with SEM of 2–8 s, which are not represented in the graphs to prevent cluttering. NU, normal untreated, $n = 17$; NT, normal treated, $n = 12$; MU, mutant untreated, $n = 9$; MT, mutant treated, $n = 9$.

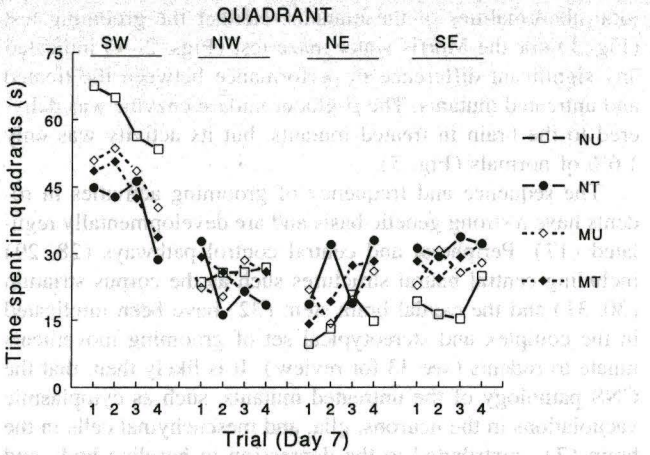

Figure 3. Time spent in each quadrant during the transfer phase of the Morris water maze test (day 7). With the platform removed from the SW quadrant, each animal was released once from each of the four cardinal points (1, 2, 3, and 4), and the amount of time spent in each quadrant was recorded. Data treatment and number of animals are as described in the legend of Fig. 2.

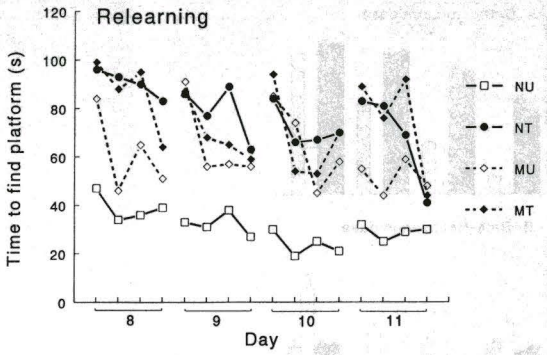

Figure 4. Latency per trial during the relearning phase of the Morris water maze test (days 8–11). From days 8–11, the platform was replaced in the original quadrant (SW). Each animal was released from each of the four cardinal points once per day. The time needed to find the platform was recorded for each trial. Data treatment and number of animals are as described in the legend of Fig. 2.

significant main effects of treatment were found in the SW quadrant ($F_{1,43} = 5.8$, $P < 0.03$), as well as the NE quadrant ($F_{1,43} = 5.04$, $P < 0.03$) and SE quadrant ($F_{1,43} = 5.50$, $P < 0.03$) but not the NW quadrant. Hence, the treated groups (normal and mutant) spent less time in the SW quadrant and more time in the NE and SE quadrants than the untreated groups (normal and mutant) (Fig. 3). Thus, the BMT treatment appeared detrimental for memory retention regardless of the genotype of the animal.

Relearning. From days 8 to 11, the mice were tested as in the "acquisition" phase for their ability to find the platform that now was replaced in the original SW quadrant. On day 8, the average time needed to find the platform of all four experimental groups was longer than those of the last day (day 6) during the acquisition phase (Fig. 2). However, over the subsequent 3 d, only the normal mice (treated or untreated) consistently decreased their search times. The two mutant groups demonstrated somewhat sporadic improvements during this relearning phase. Nevertheless, the treated normals, in spite of demonstrating a similar rate of improvement over the 4 d as the normal controls, clearly were taking much longer than their untreated cohorts to find the platform on each day (Fig. 2).

From ANOVA for repeated measures, a strong linear effect of trial ($F_{1,43} = 38.73$, $P < 0.0001$), as well as an interaction of this effect with treatment ($F_{1,43} = 3.25$, $P = 0.0786$) and with genotype ($F_{1,43} = 4.29$, $P < 0.05$) was observed. This indicates that the latency times differed significantly through the four trials each day, and that this difference was influenced by both the BMT and the genotype of the animals. Further analysis of the individual trials during each day (Fig. 4) indicated that both treated normals and treated mutants showed improvements in search times through the four trials per day in each of the 4 d tested (days 8–11); the untreated mutant mice showed similar improvements for only the first three days of testing, while no improvement was observed on the last day (day 11). The normal controls showed improvements through the four trials for the first day only. During the subsequent three days, they reached a plateau in latency times, showing no more improvements with repeated performance. Therefore, the rapidity with which the animals acquired the maximum amount of learning was in the order: normal control > mutant control > normal treated ≈ mutant treated. Unlike the normal controls, the other three groups took much longer to accomplish the task, and seemed unable to carry over their improved latency from one day to the next. The latency times for the first trial of the day were the same for all 4 d in the treated normals and treated mutants, and for the first 3 d in the untreated mutants—suggesting that each of these days was encountered as a naive experience. The ANOVA for repeated measures showed a strong main effect of treatment ($F_{1,43} = 13.54$, $P < 0.001$), indicating that BMT was a major and detrimental factor in determining the performance of the animals. This was especially evident on the first and last days of testing when both treated groups (normal and mutant) showed a higher latency time than the corresponding untreated cohorts (Fig. 4).

Reversal. On days 12 and 13 of the test, the platform was changed to the NE quadrant of the pool (Fig. 2). On the first day of this phase, all experimental groups showed latency times similar to that of the first day of the acquisition phase. On the second day, however, the normal controls quickly reduced their latency times, while the other three groups showed little improvement. ANOVA for repeated measures indicated significant main effects of genotype ($F_{1,43} = 7.91$, $P < 0.01$) and trial ($F_{1,43} = 4.45$, $P < 0.01$), while the main effects of treatment ($F_{1,43} = 3.21$, $P = 0.0800$) and day ($F_{1,43} = 3.87$, $P = 0.0556$) were significant only at the 90% level of confidence. This indicates that the latency times were affected by the genotype of the animals, by the sequence of the four trials each day, and, to a lesser degree, by the BMT as well as the day of the tests. Follow up tests (Tukey) confirmed that the performance of the normal controls on day 13 was significantly different from the other three groups ($P < 0.05$). Therefore, BMT was clearly detrimental to the cognitive performance of normal animals, as shown by the poor performance of the treated normal group. It also did not improve the performance of the transplanted mutants.

Lysosomal enzyme analysis

The effect of BMT on normalizing the deficient β-glucuronidase activity and the elevated β-hexosaminidase and α-galactosidase

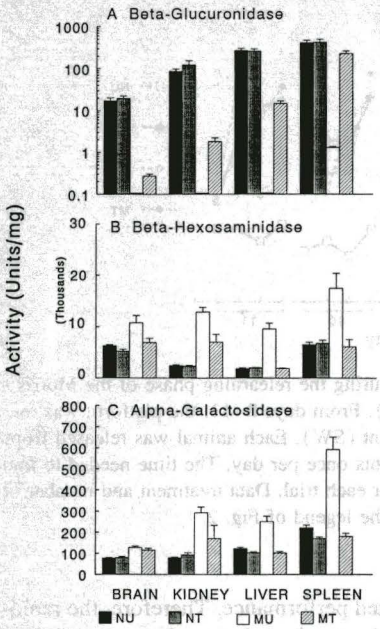

Figure 5. Lysosomal enzyme activities in various organs of the normal and gus^{mps}/gus^{mps} mice after bone marrow transplantation. At about 20 wk of age, animals were sacrificed and their organs removed for protein and lysosomal enzyme assays. Each organ was assayed in duplicate or triplicate. The means for the different animals were averaged (±SEM). NU, normal untreated, $n = 8$; NT, normal treated, $n = 6$; MU, mutant untreated, $n = 4$ (3 for the brain); MT, mutant treated, $n = 5$.

activities in the gus^{mps}/gus^{mps} mice was variable. Consistent with previous observations (10), the degree of enzymatic normalization depended on the organs studied. β-Glucuronidase activity in the brain, kidney, liver, and spleen of treated mutants was restored to 1.6, 2.1, 5.5, and 55.3%, respectively, of those of the normal controls (Fig. 5 *A*). The untreated mutants, as expected, had < 0.5% (range 0–0.35%) of normal activity. The transplanted normals showed no difference from the normal control in these biochemical profiles. ANOVA, followed by post hoc tests (Tukey) revealed that treated mutants differed from untreated mutants in all the organs tested, thus indicating the effectiveness of bone marrow transplant in restoring at least some β-glucuronidase in the mutants. When the secondary elevation of other lysosomal enzyme activities was examined, the levels of β-hexosaminidase (Fig. 5 *B*) and α-galactosidase (Fig. 5 *C*) activities also had decreased to near normal levels in all organs except for β-hexosaminidase activity in the kidney and α-galactosidase activity in the brain and kidney (Fig. 5, *B* and *C*). In these organs, even though the levels were still significantly higher than normal, they were lower than those of the untreated mutants—again confirming the biochemical efficacy of the BMT. When the peripheral blood smear of the transplanted mutants was assessed for the percent of donor cell types as an indicator of the level of engraftment in the hemopoietic system, the average±SEM was 29.7±3.9% (range 11–52%).

Discussion

Although BMT performed in neonates improved many of the biochemical (Fig. 5), clinical, and pathological abnormalities (11) of the gus^{mps}/gus^{mps} mice, it did not ameliorate the behavioral abnormalities of the mutants. Neither the grooming test (Fig. 1) nor the Morris water maze test (Figs. 2–4) indicated any significant difference in performance between the treated and untreated mutants. The β-glucuronidase enzyme was delivered to the brain in treated mutants, but its activity was only 1.6% of normals (Fig. 5).

The sequence and frequency of grooming activities in rodents have a strong genetic basis and are developmentally regulated (17). Peripheral and central control pathways (28, 29) including central neural structures such as the corpus striatum (30, 31) and the caudal brain stem (32) have been implicated in the complex and stereotypical set of grooming movements innate to rodents (see 33 for review). It is likely then, that the CNS pathology of the untreated mutants, such as cytoplasmic vacuolations in the neurons, glia, and mesenchymal cells in the brain (7), contributed to the depression in baseline body and stimulated face grooming seen here (Fig. 1) and in earlier experiments (8). That the treated mutants were not significantly different in baseline body grooming from the normal controls may be attributed to the presence of an outlier which increased the time spent in body grooming for treated mutants by 50%. However, one cannot exclude the outlier (animal M-2, level of engraftment 12%, as assessed from blood smear, unpublished observation) as there may have been variability in the degree of bone marrow engraftment and/or in the time when engraftment occurred.

Under stimulated conditions, face grooming was significantly depressed not only in the untreated mutants but also in the treated mice, compared with the normal controls. The decrease seen in the treated mutants indicates that the therapy was not effective in restoring such neurologically mediated reflex actions. However, the depression observed in treated normals suggests that the therapy itself may have been detrimental.

The performance of the animals during the Morris water maze test provides further insight to the behavior of the animals. The untreated mutants were slower than the normal controls in learning the location of the platform during the acquisition phase (Fig. 2). It has been suggested (8) that the untreated mutants are slower due to physical constraints such as synovial fluid retention in limb joints, skeletal deformities resulting in abnormal gait, and reduction in adipose tissue as reservoirs for metabolic energy (2, 7). However, the treated normals, which did not manifest these physical abnormalities, were just as slow to find the platform, suggesting that the untreated mutants indeed do have neurological deficits and that the BMT procedure is detrimental to neurological functions in general.

The latter hypothesis is supported by the results of the ''transfer'' tests on day 7 of the Morris water maze. Both treated groups (normal and mutant) spent less time in the quadrant where the platform was originally located than the untreated (normal and mutant) mice (Fig. 3). Thus the treated mice were unable to retain and transfer the memory from their previous learning experience during the acquisition phase. Hence, BMT was detrimental to memory retention regardless of the genotype of the treated animals.

The untreated mutants, and treated normals and mutants, were also unable to carry over their learned experience from one day to the next. This was particularly evident during the ''relearning'' phase, when the platform was replaced in the original quadrant (Fig. 4). In fact, the two treated groups showed no improvement in behavior over the 4 days of testing whereas the untreated mutants were able to learn at least on the

last day of testing (Fig. 4, days 8–11). When the platform was placed in a new position, only the untreated normal controls were able to adopt new strategies to locate the platform (Fig. 2). The results of the transfer, relearning, and reversal phases of the Morris water maze test not only confirm the memory deficits of the gus^{mps}/gus^{mps} mice (8), but also demonstrate that BMT was unable to restore these deficits. Even the treated normal mice were unable to perform spatial tasks requiring more complex cognitive functions as successfully as the normal controls.

Previous studies show that the brains of the transplanted mutant mice have focal areas of decreased lysosomal storage in the meninges and ependyma cells, but not in the neurons, perivascular cells, or glia (11). Consequently, the first assumption to interpret the lack of neurological functional improvement is that the level of β-glucuronidase did not reach either the critical level or the appropriate cell types in the CNS. However, this alone cannot account for the behavior of the treated mutants. Even the treated normal mice were observed to perform far below the level of the untreated normal controls and similarly to the mutants (treated or untreated), in most instances.

Hence, in general, the lack of improvement in the transplanted mutants and the regression in behavior in the transplanted normals (Figs. 1–4) can be largely attributed to the transplant procedure itself, and most likely to radiation-induced toxicity in the CNS. Mice exposed to 2 Gy showed focal thinning of the cerebellar granule cell layer of the brain (11). Furthermore, loss of Purkinje's cells, disorganization of the cortical layer, and a reduction in cerebellar mass were evident at higher radiation doses (11). In addition, the immature state of the neonate mice is particularly susceptible to radiation-induced damages (unpublished observation), even though the incomplete closure of the blood–brain barrier at this stage of development in the newborn mice (34) is a distinct advantage for the delivery of lysosomal enzymes into the brain.

In conclusion, it has been demonstrated that, even though biochemical improvement was obtained, BMT in the newborn period did not improve the neurological function of the mutant gus^{mps}/gus^{mps} mice. Furthermore, the therapy proved deleterious to the cognitive functions of even normal animals. Hence, an alternate approach to the prevention of disease progression may require temporary enzyme replacement with less invasive techniques during the newborn period (35). This can be followed by BMT after the animals have matured enough to withstand the transplant procedure (11). However, other approaches such as somatic gene therapy with autologous (14, 36) or non-autologous (37) cell implant are more benign and may well be the preferred treatment. This will certainly obviate the harmful effects of marrow ablation and still be capable of delivering β-glucuronidase immediately after birth. It is also clear from this study that the gus^{mps}/gus^{mps} mice not only provide a good model to study the restoration of biochemical and pathological deficits, but the restoration of neurological function as well. The latter, which can be determined by monitoring grooming activities and performance during the Morris water maze test, is an important adjunct when studying the efficacies of preexisting and new therapies on animal models of human neurodegenerative diseases.

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Allogeneic Gene Therapy on the Murine Model of Mucopolysaccharidosis Type VII.
Bastedo, L., Sands, M.S., Hortelano, G., Al-Hendy, A. and Chang, P.L. *To be submitted*

My specific contributions to Bastedo et al., 1995, *to be submitted.*

The concept of non-autologous somatic gene therapy involves enclosing a universal cell line engineered to secrete a desired gene product, in a biocompatible and perm-selective capsule. We received a fibroblast cell line (2A-50) that was transfected with the mouse β-glucuronidase cDNA. This cell line secreted 28 fold more β-glucuronidase than the myoblast cell line I had transfected with a different vector (see Appendix B). With the help of Dr. G. Hortelano, I encapsulated the 2A-50 cells in alginate-poly L-lysine. Dr. A. Al-Hendy then showed me how to implant these capsules into the peritoneal cavity of mice. During the course of the experiment, I routinely collected blood and urine samples from the mice. The samples were then analyzed for β-glucuronidase activity and glycosaminoglycan content respectively. I also did all secretion analysis, viability, and cell counts of naked and encapsulated cells. Mice were sacrificed at 2 weeks and 4 weeks post-implantation, various organs removed and analyzed for β-glucuronidase, β-hexosaminidase, and α-galactosidase activity. I also processed, embedded and sectioned the kidneys, livers, and spleens for histological analysis.

Allogeneic Gene Therapy on the Murine Model of Mucopolysaccharidosis Type VII.
Bastedo, L., Sands, M.S., Hortelano, G., Al-Hendy, A.and Chang, P.L. *To be submitted*

ABSTRACT

Mucopolysaccharidosis (MPS) Type VII is an autosomal recessive lysosomal storage disease caused by deficient β-glucuronidase activity. We propose to use a novel approach to somatic gene therapy by enclosing a non-autologous "universal" cell line, engineered to secrete high levels of β-glucuronidase, in perm-selective and immuno-protective microcapsules. To test its feasibility, a MPR^- mouse fibroblast cell line transfected with the mouse β-glucuronidase cDNA (gift from Dr. W.S. Sly) was enclosed in alginate microcapsules and implanted into the peritoneal cavity of mutant MPS VII mice and normal litter mates. As little as 24 hours post-implantation, 3.5% of normal β-glucuronidase activity was detected in the plasma of implanted mutants. This increased to 66% by day 24. Concomitantly, urinary glycosaminoglycan/creatinin content, normally elevated in mutants, showed a significant decrease indicating that the β-glucuronidase was exerting a therapeutic effect. β-Glucuronidase activity was detected in significant amounts in the kidney, liver and spleen, indicating that the enzyme was taken up by cells at distant sites. Deficiency in β-glucuronidase results in elevated activity of two other lysosomal enzymes, β-hexosaminidase and α-galactosidase, as well as an accumulation of undegraded glycosaminoglycan in all tissues. When mice were sacrificed at 2 and 4 weeks post-implantation, the levels of β-hexosaminidase and α-galactosidase, which were normally elevated in the mutants, had significantly decreased in the kidney, liver, and spleen. Toluidine blue stained sections of these tissues showed a dramatic reduction in lysosomal storage lesions in the spleen and a reversal of mutant pathology in the liver. By about 4 weeks post-implantation, haemorrhagic

ascites fluid was observed in the treated mutants but not the treated normal mice. Thus, we hypothesized that an immune response had been generated against the foreign protein (β-glucuronidase) and not to the non-autologous cells. In spite of the biochemical and pathologic correction, the long term efficacy of treatment has to be evaluated, particularly for diseases in which the mutation ablates the expression of a protein.

INTRODUCTION

Mucopolysaccharidosis type VII (MPS VII) is an inherited disorder of glycosaminoglycan (GAG) metabolism that is caused by a deficiency in the lysosomal enzyme β-glucuronidase [EC 3.2.1.31]. Clinical and pathologic manifestations common to both affected humans and the MPS VII mice include shortened stature, shortened lifespan, hepatosplenomegaly, corneal clouding, dystosis multiplex, and excessive intralysosomal accumulation and urinary excretion of undegraded GAGs (Sly et al., 1973; Watts and Gibbs, 1986; Neufeld and Meunzer, 1989; Birkenmeier et al., 1989; Vogler et al., 1990). The murine model of MPS VII has been extensively characterized in its genetics, biochemistry and pathobiology (Birkenmeier et al., 1989; Vogler et al., 1990; Sands and Birkenmeier, 1993). It thus serves as a good model to evaluate the efficacy of different therapies.

Since the initial characterization of the MPS VII mice, various therapies have been initiated for its treatment. Syngeneic bone marrow transplantation (BMT) into adult mice resulted in partial correction of the diseased state (Birkenmeier et al., 1991); however, BMT in neonates was more effective at reversing the mutant phenotype (Sands et al., 1993). To avoid the problem of finding HLA-matched donors (as would be the case for human BMT), mutant haematopoietic stem cells, transduced with retroviruses containing the β-glucuronidase gene, were transplanted into irradiated mutant mice. This resulted in partial correction of mutant pathology in the liver and spleen (Wolfe et al., 1992). Despite the success of BMT, the procedure used to ablate bone marrow cells had detrimental consequences. In neonates treated with BMT, there was evidence of radiation induced

toxicity in the central nervous system (CNS) (Sands et al., 1993), as well as a deterioration in neurological function as determined by behavioural tests (Bastedo et al., 1994). An alternative approach for the treatment of MPS VII involved implanting "neo-organs" composed of synthetic fibres and syngeneic primary fibroblasts engineered to secrete β-glucuronidase into the peritoneal cavity of mutant mice. This also resulted in a reduction of storage material in the liver and spleen (Moullier et al., 1993).

To avoid immune rejection, syngeneic haemopioetic stem cells (Wolfe et al., 1992) and fibroblasts (Moullier et al., 1993) were used as a source of β-glucuronidase. In humans, the procedure of obtaining a patient's own cells, propagating them *in vitro*, manipulating, characterizing and selecting the appropriate recombinants, and then implanting the cells back into the patients is labour intensive and can incur prohibitive costs. To circumvent such problems of *ex vivo* gene therapy we have proposed an alternative strategy of non-autologous somatic gene therapy (Chang 1995). This approach involves creating a "universal" cell line genetically engineered to secrete a desired gene product. The recombinant cells are then encapsulated in a biocompatible and perm-selective membrane that allows nutrient/waste and gene product diffusion. Because the encapsulated cells are also immuno-isolated, the capsules may then be implanted into different patients that require the same gene product.

The concept of implanting biological products enclosed in perm-selective artificial membranes was first described by Chang in 1964. Since then, a variety of non-recombinant cell types have been encapsulated and maintained *in vitro* or implanted into animals. For

example, microencapsulated pancreatic islet cells have been used to treat diabetic rats (O'Shea et al., 1984, Fan et al., 1990; Weber et al., 1990); encapsulated PC12 cells secreting dopamine have shown to improve the behaviour of rats with lesions in the doparminergic pathway (Winn et al., 1991); and implantation of encapsulated hepatocytes have shown to decrease bilirubin levels in hyperbilirubinemic (Gunn) rats (Sun et al., 1986; Chang, 1992).

The use of encapsulated recombinant cells for gene therapy purposes was first described Chang et al., (1993). Non-autologous fibroblasts engineered to secrete human growth hormone were shown to effectively deliver human growth hormone to the systemic circulation of rodents (Chang et al., 1993). The clinical efficacy of this approach was later demonstrated by correction of the growth retardation of the Snell dwarf mice (Al-Hendy et al., 1995). Mutation of the pit-1 transcription factor in these mice caused a down regulation of growth hormone production, resulting in a dwarf phenotype. When the mutants were implanted with C2C12 myoblasts engineered to secrete mouse growth hormone, their body weight, length, internal organs and epiphysial growth plate all showed significant increases. These studies have demonstrated that the microcapsules were able to isolate the allogeneic recombinant cells from the host's immune system, while allowing the delivery of biologically acitve recombinant gene product to correct a disease phenotype.

The MPS VII phenotype can be somewhat reversed by introducing β-glucuronidase exogenously either from neo-organs (Moullier et al., 1993) or by direct enzyme replacement therapy (Vogler et al., 1993). Hence, we hypothesized that β-glucuronidase secreting fibroblasts encapsulated in microcapsules and implanted into the peritoneal cavity

of the MPS VII mice would act as a source of corrective enzyme. We now report on the efficacy of non-autologous somatic cell gene therapy in the treatment of murine MPS VII.

MATERIALS AND METHODS

Animals and transfected cell line

Male mutants (*gus*^{mps}/*gus*^{mps}) and their normal litter mates (+/+ and +/*gus*^{mps}) obtained from the strain B6.C-H-2^{bml}/ByBir*gus*^{mps}/+) were supplied by the Jackson laboratory. Mouse L-MPR^{-} cells were transfected with the expression vector pMSXND (Lee and Nathans, 1988) which contained the mouse β-glucuronidase cDNA downstream from the metallothionein I promoter and upstream form the cDNA encoding dihydrofolate reductase (Gift from W.S. Sly). The cDNA sequences were amplified in tissue culture with 3.2 μM methotrexate.

Encapsulation of Cells

Cells were encapsulated under sterile conditions as described by Chang et al., (1994). Approximately 2 x 10^6 trypsinized cells were washed once in 0.9% NaCl and resuspended in 5 mL filter sterilized potassium-alginate (Improved Kelmar, Kelco 1KR 09847A). This mixture was drawn through a 10 cc syringe with a 27 gauge blunt needle (Popper and Sons, Inc., N.Y. #7400) and placed in a Razar A-99 syringe pump. The size of the capsules was regulated by maintaining the extrusion rate at 39.3 cc/h and air flow along the needle at 3 L/min. Capsules were extruded into 35 mL 1.1% $CaCl_2$, collected in a 50 mL conical polypropylene tube and then subjected to the following washes at 4-10°C: 0.55% $CaCl_2$ in saline; 0.28% $CaCl_2$ in saline; 0.1% CHES, 3 min; 1.1% $CaCl_2$ in saline; 0.05% poly L-lysine (PLL) in saline, 6 min; 0.1% CHES; 1.1% $CaCl_2$; 0.9% NaCl; 0.03% potassium

alginate in saline, 4 min; 0.9% NaCl; 0.055 M Sodium citrate, 6 min; 2 x 0.9% NaCl; 2 x cell media. The capsules were then maintained in regular tissue culture conditions.

Implantation

Capsules were washed 5 to 6 times in 0.9% ice cold NaCl. This was followed by final wash in TC Hanks solution (Difco), where the capsules remained on ice until needed. Each mouse was anaesthetized with a combination of isofluorane (1-chloro 2,2,2-trifluorethyl difluoromethyl ether) (Anaquest), oxygen (0.3 L/min) and nitrogen oxide (1-1.5 L/min). A catheter tube with a 16 gauge needle in the cannula was used to pierce the abdominal wall and peritoneum. This cannula was used to direct capsules from a 10 cc syringe into the peritoneum.

GAG/Creatinin Determination

The protocol was originally taken from Whitley, 1989 and modified by Moullier, 1993. Briefly 600 µL of 20 µM 1,9-dimethylmethylene blue (DMB) (Aldrich) was added to 2 µL of urine. After 20 min incubation at room temperature, absorbance at 540 nm was read. Amount of GAG present was determined against a standard prepared from chondroitin sulphate C (Sigma). To determine creatinin levels, 10 µL of a tenfold diluted urine sample was mixed with 50 µL 0.2 M NaOH and 50 µL saturated picric acid (Sigma). After 20 min incubation at room temperature, absorbance at 490 nm was read and compared to a creatinin standard (Sigma).

Blood Collection

Mice were anaesthetized as described before. One to two hundred microlitres of blood were collected retro-orbitally using 1.2 mm heparinized capillary tubes (Chase Instruments). Samples were centrifuged at 16000 *g* for 15 min at 4°C to collect plasma.

Lysosomal Enzyme Assays

Dissected tissues were either stored at -70°C or immediately homogenized in 20 mM Tris·HCl, pH 7.5/140 mM NaCl/10 mM β-mercaptoethanol/0.25% saponin homogenization buffer. Homogenates were sonicated on ice for 20 s in Kontes microultrasonic cell disrupter and then centrifuged at 16 000 *g* for 30 min. Supernatants were assayed fluorometrically for lysosomal enzyme activities by using 4-methylumbelliferyl (4-MU) substrates (Glaser and Sly, 1973). β-glucuronidase, β-hexosaminidase and α-galactosidase activities were assayed using the substrates 4-MU-β-glucuronide, 4-MU-*N*-acetyl-β-D-glucosaminide, and 4-MU-α-D-galactoside (Sigma Chemical Co., St.Louis, MO), respectively. Proteins were determined according to the method of Lowry (Lowry et al., 1951).

Secretion Analysis

All samples were taken in triplicate. Cells or intact capsules were washed twice in sterile PBS before being replaced with the appropriate pre-equilibrated media. This was considered as t = 0 h. Thereafter at regular intervals (1, 2, 4 h), an aliquot of media was

removed for appropriate enzyme analysis and the numbers of cells/capsule and total capsules used for the analysis were determined.

Histology

Dissected tissues were immediately cut into 1-1.5 mm cubes and fixed in 2% gluteraldehyde (pH 7.4) for 1 h at 4°C. This was followed with a wash in 0.2 M sodium cacodylate buffer and left overnight at 4°C. The next morning, tissues were washed twice for 5 min each in 0.2 M sodium cacodylate buffer and once in 1% osmium tetroxide for 1 h at 4°C. The tissues were then dehydrated with stepwise washes of 5 and 10 min each of 50%, 70%, 95%, 100% ethanol and 100% propylene oxide. Tissues were then infiltrated with 50:50, 25:75, propylene oxide:firm Spurr Resin for 30 min each. This was followed with two 1 h incubations with 100% firm Spurr Resin. Tissues were embedded in mold blocks with fresh 100% Spurr Resin and allowed to polymerize overnight at 65°C. Tissue blocks were trimmed and 1 μm sections were cut on a Reichert OmU2 microtome. A standard glass cutting knife was used. Sections were placed in warm Toluidine Blue for 1-3 min, rinsed 2 times with distilled water, and incubated in warm water for 1-3 min. The sections were then placed on slides and gently heated until all water had evaporated. Permount was placed over the sections followed by a coverslip.

RESULTS

2A50 cells were encapsulated in alginate-poly L-lysine at a cell density of 146 cells/capsules and a secretion rate of 522±40 nmol β-glucuronidase activity/hour/10^6cells. Five mL of the above capsules was injected into the peritoneal cavity of 11 mutant and 2 normal mice. This was considered day 1 of the experiment. Two of the treated mutant mice died 24 hours after the implantation. Post-mortem examination indicated that the mice died due to progression of the mucopolysaccharidosis.

In vivo expression of enzymatically active β-glucuronidase was determined by assessing enzyme activity in the plasma. As little as 24 hours post-implantation (Day 2), there was evidence of β-glucuronidase activity in the plasma of 4 out of 9 treated mutants (range 2.6 to 14.0 % of normal acitivty). By day 5, enzyme activity, ranging from 8.8% to 49.5% of normal, was detected in the plasma of all 9 treated mutants. β-glucuronidase was not detected in the plasma of the untreated mutants (Figure 1). The pattern of enzyme delivery to the systemic circulation varied within the treated group. Three of the treated mice initially showed increasing levels of enzyme which then started to decline between days 15 and 17. The other treated mutants showed increasing amounts of β-glucuronidase for the time points tested. By day 24 the levels of enzyme were 66% of normal activity (range 6-120% of normal activity). When the capsules were removed from the peritoneal cavity at 2 and 4 weeks, the encapsulated cells continued to secrete β-glucuronidase, showing that expression was maintained for at least 4 weeks in the mutants. Microencapsulated cells recovered from normal mice after 2 months, also secreted β-glucuronidase (Table 1). The viability of the

encapsulated cells was 59.7±5.1% upon encapsulation and had declined to 18.5±9.8% (range= 10.9 - 29.5) when retrieved from three treated mutants at 4 weeks. The viability of the cells maintained *in vitro* had also declined from 59.7±5.1% to 37.9±9.0 after 4 weeks.

To see whether β-glucuronidase secreted from the encapsulated cells was taken up by cells at distant sites, cellular extracts from the brain, kidney, liver and spleen were assayed for enzyme activity. Figure 2 shows that β-glucuronidase activity was detected in the organs of all experimental animals, while enzyme activity in the organs of untreated mutant mice remained at background levels. Statistical analysis indicated that the level of enzyme in the brains of the treated and mutant control mice were not significantly different. Enzyme activity in the kidney, liver and spleen, although lower than normal values, was significantly higher than the mutant controls ($p < 0.05$). This demonstrated that the therapy was effective in restoring some β-glucuronidase activity in the peripheral organs of the MPS VII mice. Further analysis of the liver and spleen showed an increasing amount of enzyme activity with time. The liver had 11.3% of normal activity at 2 weeks which increased to 43.5% of normal activity at 4 weeks. Likewise the β-glucuronidase activity in the spleen increased from 18.0% to 65.8% of normal activity. The kidney, on the other hand, showed more activity at 2 weeks (60% of normal activity) than at 4 weeks (20.2% of normal activity). Thus the biochemical assays demonstrated that the therapy was effective at restoring some β-glucuronidase activity to the peripheral organs of the MPS VII mice.

Deficiency of β-glucuronidase activity in the MPS VII mice results in a catabolic block in glycosaminoglycan (GAG) degradation as well as a secondary elevation of two other

lysosomal enzymes β-hexosaminidase and α-galactosidase. The secondary elevation of the hydrolases in the tissues of the brain were not reduced in the treated mice. This is consistent with the lack of significant increase of β-glucuronidase in this organ. The therapy was effective, however, at reducing both β-hexosaminidase and α-galactosidase activities in the kidney, liver and spleen (ps < 0.01) (Figure 3). Activities of both enzymes were reduced to normal levels in the spleen, while in the kidney and liver, they were still higher than normal values. These data suggested that the microencapsulated fibroblasts secreted active β-glucuronidase which was taken up by the peripheral organs and resulted in partial biochemical correction.

In both humans and mice with MPS VII, there is an excessive amount of undegraded GAGs present in the urine. To see whether *in vivo* expression of β-glucuronidase had a therapeutic effect at lowering GAG levels, urinary GAG levels expressed as micrograms GAG per milligram creatinin were assayed at regular intervals (Figure 4). Prior to implantation, urinary GAG levels in the mutants was greater than two fold of normal levels. By day 5 post-implantation, GAG levels had significantly decreased in the treated mice ($p < 0.01$), the levels being intermediate between normal and mutant values. Urine samples taken on day 17 showed no significant difference in GAG levels between normal and treated mice, confirming the effectiveness of the therapy at normalizing urinary GAG levels. When compared to the untreated mutants, urinary GAG levels had decreased by 41% after 24 days of therapy. Thus the β-glucuronidase secreted by the micro-encapsulated 2A50 cells had a positive effect in reducing urinary GAG levels in MPS VII mice.

Examination of the storage pathology in the treated MPS VII mice showed that the presence of β-glucuronidase resulted in an improvement in storage pathology of the peripheral organs. Due to the intralysosomal accumulation of GAGs, the liver of untreated mutants appear loose and uniformly disorganized (compare Figures 5A, B). Two weeks after implantation, there was a considerable reduction in lysosomal storage lesions (Figure 5C). At 4 weeks there was a dramatic reversion of mutant pathology such that the liver was undistinguishable form the normal controls (Figure 5D). Similarly, reduction of storage was quite dramatic in the spleen both at 2 and 4 weeks post-implantation (Figure 6). The fact that the pathology of the liver and spleen was closer to normal at 4 weeks than at 2 weeks suggests that increasing amounts of β-glucuronidase (Figure 2) results in a more favourable outcome. Examination of kidney sections revealed a slight reduction in GAG storage in focal areas. In the mutants the destended lysosomes are clearly evident in the renal tubular cells (Figure 7B). At 2 and 4 weks post-implantation, a slight reduction in distention was evident (Figure 7C & D). Reversion of pathology was evident in larger focal areas.

The biochemical and histopathologic results demonstrated that a functionally active β-glucuronidase was secreted from the encapsulated fibroblasts *in vivo*. Furthermore, the enzyme was taken up by cells at distant sites where it functioned to normalize GAG metabolism. However, at 3½ weeks post implantation, treated mutants developed haemorrhagic ascites. At 4 weeks, when they were sacrificed, approximately one third of the microcapsules were found adhered to the peritoneal lining and mesentery. The capsules were surrounded by inflammatory cells, identified as histiocytes, multinucleate giant cells and

lymphocytes. Wide spectrum analyses for the presence of bacteria and mycoplasma were negative indicating that contaminants were not transferred from tissue culture to *in vivo*. Normal mice implanted with the microcapsules showed no adverse effects for at least 2 months at which time they were sacrificed. The microcapsules were loose, easily retrievable with no adhering inflammatory cells.

DISCUSSION

Encapsulation of non-autologous recombinant cells has been proposed as an alternative to autologous gene therapy as a means to deliver therapeutic gene products. Fibroblasts secreting β-glucuronidase were microencapsulated in alginate-poly L-lysine, and implanted into the peritoneal cavity of mutant MPS VII mice. *In vivo* gene expression was monitored by measuring β-glucuronidase activity in the plasma. Enzyme activity in the plasma of treated mutants was 3.5% of normal 24 hours after implantation which increased to 66% of normal after 24 days. The early presence of β-glucuronidase in the plasma was probably due to lymphatic drainage of the peritoneal fluid through the portal circulation and into the systemic circulation. This demonstrated that the β-glucuronidase secreted from the encapsulated cells becomes rapidly available within the peritoneal cavity and in the circulation.

The β-glucuronidase secreted from the encapsulated cells was taken up by cells at distant sites. In this study, β-glucuronidase activity was detected in the kidney, liver and spleen both at 2 and 4 weeks after implantation. Although β-glucuronidase activity in the

brains of the treated mutants was 3.5% and 11.5% of normal at 2 and 4 weeks respectively, it was not significantly different from that of the mutant controls. This is further supported by the lack of reduction of β-hexosaminidase and α-galactosidase in this organ. This indicates that the enzyme present in the circulation of the adult mice was unable to cross the blood-brain barrier. Direct enzyme replacement studies have indicated that β-glucuronidase is able to cross the blood-brain barrier when therapy is initiated in neonates (Vogler et al., 1993). Thus, recombinant cells enclosed in microcapsules may then have to be implanted during the first few days of life when the blood-brain barrier is still incompletely developed. Alternatively, implantation of the capsules within the brain itself may facilitate more enzyme uptake by the cells of the CNS.

To demonstrate that the presence of β-glucuronidase in the peripheral organs was biologically functional, several biochemical and histological parameters were evaluated. In MPS VII mice, the levels of β-hexosaminidase and α-galactosidase activities are elevated. Two weeks after implantation, there was a significant reduction of β-hexosaminidase and α-galactosidase activities in the kidney, liver and spleen. The decrease in enzyme activities was maintained at 4 weeks. Further evidence that a functional β-glucuronidase was secreted from the encapsulated cells was derived from the urinalysis studies. Deficiency in β-glucuronidase results in an excessive excretion of undegraded GAGs in humans and the MPS VII mice. As little as 5 days after implantation, urinary GAG levels had significantly declined in the treated mutants. This indicates that the β-glucuronidase secreted from the encapsulated cells was rapidly taken up by tissues where it functioned in GAG degradation.

MPS VII mice implanted with microcapsules had 11.3% to 65.8% of normal β-glucuronidase activity in the kidney, liver, and spleen. This was associated with a reduction in histological storage lesions. Histochemical staining of the liver from one treated mutant revealed β-glucuronidase activity localized to the Kupffer cells and not to the hepatocytes (M. Sands, personal communication). In spite of this, dramatic reduction of storage lesions was observed after 2 weeks, and a reversal of mutant pathology was observed after 4 weeks of treatment. It is likely then, that intercellular GAG exchange (Yanagishita et al., 1992) between the hepatocytes and the corrected Kupffer cells resulted in the clearance of storage material in the hepatocytes. In addition, small amounts of undetected β-glucuronidase may have entered the hepatocytes via the mannose 6-phosphate receptor and contributed to GAG degradation. This phenomena was also observed after BMT (Birkenmeier et al., 1991) and primary cell neo-organ implantation (Moullier et al., 1993). In the spleen, β-glucuronidase positive cells were also found localized to the periphery of the white pulp (M. Sands, personal communication), a distribution also observed after BMT. Four weeks after implantation, a dramatic reduction in histological storage material was observed, further demonstrating that the presence of β-glucuronidase had a therapeutic effect at normalizing GAG degradation.

When the microcapsules were retrieved, the recombinant fibroblasts continued to secrete β-glucuronidase, albeit at levels lower than upon implantation. This can be explained by the lack of methotrexate selection *in vivo*. Encapsulated cells maintained *in vitro* in the absence of methotrexate (MTX) showed a decline in secretion rates over the course of the experiment. Nonetheless, the expression of β-glucuronidase was maintained *in vivo* for

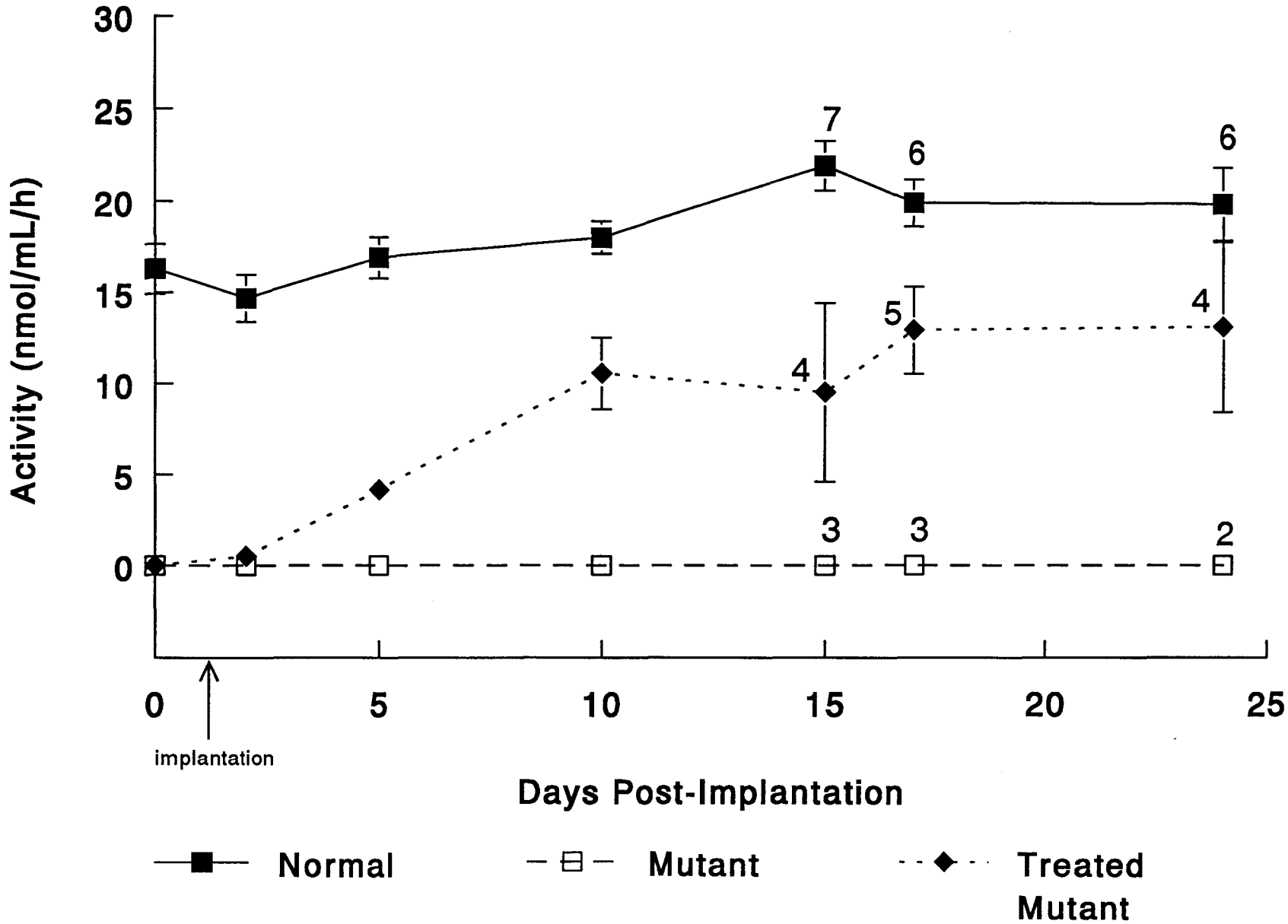
at least 8 weeks. Endogenous MTX independent β-hexosaminidase secretion from the 2A-50 cells, however, did not decline over time (Table 1), demonstrating that the decrease in β-glucuronidase expression *in vivo* was due to the absence of MTX selection.

Starting at 3½ weeks post-implantation, the treated mutant mice presented with haemorrhagic ascites, appeared dehydrated and were slow to respond. This was observed in the implanted mutants but not the implanted normal mice. Bacterial analysis demonstrated that contamination was not transferred from *in vitro* conditions or from the implantation procedure itself. Because the implanted normal mice did not suffer from any adverse effects, it is reasonable to conclude that the inflammatory reaction was not due to the allogeneic cells. As well, it demonstrates that the microcapsules are indeed biocompatible and protect the cells from immune mediators. Mice homozygous for the gus^{mps} mutation produce very low levels of β-glucuronidase mRNA and truncated protein (Birkenmeier et al., 1989; Vogler et al., 1993). It is likely then, that an immune response was generated against the β-glucuronidase. Because the source of β-glucuronidase was not eliminated, (ie: immuno-isolated within the microcapsules), an immune response of increasing magnitude resulted in severe tissue injury (Abbas et al., 1991; Golub and Green, 1991). In spite of this inflammatory response, β-glucuronidase was detected in the kidney, liver and spleen, and its presence resulted in an improved mutant phenotype.

In conclusion, the data show that this method of non-autologous somatic gene therapy effectively immuno-isolates the allogeneic cells and allows efficient delivery of the recombinant gene product. This is advantageous in that the engineered cells, of clonal origin,

can be fully characterized before implantation into many individuals, thus eliminating variability in expression during autologous manipulation. However, long term safety and efficacy of this type of therapy have to be evaluated. This is particularly important for diseases in which the mutation ablates the expression of a protein. In these cases, the introduction of normal protein may be treated as a neo-antigen by the recipient of the therapy.

Figure 1. β-glucuronidase activity in the plasma of treated mutants, normal and mutant controls. Day 0 represents baseline values before therapy and Day 1 represents the day of implantation. Data are the mean ± SEM or range (n<2). Except where indicated, n=13, normal controls; n=9, mutant controls and treated mutants.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Normal
Mutant
Treated Mutant

Table 1 Secretion rates of β-glucuronidase and β-hexosaminidase from encapsulated 2A-50 cells. Day 0 represents baseline values determined before implantation. At the start of the experiment 5 mL of capsules were implanted into 11 mutant and 2 normal mice. 18 mL of additional capsules were divided into 2 groups and retained for *in vitro* studies. One group (9mL) was cultured in the presence of methotrexate (+MTX) and the other group (9mL) was cultured in the absence of methotrexate (-MTX). Secretion rates of β-glucuronidase and β-hexosaminidase were determined for the capsules maintained *in vitro* at 0, 15, and 29 days. At 15, 29 and 56 days post-implantation, the capsules were retrieved from 6 treated mutants (T1a-T9) and 2 treated normals (N1 and N2). The capsules were then washed twice in PBS and analyzed for β-glucuronidase and β-hexosaminidase activity as described in the Methods and Materials. Data are the mean±STD.

	β-Glucuronidase activity (nmol activity/h/10^6 viable cells)				β-Hexosaminidase activity (nmol activity/h/10^6 viable cells)			
Days	0	15	29	56	0	15	29	56
Maintained in vitro								
+MTX	522±40	607±42	804±87	-	49±4	51±5	47±4	-
-MTX	522±40	337±19	258±20	-	49±4	47±5	48±8	-
Retrieved from animals								
T1a	-	289±11	-	-	-	36±6	-	-
T1	-	288±5	-	-	-	42±5	-	-
T2	-	187±5	-	-	-	39±7	-	-
T4	-	-	126±7	-	-	-	41±7	-
T5	-	-	161±13	-	-	-	39±7	-
T9	-	-	207±6	-	-	-	44±5	-
N1	-	-	-	109±8	-	-	-	44±6
N2	-	-	-	215±6	-	-	-	38±7

Figure 2. β-glucuronidase activity in the brain, kidney, liver and spleen from mice sacrificed at 2 and 4 weeks post implantation. A semi log scale was used to show background levels of enzyme in mutant controls (<0.5% of normal activity). Data are the mean ± SEM or range (n<3). At week 2: n=4, normal controls; n=3, mutant controls and n=3, treated mutants. At week 4: n=3 normal controls; n=2 mutant controls; and n=3 treated mutants.

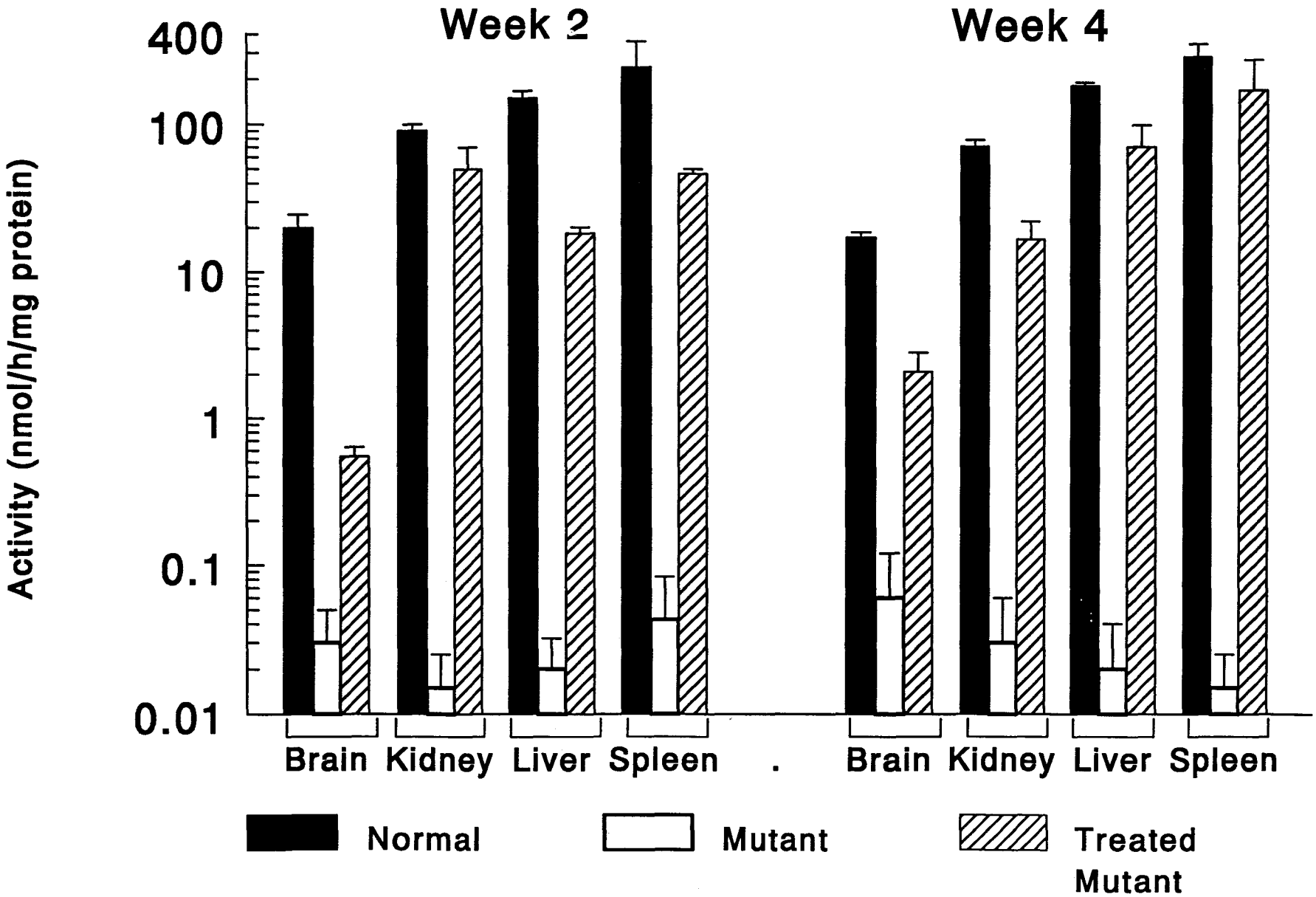

Week 2
Week 4
400
100
10
1
0.1
0.01
Activity (nmol/h/mg protein)
Brain
Kidney
Liver
Spleen
Brain
Kidney
Liver
Spleen
Normal
Mutant
Treated Mutant

Figure 3. Activities of β-hexosaminidase and α-galactosidase in the brain, kidney, liver and spleen 2 and 4 weeks after implantation. Data are the mean ± SEM or range (n<3). At week 2: n=4, normal controls; n=3, mutant controls and n=3, treated mutants. At week 4: n=3 normal controls; n=2 mutant controls; and n=3 treated mutants.

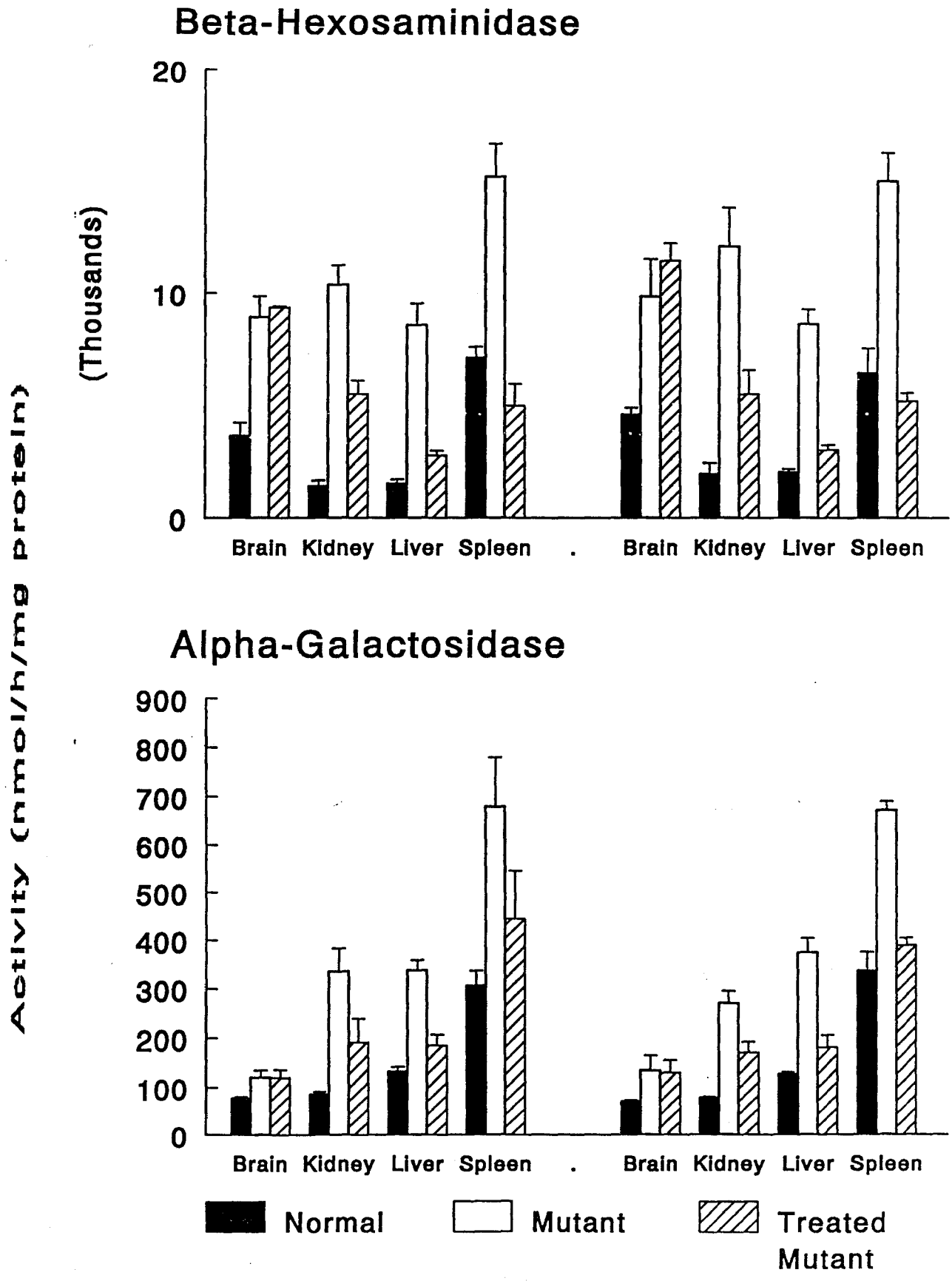

Beta-Hexosaminidase
20
10
0
(Thousands)
Activity (nmol/h/mg protein)
Brain Kidney Liver Spleen
Brain Kidney Liver Spleen
Alpha-Galactosidase
900
800
700
600
500
400
300
200
100
0
Brain Kidney Liver Spleen
Brain Kidney Liver Spleen
Normal
Mutant
Treated Mutant

Figure 4. Urinary GAG/creatinin content measured on different days post implantation. Day 0 represents baseline values before implantation and day 1 represents the day of implantation. Data are the mean ± SEM. Except where indicated, n=13, normal controls; n=9, mutant controls and n=9, treated mutants. Stars (*) indicate the time points where GAG/creatinin content was significantly reduced in the treated mutants when compared to the untreated mutants ($p<0.05$).

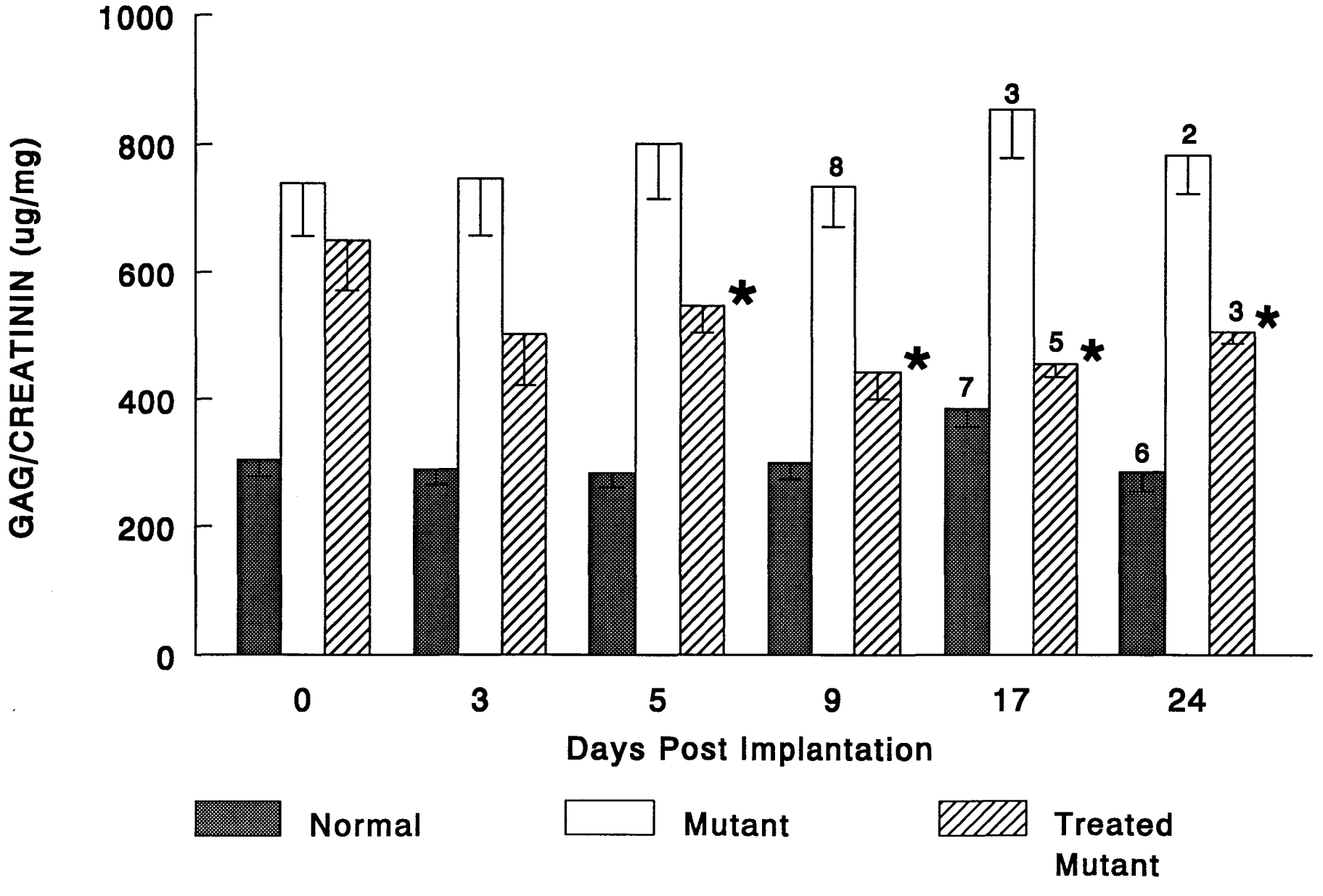
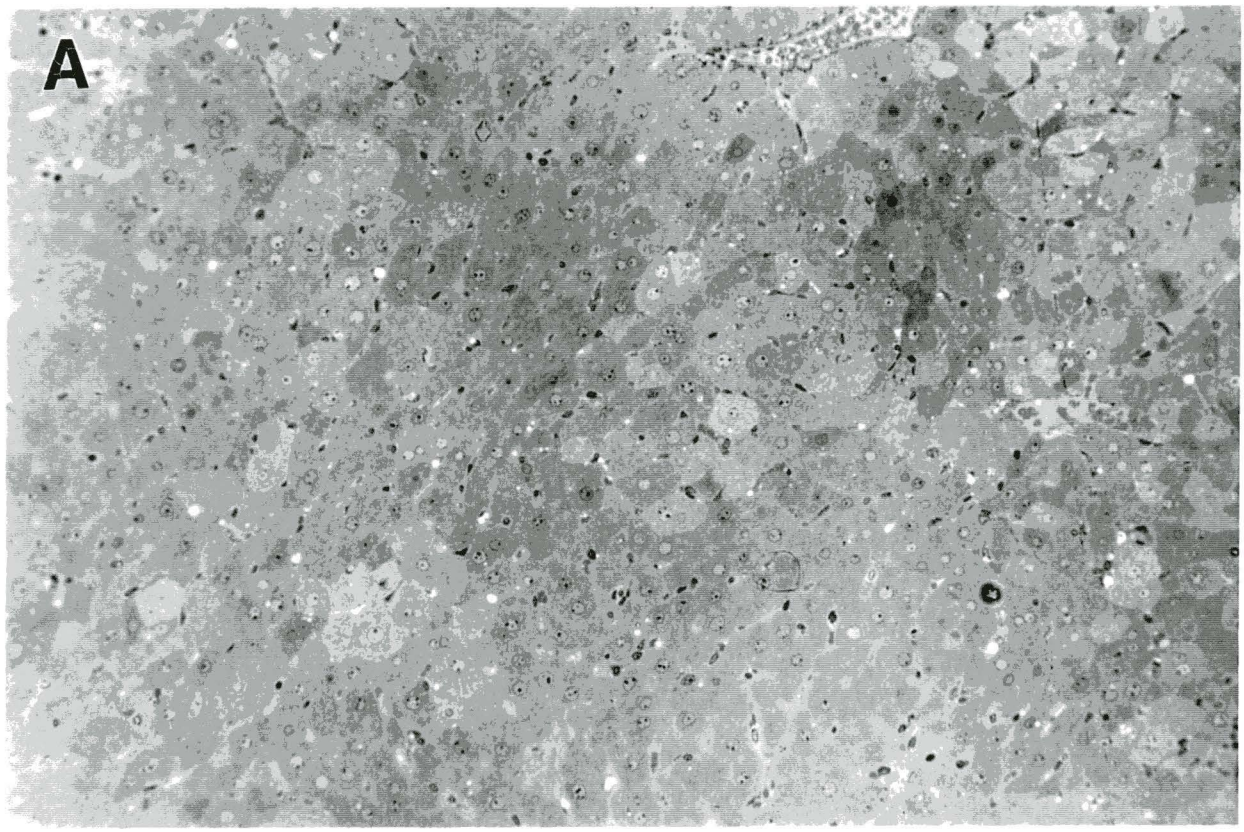
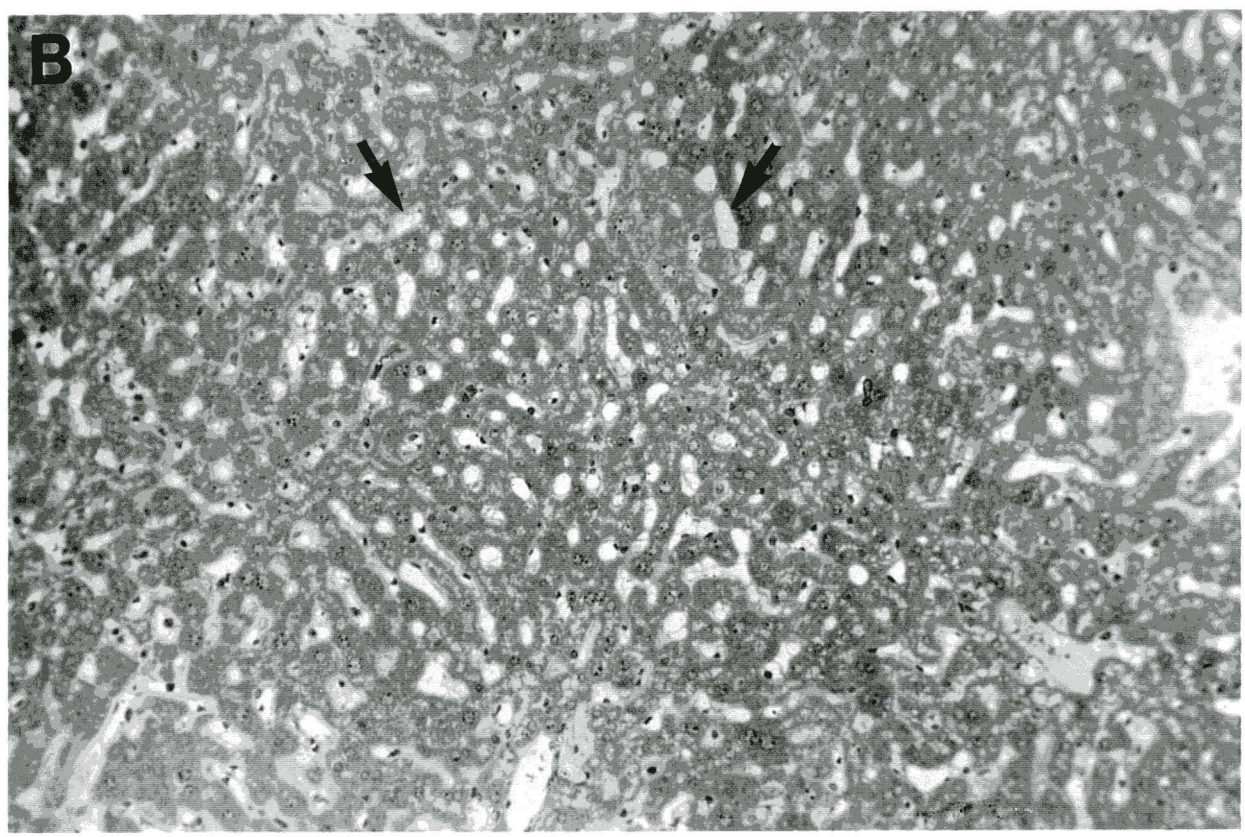

1000
800
600
400
200
0
GAG/CREATININ (ug/mg)
0
3
5
9
17
24
Days Post Implantation
8
3
2
7
5
6
3
*
*
*
Normal
Mutant
Treated Mutant

Figure 5. Toluidine blue stained sections of the liver, (A) normal control; (B) mutant control; (C) treated mutant 2 weeks post-implantation; and (D) treated mutant 4 weeks after implantation. Due to the intralysosomal accumulation (arrows) of GAG's, the liver of the untreated mutants (B) appear loose and uniformly disorganized when compared to the normal controls (A). A reduction in mutant pathology was evident 2 weeks (C) and 4 weeks (D) after implantation. Original magnification 100×.


A


B

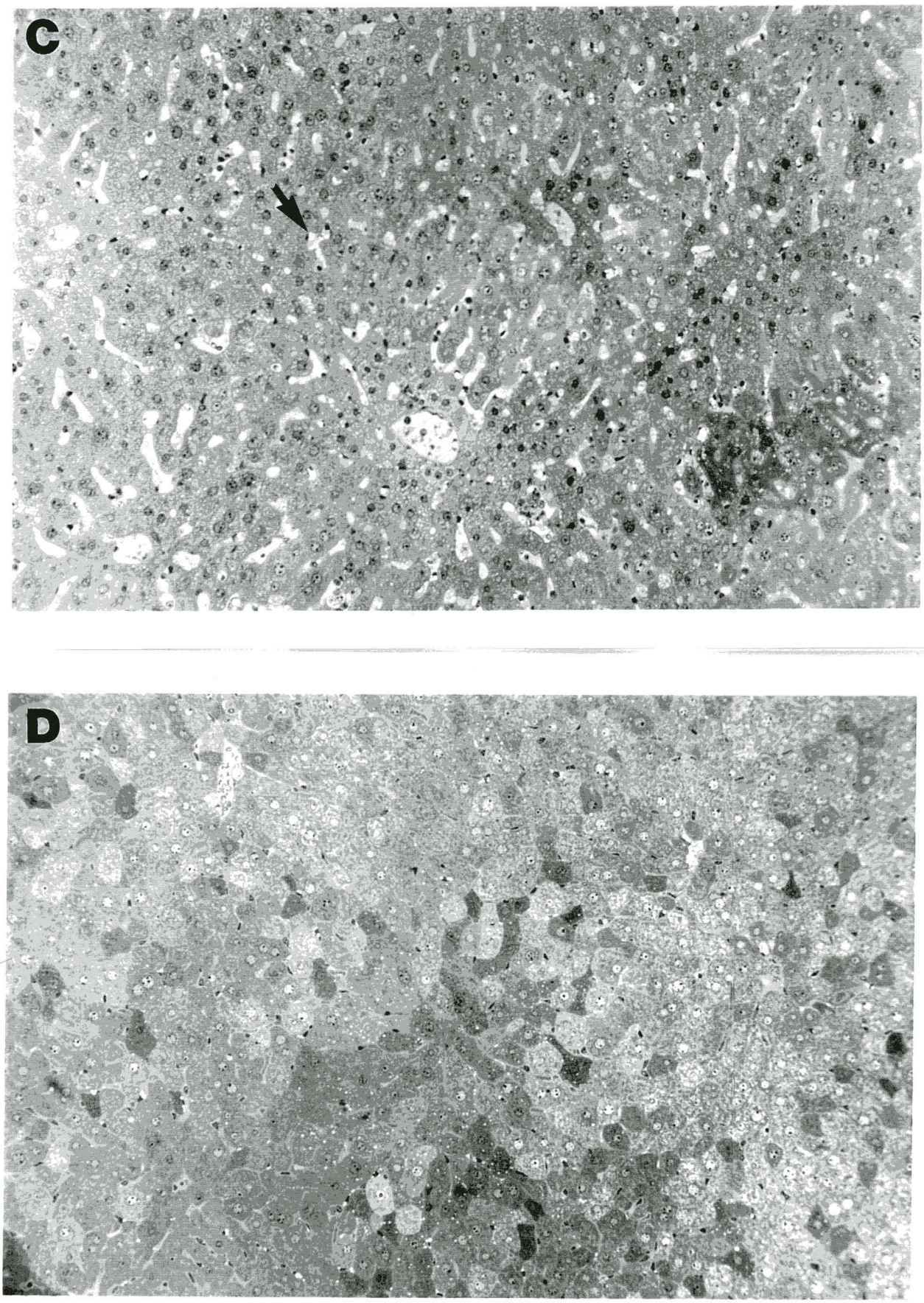
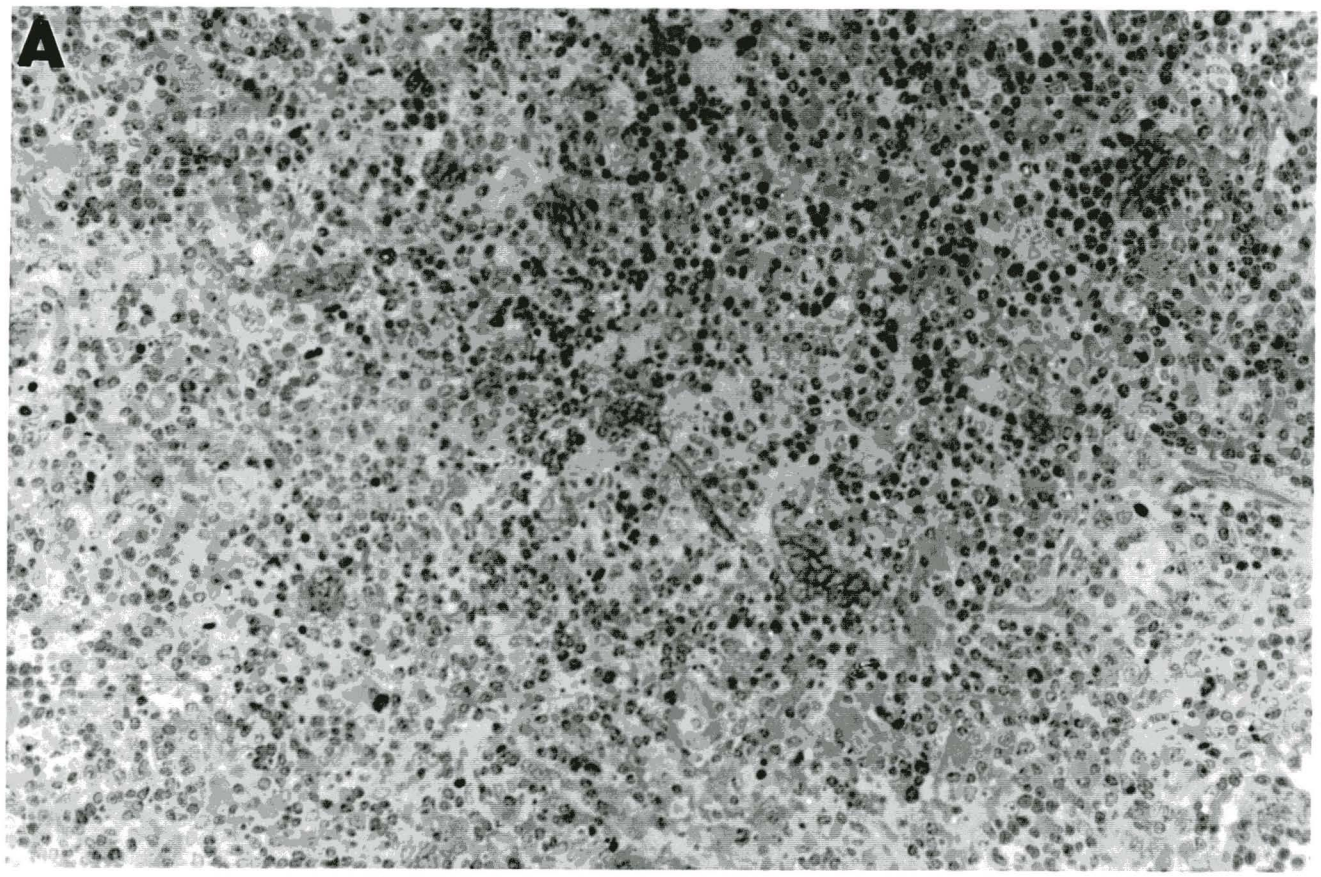

C
D

Figure 6. Toluidine blue stained sections of the spleen, (A) normal control; (B) mutant control; (C) treated mutant 2 weeks post-implantation; and (D) treated mutant 4 weeks after implantation. In the untreated mutants (B) there is marked lysosomal distention in the splenic sinusoidal lining cells (arrows). This was reduced in the treated mutants (C and D). Original magnification 160×.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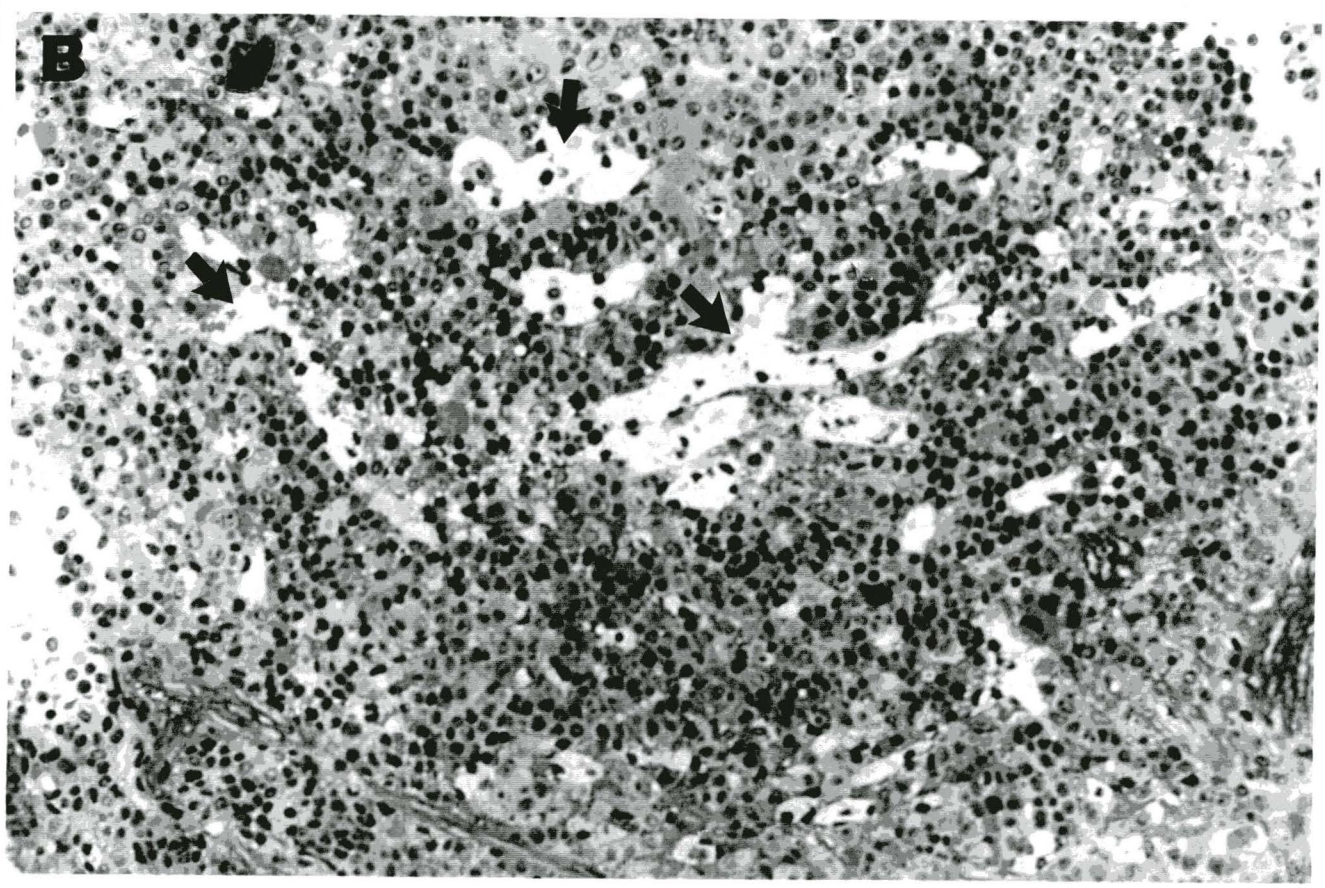
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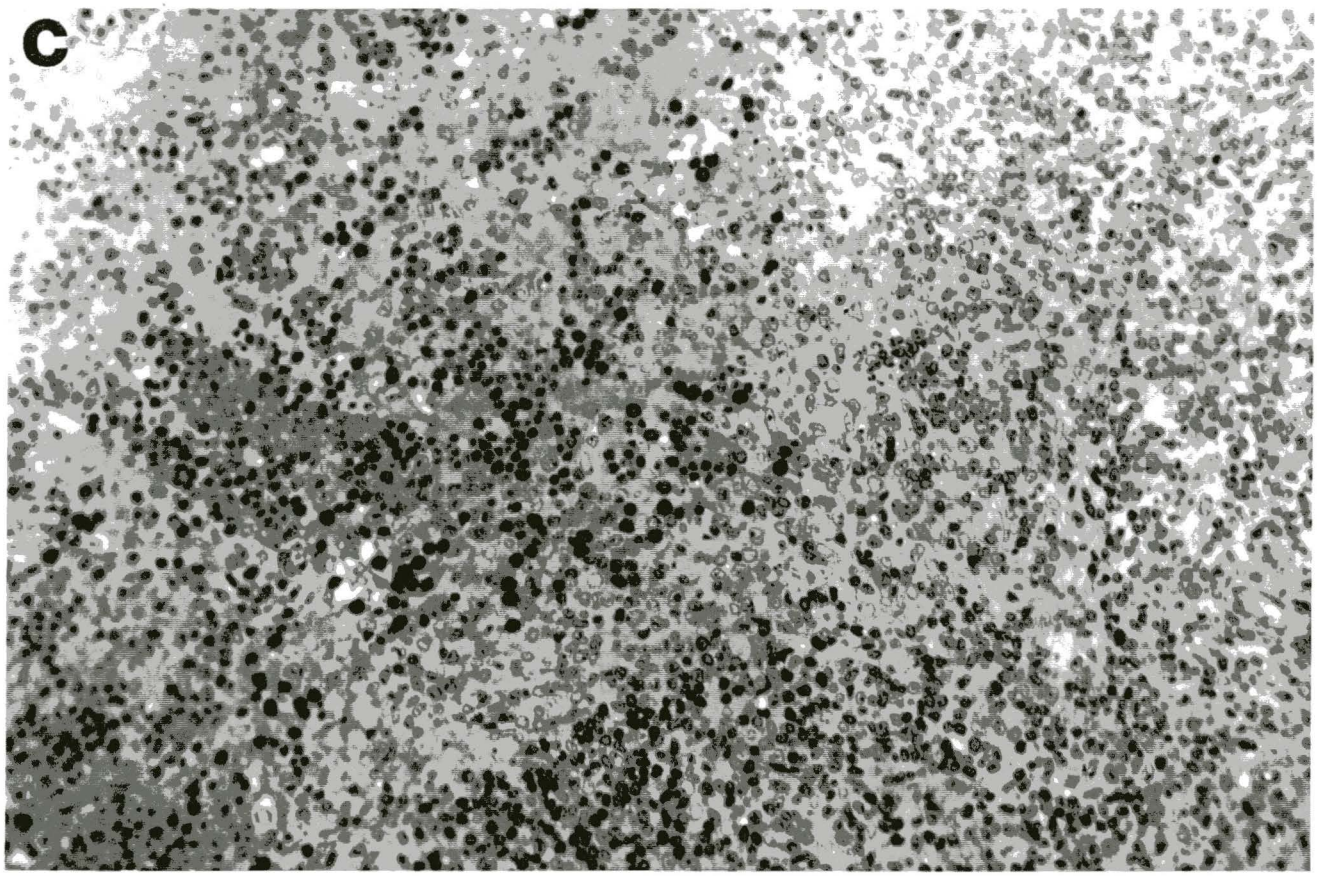
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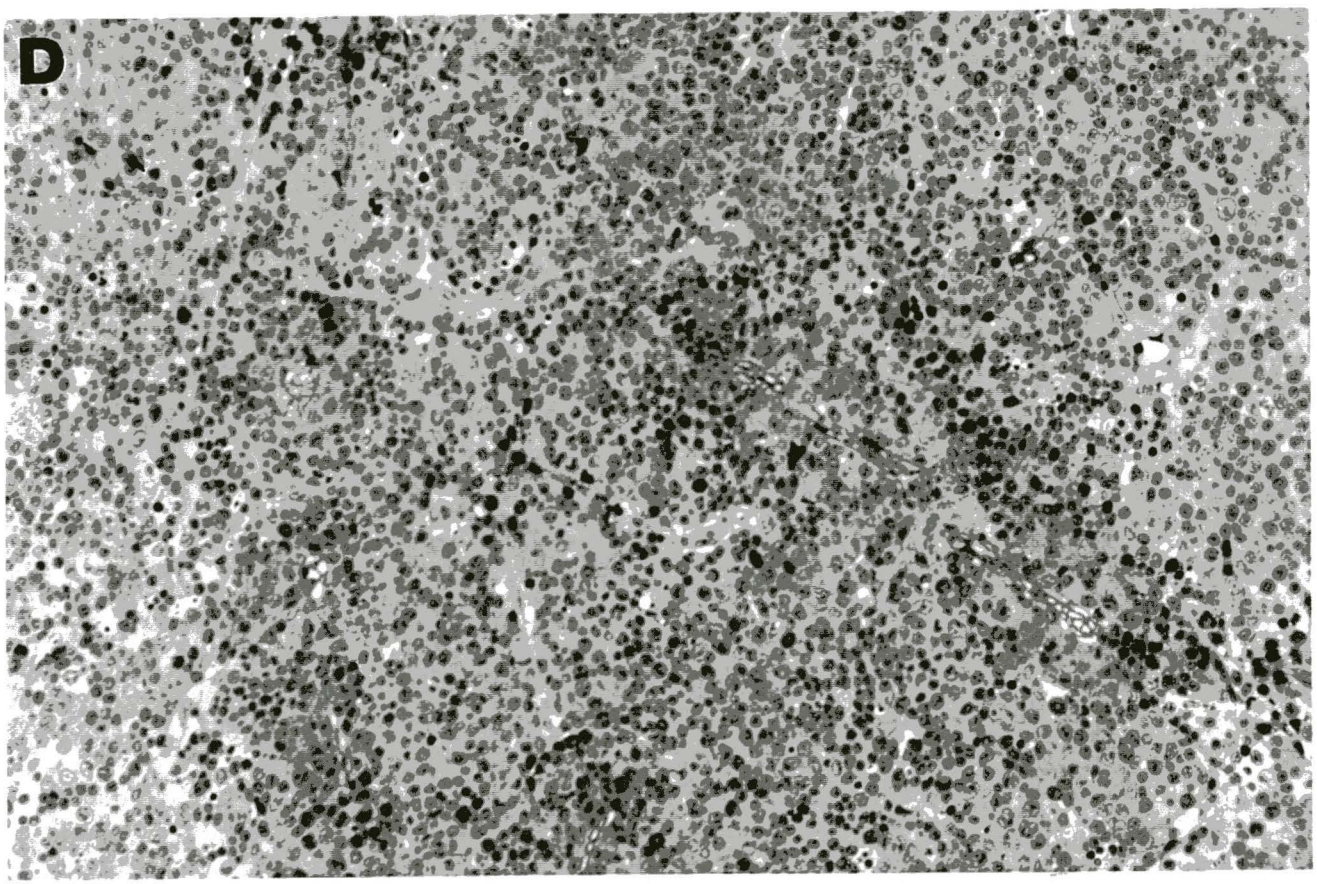
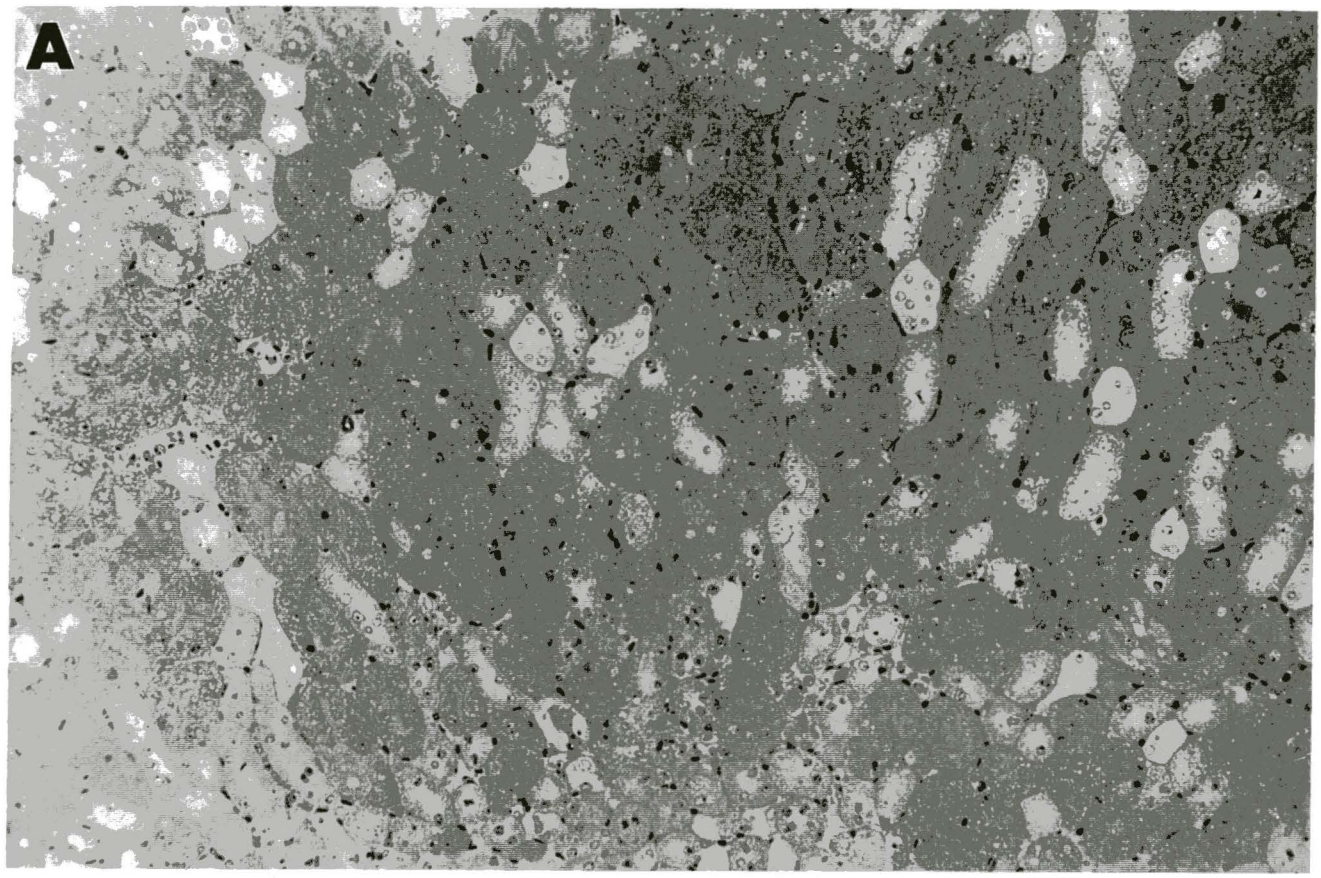

D

Figure 7. Toluidine blue stained sections of the kidney (A) normal control; (B) mutant control, (C) treated mutant 2 weeks post-implantation; and (D) treated mutatn 4 weeks after implantation. The renal tubular cells of the untreated mutant kidney (B) contain numerous distended lysosomes (arrows). At 2 weeks post-implantation, there was a reduction in storage lesions in large focal areas. At 4 weeks post-implantation, the distended lysosomes had reduced when compared to the untreated mutant.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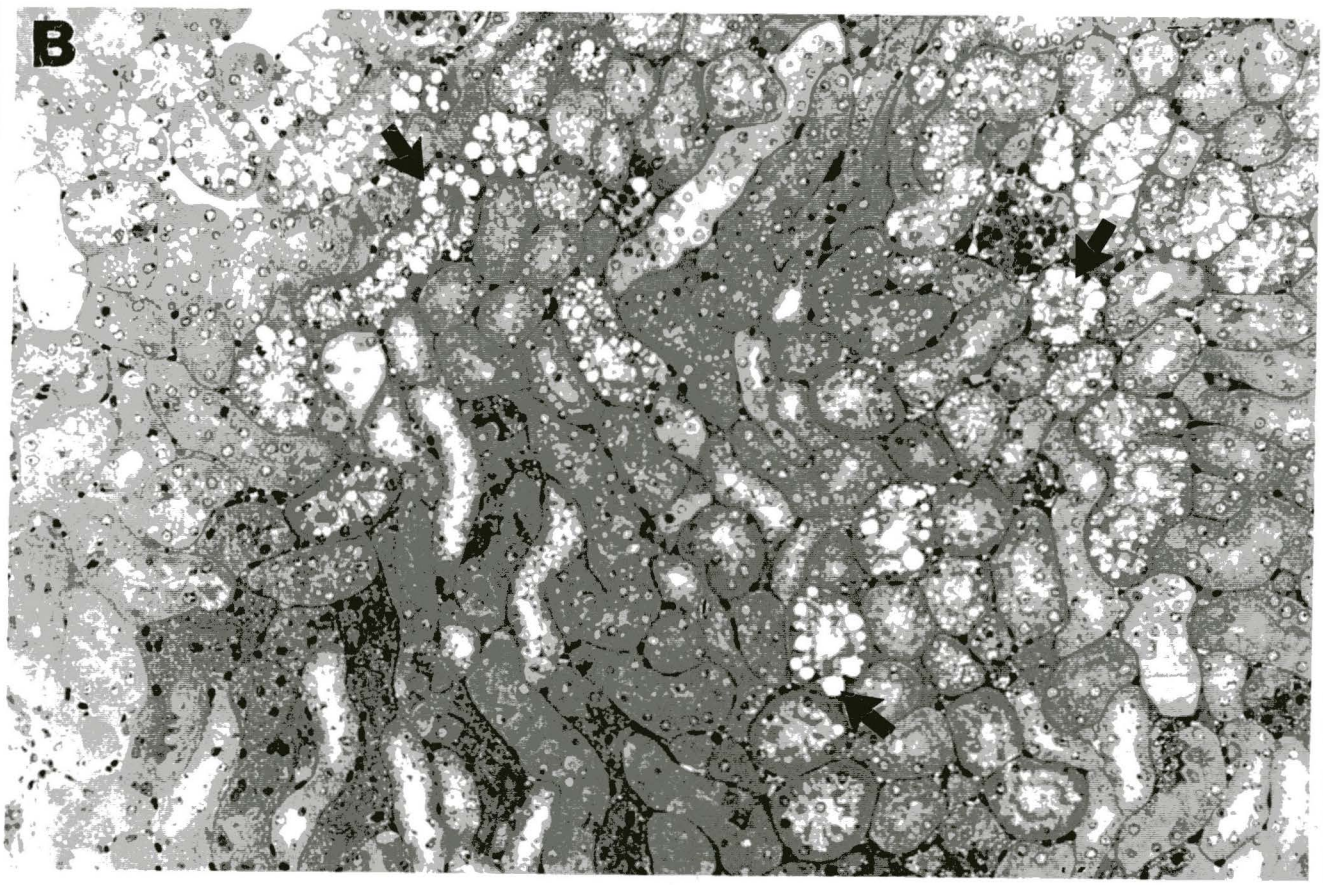
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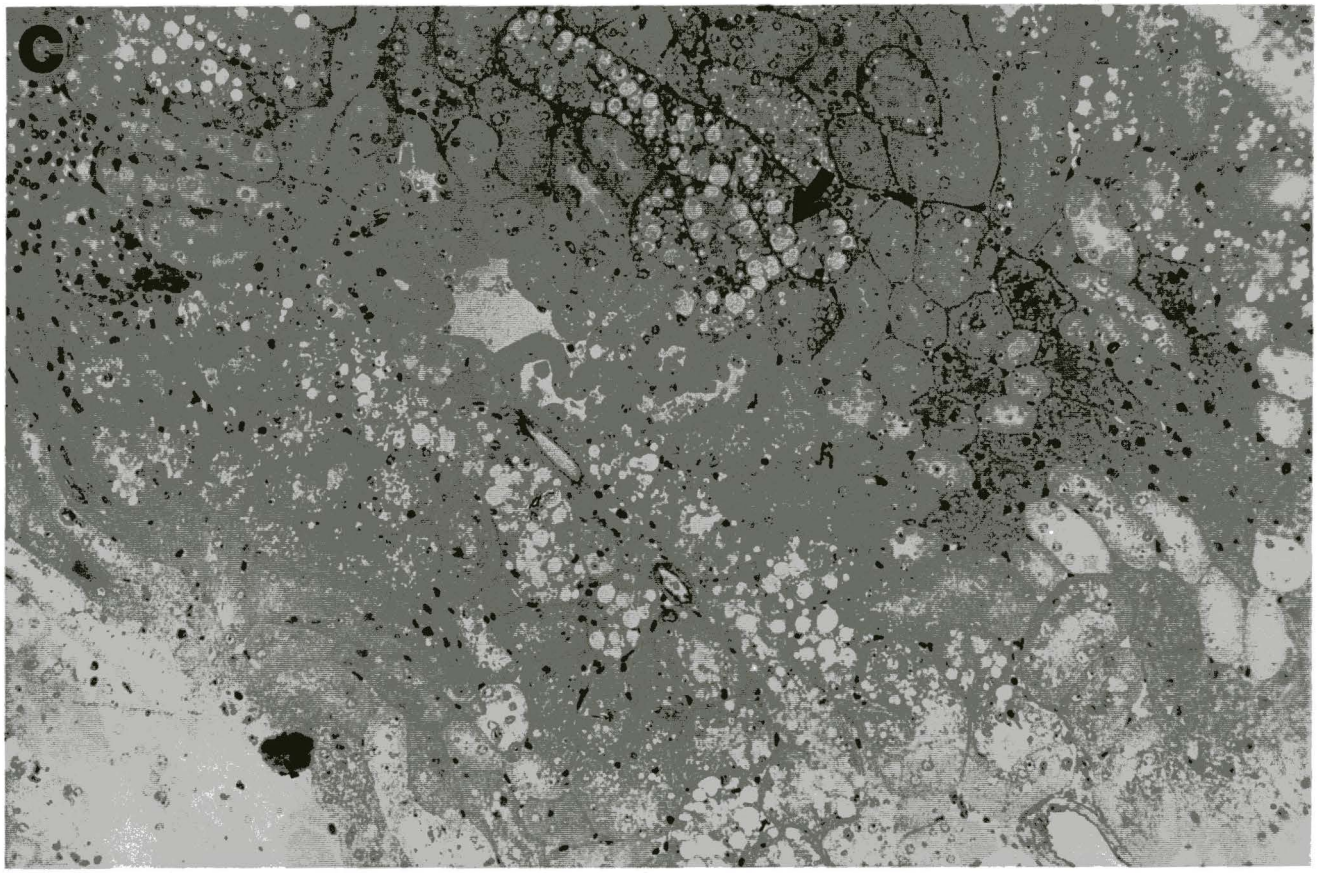
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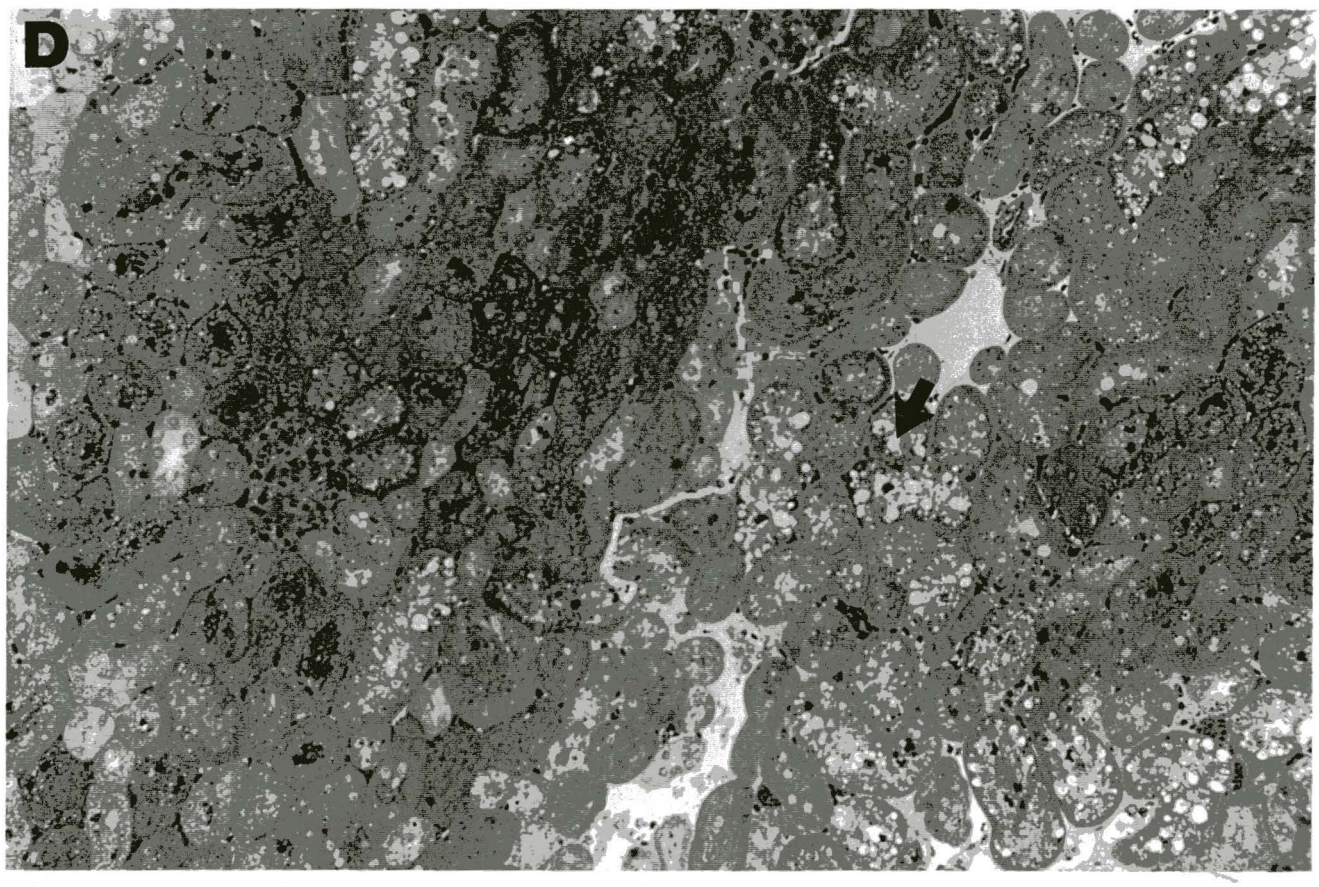
D

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5.0 DISCUSSION

Syngeneic bone marrow transplantation (BMT) and non-autologous somatic gene therapy using microcapsules were effective at correcting some of the pathobiology of murine MPS VII. For example, there was a reduction of lysosomal storage lesions in the liver, spleen and kidney. As well, the elevation of other lysosomal enzymes (β-hexosaminidase and α-galactosidase), seen in untreated mutants, had reduced as a result of the therapy. However, both therapies also had detrimental effects. BMT resulted in radiation induced toxicity in the CNS. As a consequence, neurological function was not restored in the mutant mice, and had significantly deteriorated in transplanted normal mice. Mutants treated with encapsulated fibroblasts engineered to secrete high levels of β-glucuronidase suffered high morbidity and mortality. In the discussion below, several important issues regarding the administration and evaluation of therapies will be examined with respect to BMT and gene therapy using microcapsules.

Bone marrow transplantation has been used for more than 20 years to treat various disorders, including some cancers. To date, more than 15 genetic diseases have been treated by allogeneic bone marrow transplantation. These include disorders in which the expression of the defect is manifested in haematopoietic and/or lymphoid cells such as Wiskott-Aldrich

syndrome, thalassaemia, and severe combined immune deficiency, as well as disorders that affect multiple tissues such as lysosomal storage disorders (Weinberg and Parkman, 1990).

Greater than 150 patients with lysosomal storage disorders have received allogeneic bone marrow with various degrees of success (Krivit et al., 1992b). Prior to the transplant, the patients are exposed to total body irradiation or treated with chemotherapeutic drugs (busulfan, cyclophosphamide, and/or cyclosine arabinoside) to ablate their bone marrow cells. Following the transplant, the haematopoietic system is reconstituted. In patients treated for lysosomal storage diseases, the metabolic correction is believed to occur by several mechanisms acting concurrently. First, there is a replacement of bone marrow cells deficient for a particular lysosomal enzyme with normal donor cells. This includes erythrocytes, lymphocytes, granulocytes and monocytes. The reticuloendothelial system (RES) is reconstituted from donor monocytes and consists of the Kupffer cells of the liver, the microglia of the brain, Langerhans cells of the skin, osteoclasts of bone and the macrophage system of the peritoneal, lung and lymphoid systems. Second, enzyme transfer from normal donor cells to enzyme deficient cells in the body occurs by cell-cell interaction or receptor mediated endocytosis of released enzymes. Last, for the mucopolysaccharidoses, intracellular substrate exchange between donor derived cells and enzyme deficient cells, is believed to facilitate correction (Yanagishita et al., 1992; Krivit et al., 1990; Ma, 1990).

Some of the lysosomal storage diseases treated with BMT include Niemann-Pick, Wolman's, Fabry, Gaucher, Hurler (MPS I), Hunter (MPS II), Sanfilippo (MPS III), and Maroteaux-Lamy (MPS VI) Because lysosomal storage diseases are progressive, the majority

of the BMT's have been performed on children ranging form 4 months of age to 12 years of age. In the cases where BMT was successful, the therapy either halted further progression of the disease, and/or resulted in partial correction (Hugh-Jones, 1986; Ladisch et al., 1986; Lipton et al, 1986; Rappeport et al., 1986; Whitley et al., 1986; Krivit et al., 1990; Krivit et al., 1992a; Krivit et al., 1992b; Tsai et al., 1992).

However, BMT, in itself, regardless of the disease being treated, is associated with significantly higher morbidity and mortality. Among the many treatment related complications, such as venoocclusive disease, infection, pneumonia, and organ failure, graft versus host disease is a major cause of severe morbidity and mortality (Pearson, 1986; Sanders, 1990; Furlong and Gallucci, 1994). Neurological complications occurring after BMT have also been reported. These include CNS infection, encephalopathy, peripheral nerve disorders, cerebrovascular accidents, seizures, coma, visual disturbances, musculoskeletal disorders, and confusion/disorientation (Wiznitzer et al., 1984; Patchell et al., 1985; Mohrmann et al., 1987; Sanders, 1990; Roskrow et al., 1992; Furlong and Gallucci, 1994). Research in this area is however limited, and most of the existing studies have relied on autopsy records or have been retrospective. For example, Patchell et al., 1985, reviewed 78 autopsy files of patients who had received BMT, and found CNS complications in 70% of the patients. Neurologic complications were the cause of death in five. Of the 105 patients in Mohrmann et al.'s study, 1987, who died after BMT, 32% had necrosis in significant regions of the brain and many had presented with encephalic haemorrhage and infarcts. Neurological complications arising in children after BMT have been reported by Wiznitzer

et al., 1984. Out of 57 patients, 26 suffered from CNS dysfunction, that included CNS toxoplasmosis, metabolic encephalopathy, cerebrovascular accident, and infection. In a recent study of 200 BMT patients, 52 patients experienced encephalopathy or coma, and 15 suffered from seizures. Confusion, disorientation, peripheral neuropathy and visual disturbance were also found (Furlong and Gallucci, 1994). The majority of the neurologic complications were attributed to the preparatory regimens of total body irradiation and chemotherapy (as well as immunosuppressive therapy) (Burger et al., 1981; Marcus and Goldman, 1984; Wiznitzer et al., 1984; De La Camara et al., 1991; Roskow et al., 1992; van den Berg et al., 1993; Furlong and Gallucci, 1994).

The neurological complications that arise from BMT, can conceivably result in behavioural abnormalities. This was observed in our study (Bastedo et al., 1994) where radiation induced toxicity in the CNS of transplanted mice resulted in behavioural abnormalities. It is still undetermined in humans whether impairment in cognitive function occurs after transplantation. This is largely due to the lack of adequate research in this area. For example, Sanders, 1990, mentions "..casual reports from parents..." that children pretreated with cyclophosphamide did not have problems in school. However, children pretreated with total body irradiation had learning difficulties after the transplant (Sanders, 1990). Of the 57 children who received BMT, in Wiznitzer's 1984 review, only 3 children underwent neuropsychologial testing before and after the procedure. Two additional children were tested after BMT, and only because of their poor school performance. These children

were found to have deficiencies in visual-motor skills, auditory skills and motor function. In one child, the neuropsychological dysfunction predated BMT. (Wiznitzer et al., 1984).

Of the children who received BMT for a lysosomal storage disease, a wide range of intellectual outcome was observed. In some, behavioural problems worsened and intelligence quotients declined after BMT (Krivit et al., 1990; Krivit et al., 1992b). In others, BMT either stabilized neurological deterioration for some time (Hugh-Jones, 1986; Lipton et al., 1986; Whitley et al., 1986; Krivit et al., 1990; Krivit et al., 1992b) or resulted in neurological improvement (Krivit et al., 1990; Krivit et al., 1992b). For the lysosomal storage diseases with neurological involvement, the evaluation of neurological function after BMT is complicated by several factors. Although the severity of CNS involvement and the age at which BMT is performed can affect the clinical outcome, there are two other factors which make evaluation of therapy difficult to assess. First, as just discussed, neurological complications can arise as a result of the transplantation procedure itself. It has been shown to cause mild (confusion, disorientation, visual disturbances) to severe (CNS dysfunction, encephalopathy) conditions. Whether this results in cognitive impairment is unclear because *i*) the pretreatment regimens used vary form hospital to hospital, and *ii*) little attention has been payed in this area. Secondly, for these diseases, a crucial factor as to whether the CNS pathology improves after treatment is related to whether the enzyme reaches the CNS. It is believed that donor derived monocytes cross the blood brain barrier and infiltrate the CNS (Ladisch et al., 1986; Hoogerbrugge et al., 1988; Krivit et al., 1990). Once in the CNS the monocytes aid in reversing the disease pathology by the mechanisms discussed earlier.

When neonatal MPS VII mice were treated with BMT, β-glucuronidase activity was detected in the brain (Sands et al., 1993; Bastedo et al., 1994b). As a result, CNS pathology had improved. It was clear, however, from our study, that the behavioural abnormalities typical of mutant mice had not improved (Bastedo et al., 1994b). Because normal littermates who received the same procedure demonstrated a functional deterioration in behaviour, and because CNS damage was shown, it was concluded that the transplantation procedure was detrimental. In this case, total body irradiation resulted in radiation induced toxicity to the CNS. This study shed some light on the importance of behavioural tests when evaluating the efficacy of therapy. It also implied that neuropsychological testing in humans undergoing BMT, as well as other therapies, should be strongly considered as a means to further evaluate therapeutic outcome.

Although BMT has become a life saving procedure for hundreds of children and adults, some ethical concerns are raised. For patients with malignancy and aplastic anemia, the major consideration whether BMT is necessary is survival with freedom from disease. For patients with lysosomal storage diseases with neurological involvement, survival may play a smaller role in determining whether they receive BMT. The quality of life of the patients and their families must be considered, especially if BMT improves most of the physiological conditions in the body except for the CNS. This concern is not limited to BMT, but to other procedures, and includes enzyme replacement therapy, as well as gene therapy.

Enzyme replacement therapy on neonatal MPS VII mice results in a complete reversion of mutant CNS pathology (M.S. Sands, personal communication)[12]. It will be interesting to verify whether this therapy also results in neurological improvement. When MPS VII mice were implanted with encapsulated cells engineered to secrete high levels of β-glucuronidase, partial biochemical correction and an improved disease pathology was observed. However, β-glucuronidase activity in the brain was not significantly different from that of the mutant controls. Whether the small amounts detected in some of the treated mutants resulted in neurological improvement was not ascertained as the mice became ill during the course of the experiment.

At three and a half weeks post implantation, the treated mutant mice presented with haemorrhagic ascites, appeared dehydrated, and were slow to respond. When blood samples were collected and centrifuged to collect plasma, the proportion of red blood cells to plasma fluid was reduced by one half. In humans, various clinical conditions can cause the accumulation of serous fluid in the peritoneal cavity (ascites). Ascites may develop from an inflammation of the peritoneum (peritonitis) which may be caused by a perforation of a viscus, strangulating intestinal obstruction, pelvic inflammatory disease, pancreatitis, trauma or foreign bodies. Systemic diseases such as heart failure, nephrotic syndrome, hypoalbuminemia or intra-abdominal causes such as carcinomatosis and tuberculous peritonitis, can also result in peritoneal edema. As well, liver disease, renal failure,

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hypothyroidism, bacterial toxins, obstruction of flow in portal vein or in lymphatic drainage, and inflammatory conditions can contribute to ascites. In some of these cases the ascites fluid is accompanied by cells from the blood, a condition referred to haemorrhagic ascites. This haemorrhaging results in hemodilution and a drop in red blood cell count (the latter was observed in the treated mutants). If the condition is not treated in humans, multisystem failure can occur leading to death. Severe dehydration (also observed in the treated mutants), and electrolyte disturbances result because of the loss of fluids into the peritoneal cavity. Renal failure, liver failure and adult respiratory distress syndrome follow. As well, peristalsis halts, leading to starvation (Berkow and Fletcher, 1992; Thomas, 1993).

In the treated mutants, haemorrhagic ascites may have resulted from the microcapsules obstructing normal lymphatic drainage. This, however, can be discounted as normal mice were able to tolerate the same volume of capsules. One could then argue that because the bodies of the mutant mice are more compact than the normals, the same volume of microcapsules could cause an obstruction in lymphatic drainage. However, implantation of as much as 18 mL of microcapsules into the peritoneal cavity of younger mice of a different strain did not result in peritoneal edema (G. Hortelano, personal communication)[13]. This implies that the microcapsules within the peritoneal cavity of the mutant mice did not cause an obstruction in the lymphatic drainage.

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The recombinant 2A-50 cells secrete large amounts of β-glucuronidase (Grubb et al., 1993). Thus, there was the possibility that excessive amounts of protein within in peritoneal cavity were toxic. There are two reasons why this may not be the cause of illness in the treated mutant mice. First, the encapsulated cells implanted into the normal mice had a secretion rate similar to the ones implanted into mutants; and as mentioned before, normal mice tolerated the microcapsules well. Secondly, 10-20 fold of normal β-glucuronidase activity has been either expressed in transgenic mice (Kyle et al., 1990) or administered to both normal and mutant mice (M. Sands, W.S.Sly, and E. Birkenmeier, personal communication)[14] without toxic effects. Bacterial infection from *Clostridum perfringens*, α- or β-haemolytic streptococci or meningococci can cause haemorrhagic ascites as well as a drop in red blood cell count (Berkow and Fletcher, 1992). However, a wide spectrum bacterial analysis of the peritoneal fluid from the treated mutants was negative. This ruled out bacterial infection that may have been transferred from tissue culture or from the implantation procedure itself.

Inflammatory responses within the peritoneal cavity can also result in haemorrhagic ascites. The peritoneum lining is composed of a single layer of mesothelium cells as well as bone marrow derived macrophages (Krivit et al., 1990; Moore, 1992). Macrophages can function as antigen-presenting cells (APC) during an immune response. Antigens (β-glucuronidase) are taken up by phagocytosis or receptor mediated endocytosis, proteolytically

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cleaved in the lysosomes and presented on the cell surface with class II MHC molecules. T-helper (T_H) cells recognize and interact with the antigen-MHC complexes on the macrophages, and become activated. Activation of T_H cells results in the secretion of cytokines such as IL-2, IL-5, lymphotoxin, IFN-γ, and TNF which recruits and activates other inflammatory cells of the immune system. Bidirectional "talking" occurs between the cells of the immune system, in that, activated T cells can also activate macrophages (Abbas et al, 1991; Golub and Green, 1991).

Mice homozygous for the *gus*^{mps} mutation have a greater than 200 fold reduction in β-glucuronidase mRNA levels and consequently, very little or no truncated β-glucuronidase protein (Birkenmeier et al., 1989; Sands and Birkenmeier, 1993). It is likely then, that an immune response against β-glucuronidase occurred in the treated mutant mice. This hypothesis of an immune response against β-glucuronidase is supported by enzyme replacement studies on neonatal mice. When neonatal MPS VII mice were given large weekly doses of purified mouse β-glucuronidase for a period of 6 months, they responded extremely well to the treatment. Lysosomal storage pathology was reversed in all the organs, including the brain. After 6 months, when the doses were given monthly, some of the mice suffered from what seemed like an anaphylactic reaction; some even died. Antibodies against mouse β-glucuronidase were detected in the serum. The physiology of this reaction and its

mechanism are now under study (M. Sands, W.S. Sly and E. Birkenmeier, personal communication)[15].

Once lymphocytes are activated, the function of the effector phase that follows normally leads to the elimination of the antigen. This is accompanied by local and systemic injury to normal self tissues. For example, activated macrophages release hydrolytic enzymes and toxic oxygen metabolites which results in tissue injury. Macrophages also secrete cytokines and other inflammatory mediators which in turn recruit other inflammatory cells (Abbas et al., 1991; Golub and Green, 1991). Thus, an immune response initiated within the peritoneal cavity, which has a large population of macrophages, could conceivably result in tissue damage to the peritoneum lining. Tissue damage, as well as the attendant inflammation would lead to the haemorrhagic ascites observed in the treated mutants (Berkow and Fletcher, 1992; Thomas, 1993).

The pathologic side effects of tissue injury and inflammation are normally controlled because immune responses are self-regulated and abate as the foreign antigen is eliminated. If the antigenic stimulation persists, however, the response increases in magnitude and consequently results in severe tissue injury (Abbas et al., 1991; Golub and Green, 1991). If the cells within the microcapsules are immuno-isolated from the effector mechanisms, then they would continue to secrete antigen (β-glucuronidase). When the microcapsules were retrieved, the cells did in fact demonstrate a secretion rate comparable to the encapsulated

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cells that were maintained *in vitro* for the same duration. Thus, it is likely that the severity of the inflammatory response can be attributed to the chronic antigen stimulation by the encapsulated cells *in vivo*.

The pathology of the retrieved capsules and adhering tissue indicates that the treated mutants suffered from a chronic immune response. During persistent antigen stimulation, macrophages undergo morphological changes and secrete additional cytokines and growth factors. The growth factors (PDGF and TGFβ) stimulate the proliferation of fibroblasts and augments collagen synthesis, both of which contribute to fibrosis. Morphological changes within the macrophages include an increase in cytoplasm and cytoplasmic organelles. This gives the cells an epitheloid appearance when stained with Hematoxylin and Eosin (H&E). Some macrophages may even fuse to form multinucleate giant cells (Abbas et al., 1991; Golub and Green, 1991). Approximately one third of the capsules retrieved from the treated mutants were found adhered to the peritoneum or mesentery and were surrounded by inflammatory cells. A large number of the cells resembled chronically activated macrophages in H&E stained sections. Multinucleate giant cells and lymphocytes were also observed. Sections of the retrieved capsules and adhering tissue were stained immunologically for histiocytic (macrophage) markers. However, we were unable to confirm that the surrounding cells were indeed chronically activated macrophages as none of the antibodies directed against human histiocytic markers (muramidase, α-1-antitrypsin, and α-1-antichymotrypsin) cross reacted with the murine tissue.

It is also possible that some of the tissue damage that led to the haemorrhaging resulted from the effects of cytotoxic T cells (T_{CTL}). T_{CTL}'s recognize and respond to self class I MHC molecules plus antigen presented by target cells. All nucleated cells express class I MHC molecules on their cell surfaces. Hence, it is possible that β-glucuronidase endocytosed by cells was, in some cases, processed and presented with class I MHC molecules on the cell surfaces. Upon recognition by activated T_{CTL}'s, these cells would become the target for lysis. When part of a pancreas and the adhered microcapsules were sectioned and stained with H&E, inflammation at the periphery was observed (Appendix C). Unfortunately, this was the only organ retrieved at 4 weeks post-implantation that was fixed and stained with H&E. It would have been interesting to see if there was inflammation at the periphery of the other organs such as the kidney, liver, and spleen. Vitadello et al., 1994, in a recent experiment, injected plasmids encoding the β-galactosidase gene into the thigh muscles of mice. After one month, there was a dramatic loss of transfected DNA, and mononuclear cell infiltrates were found localized around β-galactosidase positive muscle segments. It was suggested that a specific cytotoxic T cell immune response against the foreign gene product was responsible for the observed decrease in expression (Vitadello et al., 1994). Although these experiments differ in the method of enzyme delivery (microinjection of DNA versus implantation of encapsulated cells), the gene products are either expressed within the cells or endocytosed, making the above hypothesis a possibility.

In addition to an immune response against β-glucuronidase, there may have been a reaction against the 2A-50 cells themselves. Imperfections in the capsular membrane can be

caused by integration of cells into the membrane matrix during its fabrication. If these cells perforate the membrane after implantation, or become exposed to the surface of the microcapsules, it may lead to a cell mediated immune response. This response has been observed in encapsulated hapatocytes (Chang, 1992). However, because normal mice implanted with encapsulated 2A-50 cells did not mount an inflammatory response, an immune response against the cells may not have occurred. Western blot analysis in which cell surface proteins are incubated with sera from implanted mutants would confirm this.

To confirm that an immune response against β-glucuronidase occurred, and that the cells themselves were immuno-isolated, several experiments need to be done. Firstly, the presence of anti-β-glucuronidase antibody can be assessed by a Western Blot. Secondly, antibodies against murine histiocytic markers would provide a means of confirming the identity of the cells adhered to the capsules and mesentery. Cytokines such as IFN-γ are known to induce the expression of class II MHC molecules on non-lymphoid cells. Thus, detection of class II MHC molecules on cells that normally do not express these molecules constitutively, would indicate that T cells had been activated in the immediate vicinity. Lastly, the experiments need to be repeated using better controls. One control, for example, would involve the implantation of encapsulated cells which do not secrete β-glucuronidase. If no inflammatory response is observed, then one could conclude that *i*) the cells are indeed immuno-isolated and *ii*) the inflammatory response observed in the treated mutants was due to the foreign β-glucuronidase. Injection of empty microcapsules as well as naked 2A-50 cells would also serve as good controls to test that the microcapsules are biocompatible and

provide immuno-isolation. Immuno-isolation can also be determined by incubation of both naked and encapsulated cells in antiserum raised against 2A-50 cells. If the naked 2A-50 cells lyse as a consequence of cytotoxic activity and the encapsulated cells do not, it would show that the capsular membrane provides effective immuno-isolation. Alternatively, all of these experiments can be repeated in the C3H/RiJ mice which have very low levels of β-glucuronidase, but no clinical symptoms (Hoogerbrugge et al., 1987). Since this animal model would recognize β-glucuronidase as "self" then and antibody or inflammatory response would not be observed after implantation of encapsulated 2A-50 cells.

If we agree that an inflammatory response against β-glucuronidase was responsible for the haemorrhagic ascites and mortality, then how do we correlate this with Moullier et al.'s, 1993, experiments? To review, Moullier et al., (1993), transfected human β-glucuronidase cDNA into syngeneic fibroblasts, incorporated the cells into synthetic fibres to form neo-organs, and implanted the neo-organs into the peritoneal cavity. Although antibodies against the human β-glucuronidase were detected, inflammation within the peritoneal cavity was not. So, how is it that an immune response against mouse β-glucuronidase mounts an inflammatory response but an immune response against human β-glucuronidase does not? In the former study, the β-glucuronidase was being secreted from non-autologous cells, a situation which under normal conditions would provoke graft rejection and inflammation (Nakamura, 1974; Abbas et al., 1991; Golub and Green, 1991). However, no response against the encapsulated cells was observed in implanted normal mice. It stands to reason then, that the encapsulated non-autologous cells are immuno-isolated and

thus not the cause of inflammation in the mutant mice. Studies on the induction of tolerance in animals have shown that extremes of antigen concentration can induce a state of tolerance. High and very low doses of antigen are toleragenic (Golub and Green, 1991). This tolerance differs from "central tolerance", which is tolerance to "self", and is induced in developing B and T cells. "Peripheral tolerance", on the other hand, can be induced in adults after lymphocytes have matured, and is mediated by clonal anergy. The encapsulated 2A-50 cells secreted 25-84 fold more β-glucuronidase, as determined by activity, than the neo-organ implants (Moullier et al., 1993). Thus, in the latter study, T cell tolerance may have been induced as a result of low β-glucuronidase concentration. In our study and the enzyme replacement studies (mentioned above), the higher concentration of β-glucuronidase secreted by encapsulated cells or administered intravenously, resulted in an immune response against the enzyme. This hypothesis can be tested by inducing tolerance in neonatal mice by injecting β-glucuronidase and then implanting the microcapsules when they become adults. If tolerance plays a role, then no response against β-glucuronidase should occur after implantation.

As discussed in the introduction of this thesis, there are many advantages to implanting encapsulated cells as a strategy for non-autologous somatic gene therapy. First, the costs of engineering patient-specific cells lines can be reduced by engineering allogeneic "universal" cell lines. These cells can be isolated from the host's immune system with a biocompatible and perm-selective membrane. Furthermore, efficient and sustained delivery of the recombinant gene product has been clearly demonstrated. Lastly, because the

allogeneic cells are isolated, the requirement for immunosuppressive drugs such as cyclosporin, which are associated with many side effects, are alleviated (Bennett and Norman, 1986). This therapeutic strategy, however, may have its limitations, especially for diseases in which the mutation totally ablates protein expression. In this case, the recombinant gene product may initiate an immune response. This situation was observed in mutant MPS VII mice that received either enzyme replacement therapy (M. Sands, W.S. Sly, E. Birkenmeier, personal communication)[16] or that were implanted with encapsulated β-glucuronidase secreting fibroblasts.

There are very few cases in the literature that address the possibility that the "corrective" gene product of a therapy may be treated as a neo-antigen by the host. For example, in the reports on enzyme augmentation therapy for Gaucher's disease, there were no assessments of *i*) whether antibodies against the introduced enzyme were generated and *ii*) whether the patients' mutations resulted in a complete absence of protein or a protein that was either unstable, or had kinetic/functional abnormalities (Beutler et al., 1992; Fallet et al., 1992; Zimaran et al., 1993). In the PEG-ADA trials, IgG antibodies were detected against the bovine ADA, in 60% of the patients. Tolerance was induced in one patient, and enhanced enzyme clearance was observed in two patients; both of these interfered with the therapy. An autoimmune phenomenon developed in two other patients, leading to the death of one. Whether this was provoked by antibody mediated responses or immune

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dysregulation, which can occur during the early periods of therapy, remained unclear (Hershfield et al., 1993).

In some animal studies using somatic cell gene therapy, it was found that possible immune responses against foreign proteins were either not addressed (Bar and Leiden, 1991; Dhawan et a., 1991; Stratford-Perricaudet et al., 1992; Li et al., 1993; Bansal et al., 1994; Zatloukal et al., 1994) or immuno-incompetent mice were used (Dai et al., 1992; Salvatori et al., 1993). In a recent study however, where reporter gene constructs were injected into the thigh muscles of mice, a possible immune reaction against the reporter gene product was thought to have occurred (Vitadello et al., 1994). James Wilson's studies on *ex vivo* hepatic gene therapy for familial hypercholesteremia were one of the few to show concern about potential immune responses to foreign proteins. When Watanabe Heritable Hyperlipidemic (WHHL) rabbits were used to develop an approach to *ex vivo* hepatic gene therapy, the presence of antibodies to the rat low density lipoprotein receptor (LDLR) were analyzed (Chowdhury et al., 1991). No antibodies against the LDLR were found, indicating that the presence of the expressed, but dysfunctional endogenous LDLR suppressed an immune response to the introduced functional LDLR (Chowdhury et al., 1991; Yamamoto et al., 1986). A patient (FH1) homozygous for familial hyperholesterolemia was also treated using the same approach for the WHHL rabbits. Because the mutation in FH1 leads to the expression of a dysfunctional protein, it was not surprising that a Western blot analysis failed to detect antibodies to the recombinant LDLR protein (Grossman et al., 1994). Thus the

possibility that a therapeutic gene product will be recognized as a neoantigen and its possible long term effects need to be further evaluated.

6.0 CONCLUSIONS

1) Bone marrow transplantation (BMT) has been used to treat many genetic disorders including some lysosomal storage diseases. However, BMT is associated with significantly higher morbidity and mortality. Among the many complications that arise after therapy, neurological complications contribute significantly to the morbidity and mortality. Whether these complications result in cognitive impairment in humans is still unclear. Neonatal mice treated with BMT suffered from radiation induced toxicity to the CNS. Subsequent behavioural analysis demonstrated that these neurological complications resulted in cognitive impairment.

2) When encapsulated recombinant cells were implanted into rodents, the cells were immuno-isolated from the host's immune system. At the same time, it was shown that the recombinant gene products were able to diffuse out of the microcapsules. In my study, the recombinant gene product (β-glucuronidase) functioned to reverse the MPS VII phenotype. During the course of the study, urinary GAG/creatinin content decreased and a reduction in storage lesions was observed in the kidney, liver and spleen. However, at three and a half weeks, the mice became seriously ill and were speculated to mount an immune response against β-glucuronidase.

3) This thesis illustrates several important points regarding the administration and evaluation of therapies. Firstly, for any disease with neurological involvement, it is important to monitor neurological function as a result of a therapy. Secondly, for any therapy that may inflict CNS damage, regardless of whether the disease state has CNS involvement, the monitoring of neurological function provides an additional parameter in its evaluation. Lastly, for diseases in which the mutation totally ablates protein expression, the introduction of a functional gene product may initiate an immune response against that gene product. Hence the genotype of a patient may play a primary role in determining whether a therapy will be efficacious.

APPENDIX A

SOLUTIONS

2 M $CaCl_2$

$CaCl_2$ $6H_2O$	10.8 g
H_2O	to 20 mL
Filter sterilize	

2% CHES stock solution

2-(N-Cyclohexylamino)ethanesulfonic acid (CHES) (Sigma)	10 g
0.9 % NaCl	to 500 mL
pH to 8.2 with NaOH	
Filter sterilize	

0.04 M Citrate Phosphate Buffer

0.1 M Citric acid	25.2 mL
Di-sodium hydrogen orthophosphate	24.8 mL
H_2O	to 200 mL

12mM Citrate-20mM Phosphate

Do-sodium hyrogen orthophosphate	1.42 g
Citric Acid H_2O	1.15 g
pH to 4.4 with 0.1 M Citric Acid	
H_2O	to 500 mL

10 x Citrate Saline

Potassium chloride	50 g
Sodium citrate	22 g
H_2O	to 500 mL

DMB 10 x Stock Solution

95% Ethanol	10 mL
DMB dissolved in ethanol	69.5 mg
0.2 mM Sodium formate, pH 3.5	to 1 L

2 x HEPES - buffered saline (HBS)

Final Concentration	
NaCl	280 mM
KCl	10 mM
Na_2HPO_4 $2H_2O$	1.5 mM
Dextrose	12 mM
HEPES	50 mM

Homogenization buffer

Final Concentration	
Tris Cl, pH 7.5	20 mM
NaCl	140 mM
β-Mercaptoethanol	10 mM
Saponin	0.25%

2% Gluteraldehyde

4% Gluteraldehyde in H_2O	50 mL
0.2 M Sodium cacodylate buffer	50 mL

Luria Broth

Tryptone	10 g
Yeast extract	5 g
NaCl	5 g
H_2O	to 1 L
Autoclave	

LB-Ampicillin Plates

Tryptone	10 g
Yeast extract	5 g
NaCl	5 g
Agar Bacteriological (Gibco)	15 g
H_2O	to 1 L
Autoclave	
Ampicillin (when solution drops to 55°C)	50 µg/mL

1% Osmium Tetroxide Buffer

2% Osmium tetroxide in H_2O	50 mL
0.2 M Sodium cacodylate buffer	50 mL

0.05% PLL solution

Poly-L-Lysine (PLL) (Sigma P-7890)	2.5 g
0.9 % NaCl	to 500 mL
Filter sterilize	

13% PEG

PEG 8000 (BDH)	1.3 g
NaCl	0.935 g
H_2O	up to 10 mL
store at 4°C	

1.5% Potassium-Alginate

Potassium Alginate (Kelco 17703A)	3 g
0.9% NaCl	to 200 mL
Stir overnight slowly	
Filter sterilize	

Reagent A

Sodium carbonate (Na_2CO_3)	20 g
0.1 M NaOH	to 1 L

Reagent B

Cupric sulphate ($CuSO_4 \cdot 5H_2O$)	0.5 g
NaOH	1-2 pellets
1.0% Sodium tartrate	to 100 mL

Reagent C

Reagent A	50 mL
Reagent B	1 mL

RNase A in TE

RNase A	10 mg
Dissolve in 10 mM Tris, pH 7.5 and 15 mM NaCl	
Boil at 100°C for 20 min	
Cool to room temperature	
Dilute 5 µL of this solution in 5 mL TE.	

0.1M Sodium Acetate Buffer (pH 4.5)

1 M Acetic Acid	25 mL
1 M Sodium acetate	34.8 mL
H_2O	to 600 mL

0.055 M Sodium Citrate

Sodium Citrate dihydrate	8.08 g
0.9% NaCl	to 500 mL

Sodium Cacodylate Buffer

Sodium cacodylate	42.8 g
H_2O	to 1000 mL
pH to 7.4 with 1N HCL	

Solution I

0.5 M glucose	10 mL
1 M Tris HCl, pH 8.0	2.5 mL
0.5 M EDTA	2 mL
H_2O	to 100 mL

Solution II

20% SDS	1 mL
1 N NaOH	4 mL
H_2O	up to 20 mL

Solution III

Potassium acetate	29.4 g
Formic acid, glacial	11.5 mL
h_2O	to 100 mL

Spurr Resin - Firm

Vinylcyclohexane dioxide (ERL)	20 g
Diglycidyl ether of polypropylene glycol (DER)	12 g
Nonenyl Succinnic Anhydride (NSA)	52 g
Dimethylaminoethanol (DMAE)	0.8 g

1 x TE (pH 8.0)

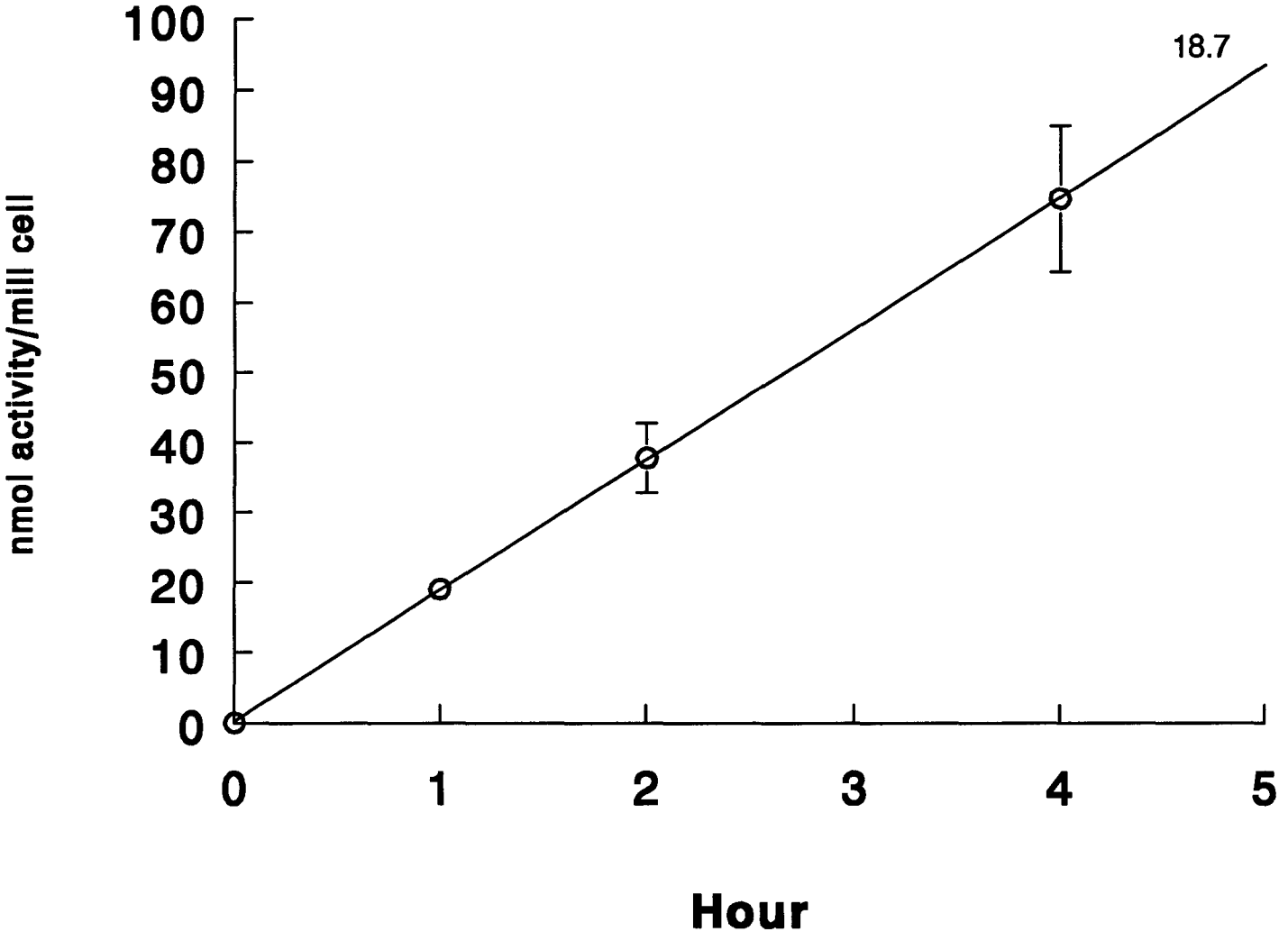
1 M Tris Cl (pH 8.0)	1 mL
0.5 M EDTA (pH 8.0)	0.2 mL
H_2O	to 100 mL

0.125% Trypsin

10 x Citrate Saline	10 mL
Trypsin (Sigma, T0134)	0.125 g
H_2O	to 100 mL
Filter sterilize	

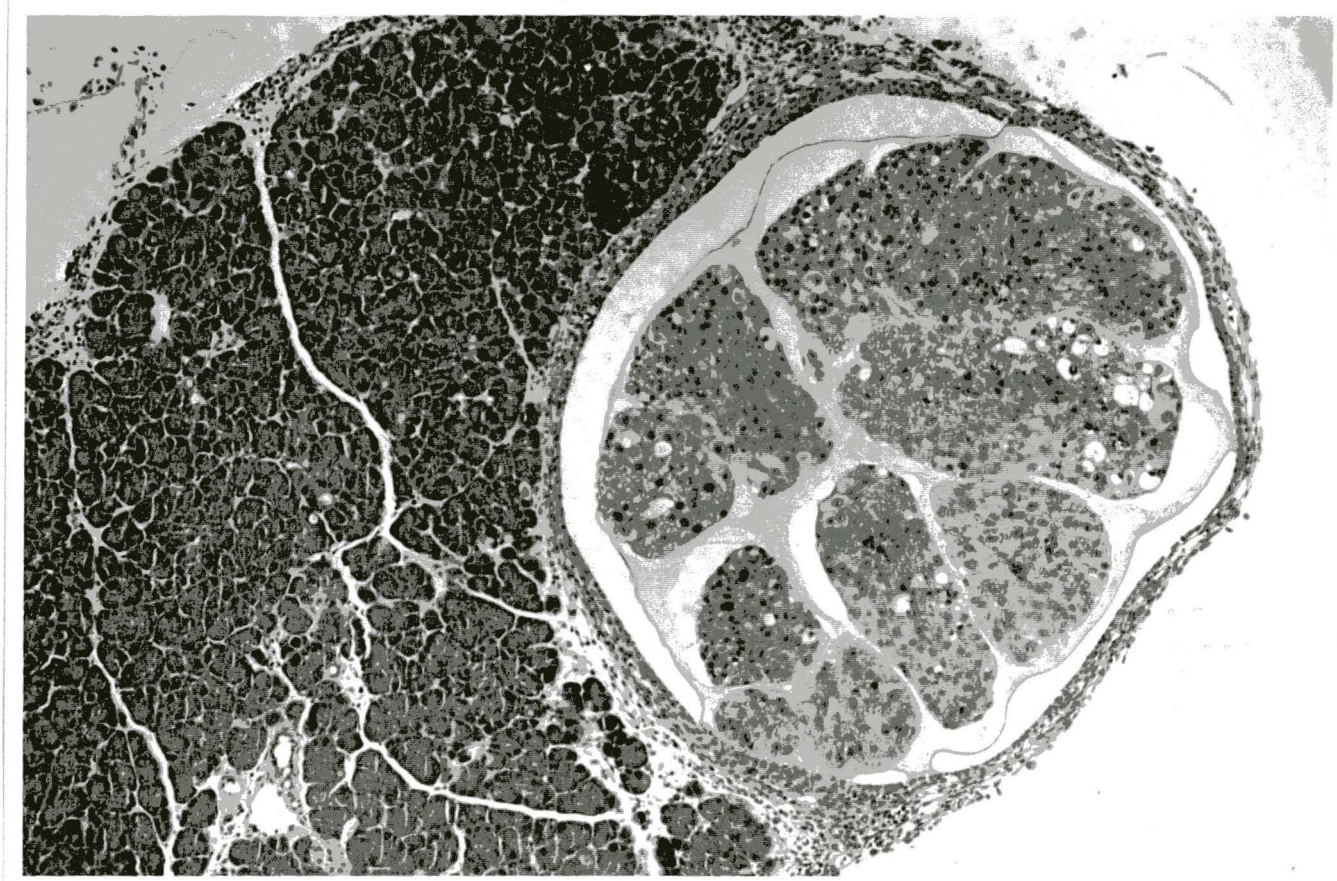
APPENDIX B

C2C12 myoblasts were cotransfected with the vectors pXBNaN and pSV2neo. pSV2 neo contains the neomycin resistance gene and pXBNaN contains the mouse β-glucuronidase cDNA downstream from the SV40 late promoter. The secretion rate for the myoblast clone with the highest secretion rate is shown. All samples for the secretion analysis were taken in triplicate; the data are the mean ±STD. The secretion rate was calculated from the slope of the best fit line and equaled 18.7 nmol of β-glucuronidase activity/h/10^6 cells.


100
90
80
70
60
50
40
30
20
10
0
nmol activity/mIII cell
18.7
0
1
2
3
4
5
Hour

APPENDIX C

A H&E section of an inflamed pancreas retrieved from a mutant mouse 4 weeks after implantation with encapsulated fibroblasts secreting β-glucuronidase. A microcapsule is observed adhered to the periphery of the pancreas. Surrounding the capsule and the periphery of the pancreas are white blood cells. Original magnification 63x.



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