IMMUNO-ISOLATION GENE THERAPY FOR A LYSOSOMAL STORAGE DISEASE.

By

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ABSTRACT

Lysosomal enzyme deficiencies are potentially catastrophic, genetic diseases. We used the β-glucuronidase deficient mouse model of mucopolysaccharidosis type VII (MPS VII) to develop an alternate approach to therapy. A "universal" cell line engineered to secrete the missing enzyme was implanted in recipients requiring the enzyme replacement. The cells, though non-autologous, were rendered immunologically tolerant by alginate microencapsulation to provide protection from immune mediators. Using this immuno-isolation gene therapy strategy, the microcapsule formulation was optimized for β-glucuronidase delivery to the peripheral organs of MPS VII mice from intraperitoneally implanted microcapsules. However, the results of this experiment revealed three primary obstacles to be overcome: the mice developed an immune response against the re-supplied mouse β-glucuronidase, the treatment did not address the early onset of the disease, and β-glucuronidase was unable to cross the blood-brain barrier into the central nervous system (CNS) to treat the neurodegeneration of this disease. The anti-β-glucuronidase immune response was transiently overcome using immunosuppression. To address the early disease onset, neonatal MPS VII mice were implanted within days of birth. Neonatal treatment delayed the onset of disease; however, the eventual development of antibodies limited the duration of the treatment. To address the third problem of gene product delivery to the CNS, small microcapsules were optimized for implantation into the rodent CNS and studied *in vivo*. Subsequently, microcapsules secreting β-glucuronidase were implanted into the CNS of MPS VII mice. This treatment reduced biochemical and histological disease parameters and improved behavior as assessed by circadian rhythm analyses. A combined peripheral and CNS treatment normalized the lysosomal storage throughout the peripheral organs and much of the CNS, an achievement not attained by other treatments of adult MPS VII mice. This novel cell-based immuno-isolation gene therapy demonstrates a potentially cost-effective and non-viral treatment alternative applicable to both peripheral and neurodegenerative lysosomal storage diseases.

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LIST OF ABBREVIATIONS

4-MU	4-methylumbelliferyl
AAV	adeno-associated virus
ADA	adenosine deaminase
ALS	amyotrophic lateral sclerosis
APA	alginate-poly-L-lysine-alginate
BBB	blood-brain barrier
BDNF	brain-derived neurotrophic factor
BHK	baby hamster kidney
BMT	bone marrow transplantation
BPA	barium-poly-L-lysine-alginate
BSA	bovine serum albumin
$CaCl_2$	calcium chloride
CD	cell determinant
CHES	2-(N-cyclohexylamino) ethanesulfonic
CNS	central nervous system
CSF	cerebrospinal fluid
CNTF	ciliary neurotrophic factor
DNA	deoxyribonucleic acid
DMEM	Dulbecco's modified eagles media
DMSO	dimethyl sulfoxide
ELISA	enzyme-linked immunosorbent assay
FBS	fetal bovine serum
G	α-L-guluronic acid
GAG	glyosaminoglycan
GFAP	glial fibrillary acidic protein
GFP	green fluorescent protein
GDNF	glial-derived neurotrophic factor
Gy	Grey
H & E	hematoxylin and eosin
HSV-I	herpes simplex virus type I
HLA	human leukocyte antigens
hGH	human growth hormone
HCl	hydrochloric acid
HIV	human immunodeficiency virus
HSV	herpes simplex virus
IM	intramuscular
IHC	immunohistochemistry
IP	intraperitoneal
ITR	inverted terminal repeat
IV	intravenous
kb	kilobase pair
kD	kilodalton
LAT	latency-associated transcript
M	1,4-β-D-mannuronic acid
MDCK	madin-darby canine kidney cells
μl	microlitre
μm	micrometre
mM	millimolar
M	molar
ml	millilitre
M6P	mannose 6-phosphate
MPR	mannose 6-phosphate receptor
MPR300	300 kD M6P-receptors
MPR46	46 kD M6P-receptors
MPS	mucopolysaccharidosis
MW	molecular weight
NaCl	sodium chloride
NGF	nerve growth factor
ng	nanogram
PAN/PVC	polyacrylonitrile/polyvinyl chloride copolymer
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PES	polyethersulfone
PLL	poly-L-lysine
RNA	ribonucleic acid
SCID	severe combined immunodeficiency
SC	subcutaneous
SD	standard deviation
SEM	standard error of the mean
TK	thymidine kinase
U	units
VEGF	vascular endothelial growth factor

1.0 INTRODUCTION

1.1 Gene Therapy

1.1.1 Principle

Gene therapy describes a method to treat or alleviate disease with the introduction of a gene into a patient. The notion of gene therapy emerged in the mid 1960s from an increasing knowledge of the role of molecular genetics in human disease. The earliest references to gene therapy came from Joshua Lederberg and Edward Tatum. In 1963 Joshua Lederberg wrote:

> The ultimate application of molecular biology would be the direct control of nucleotide sequences in human chromosomes, coupled with recognition, selection and integration of the desired genes (Lederberg 1963, in Anderson 1997).

In 1966, Edward Tatum addressed the issue of human gene therapy:

> We can anticipate… theoretical studies concerning somatic cell genetics and possibly in genetic therapy.... We even can be somewhat optimistic about the long-range possibility of therapy based on the isolation or design, synthesis, and introduction of new genes into defective cells of particular organs… This could be accomplished by directed mutation, or by the replacement of existing genes by others.... The desired new gene will be introduced, by directed mutation, from normal cells of another donor by transduction, or by direct DNA transfer. The rare cell with the desired change will then be selected, grown into a mass culture, and re-implanted in the patient (Tatum 1967, in Anderson 1997).

In the 1960s, new therapies were being developed for genetic diseases based on the manipulation of metabolic pathways; missing metabolic products were added and toxic metabolites were removed. However, Lederberg imagined therapies that aimed directly at the underlying causative genetic defects, through the introduction of normal copies of disease-causing genes into affected cells of the patient:

> Suppose we could transfer DNA extracted from one cell line to the chromosomes of another cell... What use could we make of this technology…? Repair genetic-metabolic disease? (Lederberg 1966, in Anderson 1997).

In 1968, French Anderson postulated that for "gene therapy" the desired gene from a normal chromosome would have to be isolated and amplified, and then incorporated into the genome of the defective cell (Anderson 1968, in Anderson 1997). Anderson described how gene therapy could be accomplished using a nonpathogenic virus capable of transferring genetic material to a cell.

1.1.2 Target diseases

Gene therapy can be utilized for: (i) gene replacement, to deliver genes to tissues to augment defective genes; (ii) gene supplementation, to deliver genes which will combat disease processes such as dominant or multifactorial genetic diseases, (iii) gene therapy for infectious diseases, to deliver inhibitory or supplementary genes to combat infectious diseases, (iv) and cancer gene therapy to deliver genes that will lead to tumour suppression and elimination.

The most basic gene therapy application is the delivery of a therapeutic gene to overcome a recessive single-gene deficiency. Gene therapy for this group of diseases has been applied to over 300 patients in 75 clinical trials for the treatment of 19 different single-gene recessive diseases (Table 1.1.1) (Journal of Gene Medicine 2001).

The original gene therapy goal of gene replacement has recently expanded to include gene supplementation, or the modulation of any gene's expression to combat a disease process. Conceivably any gene product could be manipulated through gene therapy. Consequently, the range of gene therapy applications has expanded. In addition to recessive single-gene disease treatments (Table 1.1.1), over 2800 patients have been involved in over 470 gene therapy clinical trials for dominant genetic diseases, multifactorial genetic diseases, infectious diseases, and cancer (Journal of Gene Medicine 2001).

Gene therapies for dominant genetic diseases are more complex than for recessive diseases, because depending on the disease, the corrective gene must either be expressed at the exact level, or the mutant gene must also be silenced. Gene therapy for dominant retinoblastoma blindness in rats utilized a simultaneous ribozyme-mediated ablation of wild-type and mutant mRNA, concurrently with a resupplied "ribozyme-protected" wild-

Table 1.1.1 Clinical gene therapy trials for recessive monogenic diseases.

Disease	Therapeutic Gene	Trials
α-1-anti-trypsin deficiency	α-1-anti-trypsin	1
Adenosine deaminase deficiency SCID	Adenosine deaminase	8
Canavan disease	Aspartoacylase	4
Chronic granulomatous disease	Gp91phox or p47phox	3
Cystic fibrosis	CFTR	20
Fabry Disease	α-Galactosidase	1
Familial hypercholesterolemia	LDL-receptor	1
Fanconi anemia	Complementation group A	3
Gyrate atrophy	Ornithine aminotransferase	1
Gaucher disease	Glucocerebrosidase	3
Hemophilia A	Factor VIII	3
Hemophilia B	Factor IX	3
Hunter's syndrome (MPS II)	Iduronate-2-Sulfatase	1
Hurler syndrome/MPS I	α-L-Iduronidase	2
Leukocyte adherence deficiency	CD18	1
Limb girdle muscular dystrophy	(α, β, γ, or δ-sarcoglycan),	1
Ornithine transcarbamylase deficiency	Ornithine transcarbamylase	1
Purine nucleoside phosphorylase deficiency	Purine nucleoside phosphorylase	1
X-linked SCID	Gamma-C cytokine receptor	2

(Adapted from the Journal of Gene Medicine 2001)

type gene on the same vector (Hauswirth *et al.* 2001). A neuroprotective strategy using CNTF has recently begun phase I clinical trials for dominant Huntington's disease and gene therapies more specifically targeted to the Huntington's disease mutation have been proposed (Bachoud-Levi *et al.* 1998; Bachoud-Levi *et al.* 2000).

Gene therapy of multifactorial genetic diseases includes the treatment of atherosclerosis, cardiovascular disease, and autoimmune disorders. For example, the treatment of atherosclerosis-related severe limb ischemia, vascular restenosis, coronary and peripheral artery diseases have been treated with neovascularization gene therapy, using angiogenic factors such as vascular endothelial growth factor (VEGF), in more than 25 clinical trials (Rosengart *et al.* 1999; Journal of Gene Medicine 2001). Potentially anti-arthritic cytokines have been used for gene therapy of autoimmune disorders such as rheumatoid arthritis and have begun clinical trials (Evans *et al.* 1996; Woods *et al.* 2000; Ghivizzani *et al.* 2000). A molecular synovectomy-based treatment for rheumatoid arthritis has also begun clinical trials using a thymidine kinase (TK) suicide gene (Journal of Gene Medicine 2001). Moreover, neurotrophic factors and glutamate receptors have been used in gene therapy clinical trials of amyotrophic lateral sclerosis (ALS), for which the exact causes of the disease are currently unknown (Aebischer *et al.* 1996b; Journal of Gene Medicine 2001; Ferrarese *et al.* 2001).

Treatments of infectious diseases make up 12% of gene therapy clinical trials (Journal of Gene Medicine 2001). Gene therapy for infectious diseases, such as HIV, utilize a range of strategies including antigen vaccination (Haubrich *et al.* 1995; Li *et al.* 2000), macromolecule viral inhibitors and transdominant genes (Morel *et al.* 1999; Todd *et al.* 2000), viral receptor decoys (Kohn *et al.* 1999), viral decoy genes (Wong-Staal 2000), anti-viral antibodies (Marasco *et al.* 1998; Marin *et al.* 2000), anti-viral antisense RNA (Mizuta *et al.* 1999), and anti-viral ribozymes (Wong-Staal *et al.* 1998; Klebba *et al.* 2000).

The treatment of cancer accounts for more than 69% of gene therapy clinical trials (Journal of Gene Medicine 2001). As the molecular basis of cancer is further understood, and alternative cancer therapies are often lacking, strategies designed to destroy tumours follow the same rational basis conceptualized for the treatment of single-gene genetic diseases. Tumour-suppressor genes have been supplied to tumour suppressor-deficient tumours to limit tumour growth (Habib *et al.* 1999; Tait *et al.* 1999), whereas the inhibition of dominant oncogenes has been used to inhibit the growth of tumours with overactive oncogenes using antisense (Holt *et al.* 1996),

antibody (Alvarez and Curiel 1997), and ribozyme strategies (Suzuki *et al.* 2000). Some cancer gene therapy clinical trials use a vaccination strategy and have entered clinical trials with a variety of tumour antigens, such as PSA antigen for prostate cancer (Journal of Gene Medicine 2001), and MART-1 antigen for melanoma (Ribas *et al.* 1997; Clay *et al.* 1999). Since many genes can cause cancer, a number of broad-based gene therapy strategies have also been developed. Anti-angiogenic factors have been used to starve a tumor's blood and nutrient supplies (Beecken *et al.* 2000). Suicide genes have been introduced into rapidly dividing neoplastic cells to stop tumour growth (Rosenfeld *et al.* 1995; Stockhammer *et al.* 1997; Herman *et al.* 1999). In addition, gene therapy mediated delivery of immunomodulatory cytokines have been widely used to stimulate the immune system into tumour recognition and destruction (Emtage *et al.* 1999; Stewart *et al.* 1999).

New applications of gene therapy are continually being developed. Gene transfer of an immunosuppressive antibody (CTLA4-Ig) has been used to modify donor organs to improve transplant survival while minimizing the need for systemic immunosuppression (Umeda *et al.* 2001). Aberrant vessel wall healing following angioplasty, vascular restenosis, has successfully been targeted by gene therapy with the delivery of cell-division inhibitors at the time of angioplasty (McArthur *et al.* 2001).

1.1.3 *Ex Vivo* Gene Therapy

Gene therapy can be divided into two broad categories depending on how a gene is transferred into patients. *In vivo* gene therapy involves the *in situ* uptake of foreign DNA directly within the patient. In contrast, *ex vivo* gene therapy entails the initial transfer of a foreign gene into cells in tissue culture, followed by subsequent injection of the modified cells into the patient.

1.1.3.1 Autologous Modified Cells

Autologous *ex vivo* gene therapy entails removing cells from a patient, followed by transduction of the cells with a therapeutic gene, and re-infusion of the cells back into the patient. Since the patient's cells are modified in cell culture, the modified cells can be selected and grown to large numbers. Since the implanted cells are originally derived from the same patient, the implanted cells are not rejected by the host immune system. Blood or bone marrow cells are often used for *ex vivo* gene therapy because they are the easiest to collect and return to the patient.

In 1980, Martin Cline of U.C.L.A. performed the first unsanctioned *ex vivo* gene therapy in humans for the treatment of β°-thalassemia. Autologous bone marrow cells were transfected with a 4.4 kb plasmid carrying the genomic human β-globin gene and reintroduced into patients (Wade 1980). Although the treatment had no effect on patients, the protocol was mired in scientific and ethical controversy. The treatment had not been effective in animals and the human protocol was performed without regulatory approval (Wade 1981a; Wade 1981b).

The first sanctioned human gene therapy trials used autologous *ex vivo* gene therapy to overcome adenosine deaminase (ADA) deficiency. ADA deficiency impairs normal immune system function. Patient T-lymphocytes were transduced with a retroviral vector carrying the human ADA gene (Culver *et al.* 1991). As a result, patients have shown improved T-cell survival and immune functions (Blaese *et al.* 1995). Subsequently, over half of all gene therapy trials have used autologous *ex vivo* gene therapy to treat a variety of diseases, including X-linked SCID, Hunter's syndrome (MPS II), chronic granulomatous disease, and Hemophilia B (Qiu *et al.* 1996; Malech *et al.* 1997; Stroncek *et al.* 1999b; Cavazzana-Calvo *et al.* 2000). However, the laborious and expensive protocols required for genetic modification and testing individual patient cell lines have limited widespread application of this method.

1.1.3.2 Non-autologous Modified Cells

In the first theoretical discussions of gene therapy in 1966, Lederberg suggested that for gene therapy it may be more efficient to transplant cells that have already been genetically modified, rather than genetically modify a patient's somatic cells (Lederberg 1966). Instead of engineering cells for each patient, the use of single, well-characterized, genetically-modified, non-autologous cell lines could improve the efficiency and reduce the costs associated with creating patient-specific cell lines (Chang *et al.* 1993b). Thus, a single genetically modified cell line could be transplanted into many patients requiring the same gene product. For example, genetically-modified stem cells show promise because they can differentiate and form many tissue types (Brazelton *et al.* 2000). However, non-autologous cells are quickly rejected by the immune system unless immunosuppression is used (Date *et al.* 1988; Brundin *et al.* 1988).

1.1.4 *In Vivo* Gene Therapy

1.1.4.1 Naked DNA

In contrast to *ex vivo* gene therapy, *in vivo* gene therapy involves the *in situ* uptake of foreign DNA directly within the patient. Conceptually, the simplest form of *in vivo* gene therapy is the direct injection of DNA into patients. Naked plasmid DNA administered by various routes can be expressed at significant levels *in vivo* (Wolff *et al.* 1990). Naked DNA is most effective for transfection of muscle, and to a lesser extent the brain, and expression has also been observed in the lung, and dermis (Wolff *et al.* 1992; Schwartz *et al.* 1996b). Rodent and non-human primate muscle have expressed plasmid genes injected intramuscularly for up to 4 months (Jiao *et al.* 1992). A phase I clinical trial for patients with severe hemophilia A is soon to begin using naked plasmid DNA targeted to skin fibroblasts (Journal of Gene Medicine 2001). However, naked DNA gene transfer is relatively inefficient because the human body has evolved to protect its cells from environmental hazards, such as the incorporation of foreign DNA into the genome.

1.1.4.2 Physical and Chemical DNA Vectors

Since gene transfer of naked DNA is limited by its relatively low efficiency, a number of chemicals, or vectors, have been used to increase cellular and nuclear DNA uptake. Synthetic vectors such as poly-lysine (Zeigler *et al.* 1996), poly-lysine-transferrin, and poly-lysine-asialoorosomucoid conjugates have been used to enhance receptor-mediated endocytosis (Wu and Wu 1987; Curiel *et al.* 1991). It is also possible to improve gene delivery when the DNA is coated in liposomes. Cationic lipids, such as lipofectin, lipofectamine and DC-cholesterol, and dioctadecylamidoglycylspermine (DOGS), polyethylenimine (PEI), have been used to greatly enhance transgene expression (Felgner *et al.* 1995). Cationic lipids have been used in clinical trials for cystic fibrosis with no adverse effects, but the transfection efficiency and duration of expression were significantly lower than what was required for disease correction (Caplen *et al.* 1995; Gill *et al.* 1997; Porteous *et al.* 1997). Although the efficiency of cationic lipid-mediated gene transfer is low, early trials have shown that gene therapy has advantages over enzyme replacement strategies (Brigham *et al.* 2000; Journal of Gene Medicine 2001). As a result, cationic lipids are currently being examined in additional clinical trials (Journal of Gene Medicine 2001). However, this strategy is still at such low efficiency that it is unlikely to be of therapeutic benefit, and recent studies have shown most cationic lipids to be less efficient than naked DNA alone (Zhang *et al.* 2000a).

A variety of other physical methods have also been used to enhance *in vivo* gene transfer. An air-powered "gene gun" can propel naked DNA bound to small gold particles directly into patients (Sun *et al.* 1998; Umeda *et al.* 2001). Ultrasound facilitates improved transduction of naked plasmid DNA into cells both *in vitro* and *in vivo* (Manome *et al.* 2000; Huber and Pfisterer 2000). Electroporation has also been used to improve DNA uptake *in vivo* (Bettan *et al.* 2000; Pradat *et al.* 2001). Hydrodynamic pressure generated from a large volume of saline injected into the mouse tail vein has been used to aid the uptake of naked DNA into the liver (Zhang *et al.* 1997; Liu *et al.* 1999; Zhang *et al.* 2000b). This hydrodynamic method of naked DNA gene transfer is a useful tool for studying *in vivo* liver gene expression, but therapeutic efficacy is limited by short-term high expression, from 1-14 days. Although improved synthetic vectors and physical delivery methods are still under development, these methods are generally inefficient and short term.

1.1.4.3 Viral Vectors

In vivo gene transfer with naked DNA or synthetic vectors is generally inefficient because cells have evolved many barriers to protect themselves; however, viruses have developed complex strategies to overcome these barriers. Viruses efficiently insert their genetic material inside cells. These properties have been utilized in viral gene therapy vectors to achieve more efficient gene transfer. In 1968, Lederberg first suggested that viruses might be modified to transfer not deleterious genes into cells, but genetic material that could complement genetic defects:

> It is a scheme that we might call "virogenic therapy"... The infection of a cell by a virus is therefore tantamount to adding some new genes to that cell....We can, however, think of extracting the DNA molecules that code say, for insulin and chemically grafting these to the DNA of an existing tempered virus. These new hybrid viruses would then have to be very carefully studied and perhaps modified even further, to select those appropriate for virogenic therapy in man (Lederberg 1968).

The wild type Shope papilloma virus was the first virus used to replace a deficient gene product in animals. Cutaneous inoculation of the Shope virus in rabbits naturally produces skin papillomas and induces viral-encoded expression of arginase in the papillomas. When this virus was administered intravenously to rabbits, the viral encoded arginase reduced serum arginine concentrations in rabbits (Terheggen *et al.* 1975). Since the Shope

papilloma virus could increase arginase activity in human arginase-deficient cells (Rogers *et al.* 1973), in 1970 the virus was tested in humans as a means to supply arginase to arginase-deficient patients. However, intravenous injection in three patients had no effect (Terheggen *et al.* 1975). Nonetheless, since then the field of viral vector design for gene therapy has swelled. Viral vectors have now been designed to carry and express therapeutic genes in many cell types with high efficiency.

In general, viral vectors are attenuated viral particles that cannot replicate in normal cells because specific viral genes that are required for replication have been replaced with a new therapeutic transgene. As a result, viral vectors must be cultured in recombinant cells that complement the deleted viral proteins to achieve viral replication. Following virus purification, the viral vector is administered and the new therapeutic gene is taken into patient cells using the natural viral entry mechanisms. A variety of viral vectors have shown increased gene transfer compared to using naked DNA alone. Viral vectors based on attenuated retrovirus, lentivirus, herpesvirus, adenovirus, and adeno-associated viruses are particularly good at incorporating foreign DNA into cells and have been studied for application to gene therapy.

1.1.4.4 Retrovirus

The RNA virus vector Moloney murine leukemia retrovirus (Mo-MLV) was the first virus broadly applied to gene therapy. These retroviral vectors are fully replication defective, deleted of all viral genes, with a foreign DNA packaging capacity of up to 8 kb. The inability of Mo-MLV to transduce non-dividing cells, short-term expression, and low yields of virus production have hindered the effectiveness of this vector in human trials. Nonetheless, retroviral vectors have been used in most human clinical trials (51% of patients), although this number is declining with the development of more efficient and safer vectors (Journal of Gene Medicine 2001).

The application of early retroviral vectors to gene therapy has been hindered because Mo-MLV retroviral vectors cannot transduce non-dividing cells, such as neurons and myofibers, and nuclear migration of the virus is dependent upon nuclear membrane breakdown during mitosis. In contrast, human immunodeficiency virus (HIV) lentivirus is a type of retrovirus that carries three additional virus-encoded proteins: matrix, Vpr, and integrase (Naldini *et al.* 1996b). These proteins form a nuclear localization complex that permits active transport of the virus through nuclear pores into the nucleus during interphase. As a result, lentiviral vectors are capable of transducing non-dividing cells in culture and *in vivo* (Naldini *et al.* 1996a; Naldini *et al.* 1996b; Kafri *et al.* 1997; Miyoshi *et al.*

1997). HIV-1-based lentiviral vectors have also shown gene therapy potential for the treatment of hemophilia B in mice, resulting in long-term, but low levels of factor IX(Park *et al.* 2000). However, it was recently found that lentivirus was not as efficient as it originally appeared, and cell division is required for effective lentiviral infection (Barrette *et al.* 2000; Park *et al.* 2000; Danos 2001; Kay 2001). In addition, vector yields using the most advanced lentiviral packaging cell lines are five logs lower than other viral vectors, which has slowed the progress of testing lentiviral vectors in large animal models (Klages *et al.* 2000). In addition, lentivirus causes acute cytokine release syndrome and efficiently transduces antigen presenting cells, which together result in acute toxicity, high levels of antigen presentation, and T-cell activation, in a manner similar to first generation adenovirus vectors (Danos 2001; Kay 2001).

1.1.4.5 Herpesvirus

Herpes simplex virus (HSV) has been widely investigated as a vector for gene therapy. HSV naturally establishes persistent latent infections in neurons and has evolved a highly efficient retrograde transport from the site of infection to the spinal ganglia. During latent infection, only one gene, the latency-associated transcript (LAT), is expressed. Thus, long-term expression was expected from foreign genes driven by either of the LAT promoters (LATP1 or LATP2) (Wolfe *et al.* 1996). However, the development of HSV vectors that exploit these properties has been difficult; expression from LATP1 was short term (Geller 1993; Dobson *et al.* 1995), and expression from LATP2, although long-term, was extremely weak (Goins *et al.* 1994). The development of improved HSV vectors continues, and if a helper-dependent system can be developed, HSV toxicity could potentially be reduced and the vector would have a theoretical insert capacity of over 150 kb, allowing the insertion of multiple or large transgenes (Marsh *et al.* 2000; Strathdee and McLeod 2000; Lilley *et al.* 2001). Overall, however, HSV is currently limited by vector toxicity and transient expression.

1.1.4.6 Adenovirus

Adenoviruses can infect a wide variety of cell and tissue types in both dividing and non-dividing cells and have been extensively used as gene therapy vectors. First generation adenoviral vectors removed the E1 and E3 genes to carry up to 7 kb of foreign DNA (Graham *et al.* 1977). However, *in vivo* transgene expression was short term because of an overwhelming immune response mounted against the virus capsid antigens and late structural antigens, which are produced because cells produce E1-like proteins that facilitate synthesis of the late viral

proteins. At the same time, peak levels of transgene expression occur within 3 days of infection, coinciding with the acute vector-derived immune response. In conjunction with the capacity of adenovirus to efficiently infect antigen-presenting cells (APCs), the transgene itself is often targeted by the over-stimulated immune system, adding to the immune response against vector-transduced cells (Danos 2001; Kay 2001). As a result, first generation adenovirus vectors result in transient gene expression and acute toxicity. Nonetheless, this vector system was examined in many clinical trials to show, as expected, short-term expression and toxicity which eventually led to the death of the first gene therapy-treated patient (Zabner *et al.* 1993; Crystal *et al.* 1994; Boucher *et al.* 1994; Bellon *et al.* 1997; Somia and Verma 2000; Teichler 2000). Most clinical trials using adenovirus have now been halted (Woo 2001).

Subsequently, the second generation of adenoviral vectors were developed using improved complementing cell lines that expressed more viral proteins, permitting the deletion of additional viral genes on the vector to improve vector capacity (10-12 kb) and reduce toxicity (Engelhardt *et al.* 1994; Fallaux *et al.* 1998; Lusky *et al.* 1998; Moorhead *et al.* 1999; Gao *et al.* 2000b). Nevertheless, persistent transgene expression has still not been achieved due to the host immune response.

The latest, third generation, of "deleted" adenoviral vectors have been constructed by deleting all of the adenovirus genes, retaining only the original virus ITRs and packaging signal (Parks *et al.* 1996). The deleted vectors utilize *Cre*/*lox*P or *Flp*/*frt* recombination to delete the packaging signal on helper virus genomes, thereby significantly reducing the production of first-generation helper virus (Parks *et al.* 1996; Ng *et al.* 1999; Parks 2000; Ng *et al.* 2001). As a result, the deleted adenovirus system shows dramatically increased expression and safety *in vivo* (Schiedner *et al.* 1998; Morsy *et al.* 1998; Morral *et al.* 1998; Morral *et al.* 1999; O'Neal *et al.* 2000). Therefore, deleted adenovirus vectors appear to be a promising vector system with long-term expression for up to a year, lower toxicity, and a large insert capacity (36 kb) However, there are still significant problems associated with this vector, primarily due to the contamination of helper virus, even after careful purification. As a result, deleted adenovirus still inflicts significant vector-derived toxicities (Cregan *et al.* 2000).

1.1.4.7 Adeno-Associated Virus

Recombinant adeno-associated virus (AAV) vectors show significant promise for human gene therapy because of their low immune response and long-term gene expression in both dividing and non-dividing cells.

Wild-type AAV is a small, stable, non-pathogenic DNA virus of the *Parvoviridae* family, containing only two genes, rep and cap (Srivastava *et al.* 1983). AAV vectors have been developed by removing all of the original viral elements except for the inverted terminal repeats (ITR) to provide a 4.6 kb capacity for foreign DNA (Samulski *et al.* 1991). The infectivity and transgene expression of AAV are very different from adenovirus and lentivirus vectors. AAV does not infect antigen-presenting cells, nor induce high levels of cytokines. Since AAV expression is delayed for weeks to months after infection, AAV-transgene expression does not coincide with potential acute vector immune responses. Thus, AAV-derived transgene expression may lead to better transgene immune tolerance and AAV transduced cells are not destroyed by the immune system (Danos 2001).

AAV vectors have recently shown significant accomplishments in the treatment of hemophilia. AAV serotype 2 (AAV2) vectors are capable of long-term gene expression lasting more than 3 years in hemophilic canines (Zammarchi *et al.* 1996; Snyder *et al.* 1999; Herzog *et al.* 1999; Daly *et al.* 1999a; Kay 2001). Preclinical AAV2 studies have produced therapeutic levels of circulating factor IX in hemophilic mice (5-7%) and dogs (5-14%) (Chao *et al.* 1999; High 2001). Although scaling up AAV-production was a technically challenging obstacle for clinical applications (Linden and Woo 1999), recently developed methods can produce high yields of helper-virus free, purified vector (10^{12} transducing units/ml), but it is a very difficult process (Drittanti *et al.* 2001). Nonetheless, this has led to the initiation of more than ten clinical trials using this gene delivery system. An intramuscular-directed AAV2 clinical trial for hemophilia B has shown no evidence of toxicity; no germ-line transmission; no inhibitory antibodies against factor IX; and indications of a modest clinical response in some patients (Fabb and Dickson 2000; Kay *et al.* 2000). Furthermore, a recently initiated liver-directed clinical trial for hemophilia B is expected to achieve 10 to 40-fold higher levels of factor IX delivery (Kay 2001). In addition, newly developed AAV serotypes (ie. AAV1) have shown to be 1000-fold more effective in factor IX delivery than AAV2 vectors (Chao *et al.* 2000). Recently, the size limitations of AAV foreign DNA capacity have been doubled with the development of duplex AAV genomes (Yan *et al.* 2000). The identification of regions within the viral capsid that are amenable to modification has begun to address the issue of AAV vector targeting to specific cell populations (Smith-Arica and Bartlett 2001). Recently, AAV has attracted significant attention with AAV-mediated restoration of visual function in a blind canine model of Leber congenital amaurosis (Acland *et al.* 2001). However, the recent finding that neonatal administration of recombinant AAV to MPS VII mice resulted in liver

tumours in 60% of mice after 1.5 years highlights the potentially serious drawbacks of an integrating viral vector (Sands *et al.* 2001).

1.1.5 Gene Therapy in the CNS

The central nervous system (CNS) is a difficult organ to target using gene therapy because of the blood brain barrier (BBB). Nonetheless, many neurodegenerative disorders such as Huntington's, Alzheimer's, Parkinson's, and neurodegenerative lysosomal storage diseases may be amenable to gene replacement or gene supplementation strategies in the CNS.

The brain is a highly complex and carefully protected organ. Inside the skull, the meninges and cerebrospinal fluid protect and form a cushioning fluid around the brain. The meninges provide three layers of protection: the dura matter is a tough thick membrane outer membrane of the meninges; the arachnoid is a thin and delicate membrane; and the pia matter is a thin membrane closely adhered to the brain.

The brain is also carefully protected within the brain's capillaries with the BBB. The BBB is a complex series of protective mechanisms controlling the internal environment of the brain by impeding the passage of most ions and large-molecular-weight compounds from the blood to the brain tissue (Saunders *et al.* 1999). Small nutrient molecules such as oxygen, glucose, and certain amino acids easily pass through the BBB. However, in order to protect the brain from a free exchange of molecules, larger molecules such as insulin pass through the barrier slowly, and some molecules are almost completely excluded. Molecules enter the brain through either passive diffusion or through carrier-mediated active transport of certain peptides with highly specific transporters (Begley 1994). The underlying morphological difference of capillaries in the brain is the presence of tight junctions between endothelial cells and between choroid plexus epithelial cells in the ventricles. Astrocytes further surround the capillaries to form a cover over the capillaries.

1.1.5.1 In Vivo CNS Gene Therapy

Naked DNA administered to the murine CNS resulted in the expression of a reporter gene; however, the efficiency was very low and the expression short, lasting no more than 5 days (Schwartz *et al.* 1996a). Although recent improvements have increased the efficiency of lipid-mediated gene transfer, most *in vivo* gene therapy strategies in the CNS have used viral vectors to improve gene transfer (Hecker *et al.* 2001).

Mo-MLV retroviral vectors are restricted to infecting proliferating cells, a property that has been applied to the treatment of cancer by targeting rapidly dividing, malignant cells in the CNS. Administration of a retroviral vector expressing herpes simplex thymidine kinase (TK) to dividing tumor cells in the brain, followed by the activation of TK by gancyclovir, selectively killed dividing tumor cells and showed complete regression of 75% of induced tumors in rats (Ram *et al.* 1993). HIV-based lentiviral vectors have been used for gene therapy in the CNS because they are capable of transducing non-dividing cells. Replication defective lentiviral vectors have shown non-toxic reporter gene expression in glial cells and terminally differentiated neurons in the CNS for up to eight months (Naldini *et al.* 1996b; Kafri *et al.* 1997). In non-human primates, lentiviral-expressed glial-derived neurotrophic factor (GDNF) prevented MPTP-induced motor deficits and induced the regeneration of dopamine-containing neurons in an aged rhesus monkey model of Parkinson's disease (Kordower *et al.* 1999; Kordower *et al.* 2000).

Since herpesvirus (HSV) naturally establishes persistent latent infections in neurons and can be taken up at nerve terminals and transferred across synapses to allow entry from the periphery into the brain, HSV has been widely investigated as a gene therapy vector for the CNS (Breakefield and DeLuca 1991). Many genes have been expressed from HSV vectors *in vivo* (Geller 1993). For example, HSV vectors have shown delivery of marker genes and nerve growth factor (NGF) to neurons; however, contrary to earlier findings, the expression from HSV is short term, and the vector carries unacceptably high levels of toxicity (Federoff *et al.* 1992; Glorioso *et al.* 2001). Nonetheless, HSV has been used in a clinical trial for the treatment of malignant glioma in the CNS with results that were suggestive of anti-tumour activity (Markert *et al.* 2000), and several gene therapy clinical trials using HSV vectors are awaiting regulatory approval for the treatment of brain tumours.

First generation adenoviral vectors can infect the CNS (Bajocchi *et al.* 1993) and when expressing tyrosine hydroxylase were capable of reducing apomorphine-induced rotational asymmetry in unilaterally lesioned rats for up to two weeks (Horellou *et al.* 1994; Horellou *et al.* 1997). Although deleted third generation vectors are remarkably improved over first generation vectors, these vectors cause moderate but significant changes to cellular function and viability in primary neuronal cultures, thereby hindering the application of adenoviral vectors in CNS therapies (Cregan *et al.* 2000).

The application of recombinant AAV vectors to the CNS has also shown promise with persistent expression in neurons with no apparent toxicity (Klein *et al.* 1998; Peel and Klein 2000). In neurodegenerative disease models, behavioral efficacy of AAV was demonstrated with the delivery of brain-derived neurotrophic factor (BDNF) in rat and primate models of Parkinson's disease (During *et al.* 1998; Klein *et al.* 1999). Promising results with AAV warranted the initiation of a phase I clinical trial using AAV-mediated delivery of astrocytic glutamate transporter EAAT2 in an attempt to restore glutamate uptake in patients with ALS (Journal of Gene Medicine 2001; Ferrarese *et al.* 2001).

1.1.5.2 Ex Vivo CNS Gene Therapy

Ex vivo gene therapy in the CNS was founded upon the use of fetal CNS transplants for the treatment of neurodegenerative diseases. Fetal dopamine neurons transplanted into rodent and primate Parkinson's disease animal models improved Parkinson's disease symptoms for at least two months (Takayama *et al.* 1995). Recently, transplantation of human embryonic dopamine neurons into the brains of patients with Parkinson's disease were shown to be slightly beneficial in some younger patients (Freed *et al.* 2001). However, whether this intervention is more effective than sham surgery in a controlled trial is not yet known. The lack of clear effects of grafts in Parkinson's patients may be due to poor grafts survival in the human brain (Gagnon *et al.* 1993).

Despite some promising preliminary results, human fetal cell CNS transplants are complicated by obtaining large amounts of human fetal tissue, ethical concerns of fetal tissue collection, regulatory approval of fetal tissue research, political issues, and the symptomatic effects are incomplete and vary widely between patients (Lindvall 2000). Since the brain is not completely immunologically privileged (Date *et al.* 1988; Brundin *et al.* 1988), grafted fetal tissue is capable of provoking a cellular immune response which can destroy fetal transplants within two weeks (Date *et al.* 1988; Zhou *et al.* 1993; Hudson *et al.* 1994). The immune response is mediated by microglia, dendritic cells, and lymphocytes, and the transplanted cells are killed by host cytotoxic lymphocytes after encirclement by host microglial cells (Lawrence *et al.* 1990). This response may be reduced with immunosuppression (Finsen *et al.* 1988; Brundin *et al.* 1988; Zhou *et al.* 1993) to allow even xenogeneic fetal neural cells to survive, mature, and integrate into the host (Jacoby *et al.* 1997).

1.1.5.2.1 Autologous Cells

Genetic modification of a patient's own cells could help avoid the myriad of ethical, regulatory, political, procurement, and immunological problems associated with fetal human tissue transplantation. Fred Gage first proposed grafting genetically modified cells into the CNS in 1987 (Gage *et al.* 1987). Primary autologous fibroblasts transfected with a plasmid expressing human growth hormone have shown hormone delivery for up to 4 weeks in rodents, with decreased levels after 6 and 8 weeks (Doering and Chang 1991).

Nerve growth factor (NGF) has been widely examined as a treatment for neurodegenerative disorders using *ex vivo* autologous gene therapy in the CNS. In rats and monkeys, genetically modified autologous fibroblasts produced NGF in the brain for up to a year, and enhanced the survival and function of cholinergic nerve cells, and prevented neuronal degeneration in an age-related neurodegenerative disorder (Smith *et al.* 1999). In April 2001, Mark Tuszynski of U.C.S.D. initiated the first human gene therapy clinical trial for a neurodegenerative disorder (Beasley 2001). Half a million genetically modified autologous fibroblasts expressing NGF were implanted into each of five sites in the brain of one patient for the treatment of Alzheimer's disease (Okie 2001). The trial is currently ongoing and will include seven additional patients. The treatment is not expected to cure the disease, but is expected to slow the mental decline and preserve the capacity to learn and remember (Beasley 2001).

Ex vivo autologous gene therapy in the CNS has raised a variety of concerns. A clinical trial in which purified NGF was infused into the cerebrospinal fluid produced unexpected severe side-effects by stimulation of cells in several parts of the nervous system, causing pain, and weight loss (Eriksdotter *et al.* 1998). Similar side-effects were later observed in animals, along with fear-related behaviors and adverse neuroproliferative changes (Winkler *et al.* 2000; Hao *et al.* 2000). Although such side-effects were not expected to occur with the NGF gene therapy trial because the dose was lower and the treatment did not target the CSF (Tuszynski 2000; Beasley 2001), if complications did occur, there would be no simple way to remove the genetically modified fibroblasts once they have been implanted in the brain. Furthermore, the costs associated with creating patient-specific genetically modified cell lines are prohibitive for large-scale application.

1.1.5.2.2 Non-autologous Cells

The use of non-autologous cells for gene therapy in the CNS could circumvent the high cost of developing patient-specific, genetically-modified cell lines and is not restricted by limited tissue availability of primary cells or fetal tissues. A variety of neurodegenerative animal models have been treated with genetically modified non-autologous cells.

Neural stem cells may also be suitable as alternative tissue sources for non-autologous gene therapy in the CNS. Neural progenitor cells are multipotent neural stem cells capable of differentiating into neurons, and may be clonally expanded in tissue culture to provide a renewable supply of material for transplantation (Snyder and Macklis 1995; Pincus *et al.* 1998; Yamaguchi *et al.* 2000). Human neural progenitors expanded *in vitro* have been modified with recombinant adenovirus to express a reporter gene for up to 12 days in rats (Sabate *et al.* 1995). Recently, in the search for novel neuronal stem cells, Helen Blau at Stanford found that GFP-marked bone marrow cells could migrate into the brain, differentiate into neurons, and begin to function like the native neurons (Brazelton *et al.* 2000). Although there is no means yet to identify which stem cells in the bone marrow develop into neurons, these findings show that such stem cells exist and that bone marrow could be used to replace those damaged or lost neurons.

Several problems remain with the use of non-autologous cells in the CNS. As with fetal tissue transplants, non-autologous cells are soon rejected by the immune system, even in the immunopriviledged CNS, unless immunosuppression is used (Date *et al.* 1988; Brundin *et al.* 1988). Moreover, the use of immortalized cells introduces the risk of tumour formation in the brain. In addition, as with autologous and fetal transplants, if complications occur, there is no simple way to remove the genetically modified cells once they have been implanted.

1.2 Immuno-Isolation.

1.2.1 Principle

An emerging form of therapy using non-autologous somatic cells has been made possible with immuno-isolation, in which a primary cell line or tissue is enclosed in an implantable device with selective permeability. The use of a selectively-permeable, artificial membrane to enclose aqueous suspensions of enzymes and cells was first described in 1964 by T.M.S. Chang (Chang 1964). The unique biocompatible and permselective features of an immuno-isolation barrier protect foreign cells from immune rejection after transplantation, while permitting the passage of a desired therapeutic product from the cells as well as small molecules such as oxygen, nutrients and waste products necessary for maintaining the viability of the enclosed cells (Chang *et al.* 1993b). The permeability barrier inhibits entry of large molecular or cellular moieties such as complement, macrophages and lymphocytes. The immune mediators responsible for graft rejection are thus prevented from contact with the enclosed recombinant cell. Hence, the same cell line can be used to treat different patients regardless of the recipients' histocompatibility status and cells from a variety of sources can be transplanted without the need for immunosuppression, including non-autologous or xenogeneic cells. Recently, the potential application of immuno-isolation has significantly expanded with the development of immuno-isolation gene therapy, using recombinant cells to express a desired gene product (Section 0).

1.2.2 Immuno-Isolation Devices

Several devices have been developed for immuno-isolation. These devices carry the requisite biocompatibility to avoid host inflammatory responses, lack of cytotoxicity to permit cell survival, and chemical flexibility to engineer the pore-size and permeability of the device. The pores must be small enough to prevent the immune system from destroying the enclosed cells, while large enough to maintain cell viability with sufficient waste and nutrient exchange, as well as permit the export of the desired therapeutic gene product. It has been found that the entry of smaller immune molecules into the immuno-isolation device is tolerable since this does not lead to rejection of the transplanted cells (Chang *et al.* 1993b; Awrey *et al.* 1996; Brissova *et al.* 1996). The two general types of immuno-isolation devices are preformed chambers and microcapsules.

1.2.2.1 Immuno-Isolation Chambers

Preformed immuno-isolation chambers enclose cells between two semi-permeable, fixed pore size membranes made of polytetrafluoroethylene or acrylic (Carr-Brendel *et al.* 1997). These chambers have been able to maintain the viability and secretion from xenogeneic cells for more than a year in immunodeficient rodents (Brauker *et al.* 1998; Josephs *et al.* 1999). Hollow fibers made from polyethersulfone (PES) and polyacrylonitrile/polyvinyl chloride copolymer (PAN/PVC) membranes are immuno-isolation tubes that can be filled with cells, sealed, and subsequently implanted. Subcutaneous xenografts of acrylic copolymer encapsulated islets in diabetic mice have maintained normoglycemia for up to 60 days (Lacy *et al.* 1991; Lacy 1995). Similarly, experiments with immuno-isolation fibres have also demonstrated immuno-isolation for xenogeneic cells (Aebischer *et al.* 1991a).

1.2.2.2 Immuno-Isolation Microcapsules

Microcapsules are another type of immuno-isolation device that are formed through the gelation of a biopolymer around cells or tissues. Several naturally occurring and synthetic polymers have been used to formulate microcapsules. The most commonly used microcapsule polymer is the seaweed extract, alginate, cross-linked with divalent cation such as calcium (Smidsrod and Skjak-Braek 1990).

1.2.2.2.1 Alginate Microencapsulation

Alginate is an anionic polysaccharide composed of 1,4-β-D-mannuronic (M) and α-L-guluronic (G) acid units joined to form unbranched chains (Otterlei *et al.* 1991). Alginate occurs naturally in a gel form in the intracellular matrix of algae where it acts as a flexible skeleton. Alginate can form a gel following interchain chelation of divalent cations, such as Ca^{2+} and Ba^{2+}, with G-blocks in the alginate (Martinsen *et al.* 1989; Otterlei *et al.* 1991). The resulting gel strength increases in proportion to the square of the alginate concentration, with higher proportions and lengths of G-blocks in the alginate resulting in further increased gel strength (Martinsen *et al.* 1989). For a microcapsule to perform effectively, it requires a biocompatible, semipermeable membrane that completely surrounds implanted cells with an optimal immunoisolating molecular cutoff that also provides an environment compatible with cell growth.

Alginate microencapsulation generally involves a three-step process using a 1.5% alginate-cell solution. Initially, a mixture of cells in alginate solution is extruded through a 27-gauge needle to form alginate droplets

containing cells. A concentric airflow is applied to the needle tip to control droplet size. The droplets fall into a calcium chloride or barium chloride bath and form alginate beads upon alginate gelation with divalent cations cross-linking the alginate. The second step involves the application of a poly-L-lysine (PLL) coat. The PLL interacts with the alginate through ionic interactions and serves to increase the structural stability of the microcapsules, as well as designate the molecular weight cut-off of the microcapsule membrane (Goosen *et al.* 1985). Since the PLL may elicit an immune response, the final step is the addition of another layer of alginate to maintain the biocompatibility on the microcapsule surface. The result is an alginate-poly-L-lysine-alginate (APA) microcapsule (Liu *et al.* 1993; Chang *et al.* 1993b; Chang 1995). The capsule's alginate core may be liquefied using sodium citrate, leaving cells to float freely in the centre of the capsule.

The parameters for the production of optimized strong, appropriately permeable, and non-immunogenic alginate microcapsules have been well characterized from the study of alginates isolated from different types of algae. Alginates with high-G content have the highest porosity (Smidsrod and Skjak-Braek 1990), are the most biocompatible (de Vos *et al.* 1997a), and are the strongest formulation because only the G units of alginate participate in ionic cross-linking interactions with calcium ions (Thu *et al.* 1996). However, alginate purity appears to be the most critical factor in determining alginate biocompatibility (Arita *et al.* 1997).

In addition to APA microcapsules, alternative alginate microcapsules have been investigated, such as alginate-polyphosphazene microcapsules (Van Raamsonk *et al.* 2001), uncoated alginate microspheres (Lanza *et al.* 1995; Lanza *et al.* 1996), alginate-protamine-heparin capsules (Tatarkiewicz *et al.* 1995), alginate-poly-L-ornithine capsules ((Calafiore *et al.* 1996; Calafiore *et al.* 1997), alginate-cellulose sulfate-poly-methylene-co-guanidine capsules (Wang *et al.* 1997). Moreover, higher alginate concentrations, different cross-linking reagents, omitting the poly-L-lysine and second alginate layers, or omitting core solubilization, and using agarose have also been studied *in vivo* in order to improve microcapsules mechanical stability without compromising immuno-protective properties. (Zekorn *et al.* 1992; Soon-Shiong *et al.* 1992b; Klock *et al.* 1994; Tashiro *et al.* 1998).

1.2.3 Application of Immuno-Isolation

The first therapeutic application of alginate microcapsule immuno-isolation was demonstrated for the treatment of diabetic mice, showing long-term survival of xenogeneic pancreatic islet cells implanted into the peritoneal cavity (Chick *et al.* 1977; Lim and Sun 1980). In rats and dogs, encapsulated islets were able to secrete

insulin and maintain normoglycemia for up to 2 years (Lum *et al.* 1991; Lum *et al.* 1992; Soon-Shiong *et al.* 1992a; Soon-Shiong *et al.* 1992b; Soon-Shiong *et al.* 1992c; Soon-Shiong *et al.* 1993; de Vos *et al.* 1997b; Lanza *et al.* 1999). Subsequently, in a type 1 diabetic patient receiving an intraperitoneal injection of encapsulated human islets insulin independence was maintained for 9 months, demonstrating the first successful clinical application of alginate immuno-isolation in humans (Soon-Shiong *et al.* 1994).

1.2.4 Immuno-Isolation in the CNS

Immuno-isolation also provides a novel means of achieving long-term graft survival in the CNS. The feasibility of encapsulated cell transplantation in the CNS was first demonstrated in rats with the survival of PAN-PVC encapsulated xenogeneic neural tissues for up to 12 weeks (Aebischer *et al.* 1988). Since then, immuno-isolation has been investigated for the treatment of several animal models of Parkinson's disease using PC-12 cells that naturally produce dopamine. Striatal implants of thermoplastic, and PAN-PVC encapsulated PC-12 cells could survive and release dopamine for up to 6 months to improve rotational behavior in CNS-lesioned rodents (Winn *et al.* 1991; Aebischer *et al.* 1991c; Emerich *et al.* 1992; Tresco *et al.* 1992a; Tresco *et al.* 1992b). In addition, polymer-encapsulated PC-12 cells could survive for up to five months in the CNS of hemiparkinsonian primates and alleviate the neurological deficits (Aebischer *et al.* 1994; Yoshida *et al.* 1999). Polymer-encapsulated PC12 cells have also been used to ameliorate the motor deficits of aged rats for up to three weeks following intra-striatal implantation (Emerich *et al.* 1993; Emerich *et al.* 1994b).

The treatment of chronic pain has been investigated with immuno-isolation in the CNS. Pain sensitivity was reduced for up to three months in rodents using PAN-PVC encapsulated bovine chromaffin cells that release pain-reducing neuroactive compounds, including catecholamines and opioid peptides (Sagen *et al.* 1993). Scale-up of this approach has also been demonstrated in sheep (Joseph *et al.* 1994). Subsequently, the clinical potential of this approach was evaluated in humans. Hollow-fiber encapsulated bovine chromaffin cells implanted in the subarachnoid space of seven patients with severe chronic pain reduced morphine intake and improved pain ratings in several patients, representing the first successful human trial of encapsulated cells in the CNS (Buchser *et al.* 1996).

1.3 Immuno-Isolation Gene Therapy

1.3.1 Principle

A unique approach to therapeutic protein delivery combines immuno-isolation and gene therapy. The use of immuno-isolated recombinant cells for gene therapy was first described in 1993 (Chang *et al.* 1993b). This approach is similar to *ex vivo* gene therapy because cells are engineered *ex vivo* to secrete a therapeutic protein prior to implantation in patients. However, instead of engineering the patient's own cells, this approach involves immuno-isolation of a standard "universal" cell line that has been genetically engineered to secrete a therapeutic protein. Thus, the need for patient-specific genetic engineering and immunosuppression required with conventional autologous or non-autologous *ex vivo* gene therapies, respectively, are removed using immuno-isolation gene therapy. Moreover, the encapsulation of recombinant cells significantly expands the scope of immuno-isolation technology from dependence on naturally occurring cells or tissues, to cells genetically engineered to deliver a therapeutic recombinant protein of choice. With the recent completion of the human genome sequence projects (Lander et al. 2001; Venter et al. 2001), the scope of immuno-isolation gene therapy is likely to expand (Chang *et al.* 1999).

1.3.2 Application of Immuno-Isolation Gene Therapy

The feasibility of immuno-isolation gene therapy has been demonstrated using alginate-poly-L-lysine-alginate (APA) microcapsules. *In vitro* studies have shown that genetically-modified fibroblasts remain viable within alginate microcapsules and can deliver recombinant gene products such as human growth hormone, factor IX, and lysosomal enzymes (Liu *et al.* 1993; Chang *et al.* 1994; Awrey *et al.* 1996). *In vivo* studies have shown successful delivery of recombinant human growth hormone and adenosine deaminase to rodents (Tai and Sun 1993; Chang *et al.* 1993b; Huges *et al.* 1994; Ross *et al.* 1999). In addition, human growth hormone has been successfully delivered to the circulation of large canine animal models (Peirone *et al.* 1998a; Stockley *et al.* 2000).

Immuno-isolation gene therapy has demonstrated clinical efficacy in treating several animal models of disease. The treatment of inherited genetic disorders has also shown clinical efficacy. The delivery of mouse growth hormone corrected the growth retardation in the growth hormone deficient Snell dwarf mouse (Al-Hendy

et al. 1995). The clotting deficiency of hemophilia B mice was corrected with the delivery of human factor IX (Hortelano *et al.* 1996; Van Raamsonk *et al.* 2001). In addition, the lysosomal storage disease pathology of Mucopolysaccharidosis VII mice was corrected with delivery of β-glucuronidase in this thesis (Chapters 3.2, 3.3) (Ross *et al.* 2000a; Ross *et al.* 2000b). Recent reports also show that alginate encapsulated cells can inhibit tumour growth. Potent immune-modulation using an anti-HER2 monoclonal antibody-IL-2 fusion protein delivered from alginate microcapsules has suppressed tumour growth and improved survival in mice (Cirone *et al.* 2000). Similarly, the delivery of endostatin from alginate microcapsules inhibited tumor neovascularization and tumour growth in several mouse models of malignant glioma (Read *et al.* 1999; Read *et al.* 2001; Joki *et al.* 2001). These studies illustrate a new paradigm of treatment via immuno-isolation gene therapy technology using alginate microcapsules.

1.3.3 Immuno-Isolation Gene Therapy in the CNS

Immuno-isolation gene therapy also provides a novel means of achieving continuous delivery of therapeutic molecules to the CNS. The use of encapsulated PC-12 cells secreting dopamine provided the foundation for these studies. Prior to the research of this thesis, immuno-isolation gene therapy in the CNS had been demonstrated with only polymer-encapsulated cells for the delivery of NGF, and the feasibility of using alginate microcapsules in the CNS had not been studied. Subsequently, the CNS-implantation of several immuno-isolation devices created from different polymer formulations have been investigated for the treatment of a variety of neurological conditions.

1.3.3.1 β-endorphin

The treatment of chronic pain has been ameliorated using immuno-isolation gene therapy in the CNS. As an alternative to implanting of a limited supply of encapsulated primary bovine chromaffin cells (Sagen *et al.* 1993; Buchser *et al.* 1996), immuno-isolation gene therapy using recombinant cells engineered to secrete β-endorphin has been used to alleviate pain in animals. Intrathecally-implanted PAN-PVC copolymer-encapsulated mouse neuroblastoma cells secreting β-endorphin significantly reduced pain sensitivity in rats (Saitoh *et al.* 1995a; Saitoh *et al.* 1995b; Saitoh *et al.* 1998).

1.3.3.2 NGF

The delivery of neurotrophic molecules to the CNS has gained considerable attention as a potential treatment strategy for several neurodegenerative disorders. For the treatment of Parkinson's or Alzheimer disease, polymer encapsulated cells engineered to produce NGF implanted into the striatum or ventricle produced nanogram levels of NGF and prevented the degeneration of both cholinergic and non-cholinergic striatal neurons of fimbria-fornix lesioned rats and primates (Hoffman *et al.* 1993; Kordower *et al.* 1994; Winn *et al.* 1994; Emerich *et al.* 1994c; Schinstine *et al.* 1995; Lindner *et al.* 1995; Winn *et al.* 1996; Kordower *et al.* 1996). NGF from polymer-encapsulated cells also significantly reduced apomorphine-induced rotation in hemiparkinsonian rats (Emerich *et al.* 1994a; Date *et al.* 1997) and attenuated the age-related cognitive deficits of aged rats (Lindner *et al.* 1996).

1.3.3.3 GDNF and CNTF

In progressive motoneuronopathy (PMN) mice, which exhibit a loss of motor fibres and motoneuron cell bodies similar to Amyotrophic Lateral Sclerosis (ALS), glial cell line-derived neurotrophic factor (GDNF) or ciliary neurotrophic factor (CNTF) delivered from polypropylene polymer encapsulated cells delayed disease progression, and CNTF also increased survival time by 40% (Sagot *et al.* 1995; Sagot *et al.* 1996). In quinolinic acid or axotomy rodent models of neurodegeneration, PAN-PVC copolymer-encapsulated baby hamster kidney (BHK) cells secreting GDNF or CNTF in the CNS inhibited striatal neuron degeneration and nearly normalized rotational behaviour within three weeks (Deglon *et al.* 1996; Tan *et al.* 1996; Emerich *et al.* 1996a; Emerich *et al.* 1996b; Sautter *et al.* 1998; Emerich *et al.* 1998). After similar results were observed in primates (Emerich *et al.* 1997), phase I and II clinical studies were initiated in humans. ALS patients were implanted with PAN-PCV encapsulated BHK cells releasing 0.5 μg of human CNTF per day within the lumbar intrathecal space (Aebischer *et al.* 1996a). Nanogram levels of CNTF were measured within the CSF for several weeks in 75% of patients, and for up to 20 weeks in 10% of the patients. The treatment was not associated with adverse side effects, except for the generation of antibodies against components of the media used to culture the encapsulated cells prior to implantation (Aebischer *et al.* 1996b; Zurn *et al.* 2000). These studies demonstrated the first human clinical application of immuno-isolation gene therapy in the CNS.

A similar phase I clinical trial using hCNTF-secreting encapsulated BHK cells implanted into the striatum has since been initiated for the treatment of Huntington's disease (HD) after the treatment successfully restored neostriatal function in a primate model of HD (Bachoud-Levi *et al.* 2000; Mittoux *et al.* 2000).

1.3.3.4 Endostatin

The first report of using alginate microencapsulated cells for immuno-isolation gene therapy in the CNS is described in this thesis (Chapter 3.5) (Ross *et al.* 1999). Recently, alginate microcapsules have also been used to deliver anti-angiogenic endostatin to the CNS to inhibit tumor neovascularization and growth, resulting in increased survival in several mouse models of malignant glioma (Read *et al.* 1999; Read *et al.* 2001; Joki *et al.* 2001).

1.4 Lysosomal Storage Diseases

1.4.1 Lysosomes

Lysosomes are membrane-bound cytoplasmic organelles involved in intracellular protein degradation within the vacuolar system of eukaryotic cells. Lysosomes are the final destination for many endocytic, autophagic, and secretory materials targeted for destruction. Lysosomal degradation is required for normal cellular protein turnover, disposal of abnormal proteins, down-regulation of surface receptors, release of endocytosed nutrients, inactivation of pathogenic organisms, and antigen processing (Gieselmann 1995b).

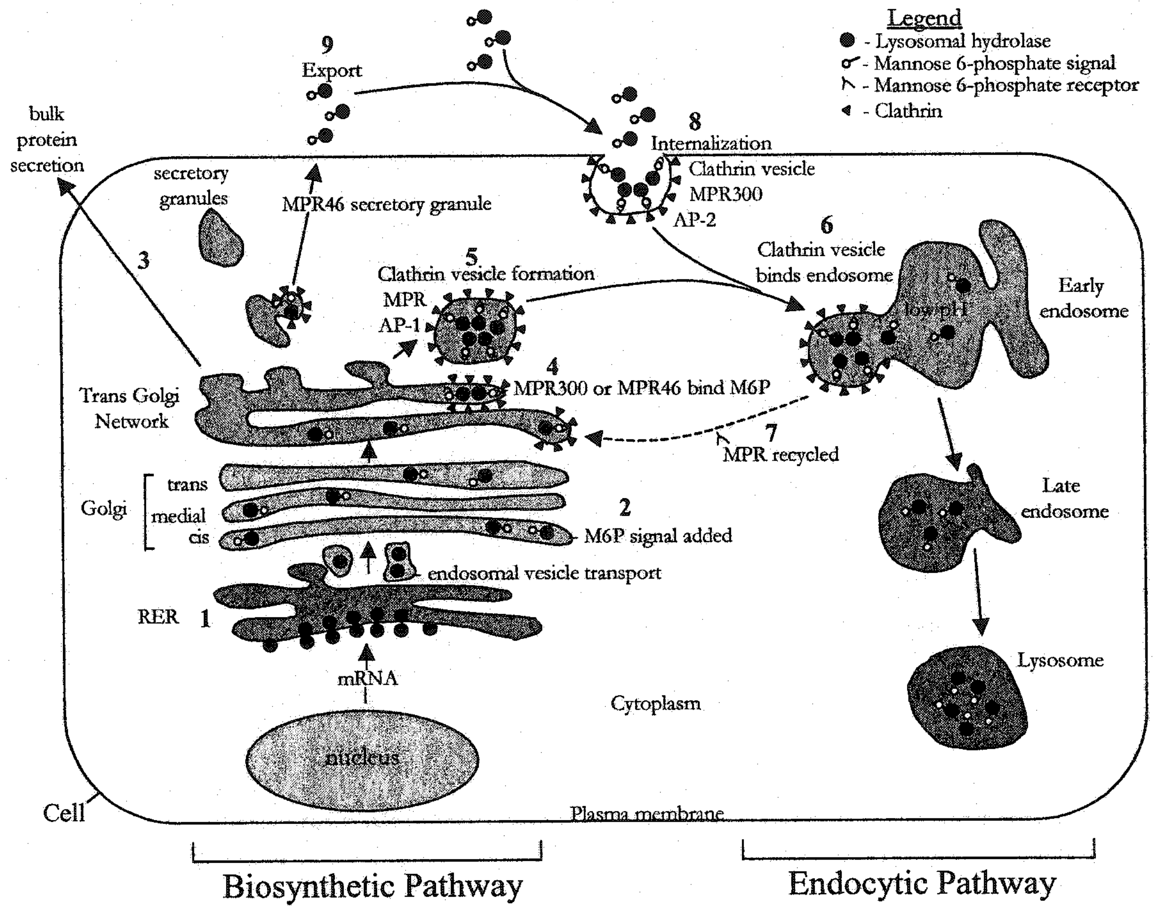
The delivery of materials to the lysosome occurs through a variety of different pathways. Materials may be endocytosed, such as cell-surface receptors targeted for destruction, and trafficked to lysosomes, or cytoplasmic proteins may be internalized within lysosomes. Various models of lysosome formation have been proposed. In the "maturation" model lysosomes mature from endosomal compartments by combining vesicles from the plasma membrane with vesicles derived from the trans-Golgi network, converting endosomes to late endosomes, and eventually lysosomes (Murphy *et al.* 1993). The ``vesicle-transport" model proposes that early endosomes, late endosomes, and lysosomes are separate, stable, compartments transporting materials from early endosomes through an endosomal carrier vesicle to late endosomes, which mature into lysosomes (Dell'Angelica *et al.* 2000), or to mature lysosomes directly (Murphy *et al.* 1993). The ``kiss and run" model proposes that endosomes and lysosomes undergo repeated cycles of fusion and fission permitting material transfer (Storrie and Desjardins 1996). The fusion-fission model is a variation of this, in which late endosomes fuse with lysosomes, to produce a hybrid that matures into lysosomes (Luzio *et al.* 2000). It is possible that cells employ more than one of these processes in lysosomal biogenesis (Mullins and Bonifacino 2001).

1.4.2 Lysosomal Enzymes

Lysosomes maintain a broad spectrum of soluble, acid-dependent, hydrolase degradative enzymes responsible for the digestion of materials in lysosomes (Dell'Angelica *et al.* 2000). Lysosomal hydrolases are carried by vesicular transport from their sites of synthesis in the endoplasmic reticulum, to the Golgi, and through endosomal vesicles to the lysosome (Figure 1.4.1.).

Figure 1.4.1. Lysosomal hydrolases are carried by vesicular transport from the site of synthesis in the endoplasmic reticulum (1) to the Golgi. Initial transport of lysosomal hydrolases to the Golgi is shared by all secretory proteins in bulk flow. Newly synthesized lysosomal hydrolases are modified in the cis-Golgi to receive a M6P signal (2). In the trans-Golgi network most lysosomal hydrolases are actively separated away from the main secretory pathway (3) via the M6P-signal binding MPR300 and MPR46 receptors in clathrin-coated pits (4). MPR46 or MPR300 interaction with AP-1 form clathrin-coated vesicles (5), which are transported to endosomes where they fuse with early or late endosomes (6). The M6P-receptors release bound lysosomal hydrolases due to the low pH and the M6P-receptors are recycled (7). MPR300 is also responsible for lysosomal enzyme internalization from the cell surface in conjunction with AP-2 (8) to form clathrin-coated vesicles at the cell-surface that recapture newly synthesized lysosomal enzymes that escaped sorting in the trans-Golgi network or that were actively exported in secretory granules by MPR46 (9). (Adapted from Borgne and Hoflack, 1998)

Figure 1.4.1 Lysosomal enzyme synthesis and transport.



The initial transport of lysosomal hydrolases to the Golgi is shared by all secretory proteins in bulk flow (Hille-Rehfeld 1995). However, newly synthesized lysosomal hydrolases are modified in the cis-Golgi with N-acetylglucosamine-1-phosphotransferase to receive a mannose 6-phosphate (M6P) signal on oligosaccharide chains (N-acetylglucosamine-1-phosphate on mannose residues) (Kaplan *et al.* 1977; von Figura and Klein 1979). In the trans-Golgi network the lysosomal hydrolases are actively separated away from the main secretory pathway via the M6P-signal binding M6P-receptors in clathrin-coated pits (Campbell and Rome 1983; Schulze-Lohoff *et al.* 1985; Geuze *et al.* 1985a; Geuze *et al.* 1985b; Lemansky *et al.* 1987; Lobel *et al.* 1988; Pohlmann *et al.* 1995; Munier-Lehmann *et al.* 1996).

The two M6P-receptors have been described according to their mass of 300 kD (MPR300) (Sahagian *et al.* 1981) and 46 kD (MPR46) (Hoflack and Kornfeld 1985). Both receptors are type I integral membrane proteins that expose an N-terminal ligand-binding domain into the lumen of membrane vesicles or the cell surface. The M6P-receptors are both involved in intracellular sorting of lysosomal enzymes into clathrin-coated vesicles for transport to lysosomes. Clathrin vesicle internalization involves binding signals in the cytoplasmic domains of MPR46 or MPR300 with a specific adaptor protein (AP-1), which connect clathrin triskelia to the cytoplasmic face of the membrane to form coated vesicles. The resulting clathrin vesicles are transported to endosomes. These vesicles specifically fuse with early or late endosomes through pairing of unique vesicle and target membrane SNARES (N-ethylmaleimide-sensitive factor attachment protein receptors) (Rothman and Wieland 1996), whereupon the M6P-receptors release bound lysosomal hydrolases due to the low pH (Griffiths *et al.* 1988). The M6P-receptors are then recycled back to the trans-Golgi network (Park *et al.* 1991). A second, and to date uncharacterized, M6P-independent sorting of lysosomal enzymes has also been described in the cells of patients with I-cell disease, which cannot generate M6P-signals on lysosomal enzymes (Glickman and Kornfeld 1993).

MPR46 contains one M6P-binding site (Tong and Kornfeld 1989) and, in addition to intracellular sorting, is also responsible for lysosomal enzyme export from cells (Chao *et al.* 1990). In contrast, MPR300 contains two M6P-binding sites (Tong *et al.* 1989) and is more efficient at intracellular sorting than MPR46. The MPR300 is a bifunctional molecule that binds and is important in the clearance of insulin-like growth factor II independently of M6P-ligands (Tong *et al.* 1988). In addition, MPR300 is responsible for lysosomal enzyme internalization from the cell surface in conjunction with AP-2 to form clathrin-coated pits that recapture newly synthesized lysosomal

enzymes that escaped sorting in the trans-Golgi network or that were actively exported by MPR46 (Chao *et al.* 1990; Watanabe *et al.* 1990; Koster *et al.* 1994). Clathrin-coated vesicles that form at the cell-surface are also transported to early endosomes and eventually lysosomes. As a result, lysosomal enzymes can be transferred from cell to cell, as first demonstrated by the cross-correction of cells from patients with different lysosomal storage diseases (Fratantoni *et al.* 1968). Thus, cells can secrete high levels of lysosomal enzymes which can be endocytosed by other cells via MPR46 and MPR300 (O'Brien et al. 1989; 1993) which also mediate intracellular lysosomal enzyme sorting and export.

Lysosomal enzymes can also be transported between cells independently of M6P-receptors. Lysosomal enzyme uptake in cells can be inhibited using an antibody against the M6P-receptor, but this has no effect on enzyme transfer between lymphocytes and fibroblasts that are in direct contact (Bou-Gharios *et al.* 1991). In contrast, the presence of an antibody that prevents cell adhesion inhibits lysosomal enzyme transfer. This mechanism of transfer involves uncoated vesicles localized near the site of cell contact (Bou-Gharios *et al.* 1991). Thus, lysosomal enzymes can also be transferred between cells through a contact-mediated mechanism.

Once transported to the lysosome, lysosomal hydrolases are responsible for the degradation of materials delivered into the lysosome, such as glycolipids, glycoproteins, and glycosaminoglycans. Abnormalities in normal hydrolase function may produce harmful accumulations of undegraded materials, resulting in a lysosomal storage disease.

1.4.3 Lysosomal Storage Diseases

Lysosomal storage diseases are inborn genetic diseases manifesting with excessive storage of undegraded metabolites in lysosomes. These metabolites accumulate in lysosomes because the normal hydrolytic cleavage of these complex molecules in lysosomes has been interrupted (Gieselmann 1995b). Due to the ubiquitous presence of lysosomes in almost all cell types, deficiencies of these enzymes cause multi-system anomalies, often with catastrophic consequences, and frequently result in early death. Although the incidence of each individual enzyme deficiency is rare, together the lysosomal storage diseases represent a significant burden of genetic disease at 1 in 7700 live births (Meikle *et al.* 1999).

1.4.4 Classification

The lysosomal storage diseases are grouped into four main categories based upon the affected pathway and the accumulated substrate: sphingolipidoses (sphingolipids), glycoproteinoses (glycoproteins), glycogenoses (glycogen), and mucopolysaccharidoses (glycosaminoglycans). There are more than 50 different lysosomal storage diseases for which a variety of different therapies have been investigated, including gene therapy (Table 1.4.1) (Gieselmann 1995a).

Sphingolipidoses are a heterogeneous group of diseases interfering with sphingolipid catabolism. This group encompasses neurodegenerative diseases, such as GM2 gangliosidoses metachromatic leukodystrophy, or type 2 and 3 Gaucher's disease, as well as non-neuropathic diseases, such as type 1 Gaucher's disease. The diseases may also affect bone marrow, spleen, liver and bone resulting in massive hepatosplenomegaly, anaemia, thrombopenia and bone lesions (Desnick and Bishop 1995)

Glycoproteinoses are rare lysosomal diseases due to an abnormal catabolism of oligosaccharide chains from glycoproteins and glycolipids. Storage of these materials manifest clinical features such as neurodegeneration, hepatomegaly, coarse facial features, and dysostosis multiplex (Thomas and Beaudet 1995).

Glycogen storage disorders are characterized by the intralysosomal accumulation of glycogen in numerous tissues (Hirschhorn 1995). The infantile form, Pompe's disease, is rapidly fatal with massive cardiomegaly and progressive muscle weakness, while the adult form, Glycogenosis type II, is a slower progressive disorder with manifestations restricted to skeletal muscle.

Mucopolysaccharidoses (MPS) affect the degradation of glycosaminoglycans, which progressively accumulate in the lysosomes. The different MPS diseases share many clinical features to variable degrees, including organomegaly, dysostosis multiplex, and coarse facies. In addition, vision, hearing, cardiovascular function and joint mobility can be affected. Neurodegeneration occurs in MPS I (Hurler syndrome), MPS II (Hunter's syndrome), MPS III (Sanfilippo's syndrome), and MPS VII (Sly), but is absent in MPS IV (Morquio) and MPS VI (Maroteaux-Lamy) (Neufeld and Muenzer 1995).

Table 1.4.1 Features of the lysosomal storage diseases.

Storage Disease	*Enzyme deficiency*	*Clinical Features*
I. Sphingolipidoses		
Niemann-Pick Type A and B	Sphingomyelinase	IA: Neuropathic, hepatosplenomegaly, often fatal in infancy. IS: Non-neuropathic
Farber's disease	Ceramidase	Neuopathic, death by 2 yrs, organomegaly
Fabry disease	α-galactosidase A	Neuropathic, Pain in extremities, myocardial ischemia
Gaucher's disease	β-glucosidase	Type 1: Non-neuropathic, bone pain Type 2: Neuropathic, infantile, death by 1yr Type 3: Neuropathic, death in childhood
GM1-Gangliosidosis (Landing's)	β-galactosidase	Type 1: Neuropathic, seizures. Type 2: Slow motor development
GM2-Gangliosidosis (Tay Sachs)	β-hexosaminidase A	Motor weakness, deafness, blindness
Krabbe's Disease	Galactosyl-ceramidase	Psychomotor retardation, hyperthermia
Metachromatic leukodystrophy	Arylsulfatase A	Neuopathic, progressive dementia
Sandhoff Disease	β-hexosaminidase A & B	As Tay Sach's plus mild neuropathy
II. Glycoproteinoses		
Aspartyl-glucosaminuria	N-acetyl-β-glucosaminidase	Neuropathy, motor retardation, skeletal
Fucosidosis	α-fucosidosis	Type 1: Cardiomegaly motor retardation. Type 2: Mild, lesions
Galactosialidosis	Cathepsin A	Neuopathic, coarse facies, organomegaly
α-Mannosidosis	α-mannosidase	Hepatomegaly, bone changes, psychomotor retardation
β-Mannosidosis	β-D-mannosidase	Hepatomegaly, bone changes, retardation
Schindler's disease	α-N-acetyl-galactosaminidase	Developmental delay, motor funct., blindness
Sialidosis	Neuraminidase	Type 1: Neuropathic dysmorphic Type II: Red spots on macula
III. Glycogen Storage		
Pompe's Disease	α-1,4-glucosidase	Cardiomegaly, infantile to adult presentation

Table 1.4.1 Features of the lysosomal storage diseases (cont'd)

Storage Disease	*Enzyme deficiency*	*Clinical Features*
IV. Mucopolysaccharidoses		
MPS I (Hurler)	α-L-iduronidase	Neuropathic, corneal opacity, progressive physical debilitation, organomegaly, coarse facies
MPS I (Scheie)	α-L-iduronidase	Mild MPS I, corneal opacity, mild or absent mental retardation
MPS II (Hunter)	Iduronate-2-sulfate sulfatase	As MPS I, can be neuropathic
MPS IIIA (Sanfillipo)	Hepara*n* *N*-sulfaminidase	Severe neuropathy
MPS IIIB (Sanfillipo)	N-acetyl-α -glucosaminidase	Severe neuropathy
MPS IIIC (Sanfillipo)	α-glucosamide-*N*-acetyltransferase	Severe neuropathy
MPS IIID (Sanfillipo)	*N*-acetyl-glucosa-mine-6-sulphatase	Severe neuropathy
MPS IVA (Morquio)	N-acetylgalactos-amine-6-sulfatase	Non-neuropathic, skeletal abnormalities, small stature
MPS IVB (Morquio)	β-galactosidase	Non-neuropathic, cloudy corneas
MPS VI (Maroteaux-Lamy)	Arylsulfatase B	Non-neuropathic, corneal opacity, short stature, dysostosis multiplex
MPS VII (Sly)	β-glucuronidase	Neuropathy, skeletal abnormalities, coarse facies, organomegaly, corneal clouding
MPS IX	Hyaluronidase-1	Tissue masses, stature
V. Disorders of Multiple Storage Products		
Type 1 Ceroid Lipofuscinosis	Palmitoyl protein thioesterase	Neuropathic, infantile, blindness, seizures
Type 2 Ceroid Lipofuscinosis (Jansky-Bielsch.)	Tripeptidyl peptidase I	Neuropathic, seizures, late infantile, blindness
Mucolipidosis II (I-cell)	N-acetyl-glucosaminl-phosphotransferase	Neuropathic, thoracic deformities, hepatosplenomegaly
Mucolipidosis III	UDP-N-acetyl-glucosemia glyco protein	Mild I-cell
Mucolipidosis IV	Mucolipin-1	Retinopathy,hypotonia
Multiple sulfatase deficiency (Austin's disease)	Arylsulfatases A, B, and C	Neuopathic, skeletal deformities, hepatosplenomegaly
Wolman's disease	Lysosomal Acid Lipase	Hepatosplenomegaly, death in infancy
Pycnodysostosis	Cathepsin K	Osteosclerosis, stature
Cystinosis	Cystinosin	Nephropathic, retina
Sialic acid storage diseases (Salla)	Sialic acid carrier	Hepatosplenomegaly, coarse facies
Niemann Pick C	NPC1, NPC2	Ataxia, death 12-20 yr

(Partially adapted from Neufeld and Muenzer 1995; Ross and Chang 1999)

1.4.5 Animal Models

1.4.5.1 Sphingolipidoses

Animal models of many human lysosomal storage diseases are available, providing a range of models upon which to investigate different therapies. At least ten animal models of the sphingolipidoses have been described. The Niemann-Pick disease mouse exhibits severe neurodegeneration and death by eight months of age (Otterbach and Stoffel 1995; Horinouchi *et al.* 1995). Krabbe disease in the twitcher mouse, dog, and rhesus monkey have severe tremors, hypertonia, and incoordination with very early death (Suzuki 1995; Baskin *et al.* 1998; Wenger *et al.* 1999). Metachromatic leukodystrophy mice exhibit mild lipid storage features causing deafness and neuromotor abnormalities (Gieselmann *et al.* 1998). GM1-gangliosidosis mouse and canine models resemble the human disease (Ahern-Rindell *et al.* 1996; Oshima 1998). The Fabry disease mouse displays lipid accumulation similar to that of patients (Ohshima *et al.* 1997). The Sandhoff disease mice have fatal neurodegeneration and muscle weakness (Phaneuf *et al.* 1996). There are three mouse models of Tay-Sachs disease with a phenotype that is milder than human patients (Yamanaka *et al.* 1994; Cohen-Tannoudji *et al.* 1995; Liu *et al.* 1997). A mouse model of Wolman's disease was generated with a phenotype that closely resembles the human disease with massive accumulation of triglycerides and cholesterol esters (Du *et al.* 1998).

1.4.5.2 Glycoproteinoses

For the glycoproteinoses, animal models have been described for α-mannosidosis in the cat and goat (Vandevelde *et al.* 1982; Jezyk *et al.* 1986; Pearce *et al.* 1990), and canine fucosidosis models have been described in the U.K., U.S.A., and Australia (Hartley *et al.* 1982; Littlewood *et al.* 1983; Taylor *et al.* 1989a; Skelly *et al.* 1996; Smith *et al.* 1996; Occhiodoro and Anson 1996). A mouse model is also available for aspartylglucosaminuria with a phenotype consistent with the human pathogenesis (Kaartinen *et al.* 1996; Jalanko *et al.* 1998). Mouse models also exist for Sialidosis (Rottier *et al.* 1998), Galactosialidosis (Zhou *et al.* 1995), and GM1 Galactosialidosis (Itoh *et al.* 2001).

1.4.5.3 Glycogenoses

An animal model of Pompe's disease has been described in Japanese quail (Van Hove 1998).

1.4.5.4 Mucopolysaccharidoses

Several animal models have been described for the mucopolysaccharidoses, including MPS I in the mouse, cat, and dog which all exhibit an affected phenotype (Haskins *et al.* 1979a; Haskins *et al.* 1979b; Shull *et al.* 1982; Spellacy *et al.* 1983; Clarke *et al.* 1997). MPS IIIA and IIIB models of Sanfilippo syndrome exists in mice (Bhaumik *et al.* 1999; Yu *et al.* 2000) and an MPS IIID model was found in a Nubian goat (Thompson *et al.* 1992). Mouse, rat, and cat models of MPS VI Marteaux-Lamy syndrome display skeletal abnormalities and lysosomal lesions (Jezyk *et al.* 1977; Haskins *et al.* 1980; Gasper *et al.* 1984; McGovern *et al.* 1985; Yoshida *et al.* 1993; Evers *et al.* 1996). Dog and cat models of MPS VII closely resemble the human disease with hepatomegaly and vacuolated cytoplasm in many cell types including neurons (Haskins *et al.* 1984; Schuchman *et al.* 1989; Haskins *et al.* 1991; Sheridan *et al.* 1994; Gitzelmann *et al.* 1994; Ray *et al.* 1998; Fyfe *et al.* 1999; Ray *et al.* 1999). However, the most extensively characterized animal model of all lysosomal storage diseases is the murine model of the MPS VII, and is described in further detail in Section 1.4.6.6 (Birkenmeier *et al.* 1989).

1.4.6 Mucopolysaccharidosis VII

Mucopolysaccharidosis VII (MPS VII, Sly Syndrome, 253220) is a progressively degenerative autosomal-recessive lysosomal storage disease caused by a deficiency of β-glucuronidase.

1.4.6.1 Clinical Features

MPS VII presents in early childhood, often before 4 years of age, with a range of phenotypes and clinical severity. The major clinical disabilities include coarse facies, growth retardation, neurodegeneration, corneal clouding, hepatosplenomegaly, skeletal abnormalities, developmental delay, and decreased life span (Neufeld and Muenzer 1995). Unusually mild cases have been reported in patients reaching over 19 years of age (Gitzelmann *et al.* 1978; de Kremer *et al.* 1992; Neufeld and Muenzer 1995). However, with complete enzyme deficiency severe neonatal forms result in hydrops fetalis and fetal death (Kagie *et al.* 1992; Stangenberg *et al.* 1992). The leading cause of death among all MPS patients is anesthetic complications and coronary artery disease leading to ischemia and infarction with sudden cardiovascular collapse (Neufeld and Muenzer 1995). A phenotypically normal, compound-heterozygous individual was identified with 6-10% of normal β-glucuronidase activity, suggesting that this level of enzyme could be curative (Chabas *et al.* 1991).

1.4.6.2 Genetics

The gene for human β-glucuronidase was first localized to chromosome 7 using somatic cell hybrids and to 7q11.23-q21 by dosage analysis of chromosomal aberrations (Knowles *et al.* 1977; Ward *et al.* 1983). The gene was further localized to 7q21.11 using large chromosomal deletions in patients with either normal or deficient β-glucuronidase activity (Frydman *et al.* 1986; Fagan *et al.* 1989; Schwartz *et al.* 1991). The human β-glucuronidase cDNA was isolated and sequenced before the exact position of the gene was known (Guise *et al.* 1985; Oshima *et al.* 1987). At the genomic level the β-glucuronidase gene spans 21 kb and contains 12 exons ranging from 85 to 376 bp in length (Miller *et al.* 1990). Two forms of β-glucuronidase mRNA arise by alternate splicing of exon 6 with the shorter transcript encoding a non-functional enzyme (Oshima *et al.* 1987; Miller *et al.* 1990).

1.4.6.3 β-glucuronidase

β-glucuronidase (75 kD, 651 amino acids) is a homotetrameric (300 kD) glycoprotein acid hydrolase that is normally localized to lysosomes where it degrades chondroitin-, dermatan-, and heparan sulfate glycosaminoglycans by removing terminal β-glucuronic acid residues (Figure 1.4.2) (Levvy 1953; Tomino *et al.* 1975). The half-life of β-glucuronidase in tissues is 2-3 days (Smith and Ganschow 1978; Vogler *et al.* 1993). The X-ray structure of β-glucuronidase has been determined to 2.6 Å resolution (Jain *et al.* 1996). The active site of the enzyme is formed from a large cleft at the interface of two monomers where residues Glu 451 and Glu 540 are proposed to be important for catalysis (Jain *et al.* 1996).

1.4.6.4 Pathology

β-glucuronidase deficiency in MPS VII patients manifests with large distended lysosomes engorged with chondroitin-, dermatan-, and heparan sulfate glycosaminoglycans (Sly *et al.* 1973; Watts and Gibbs 1986; Neufeld and Muenzer 1995). The excessive accumulation of undegraded glycosaminoglycans eventually leads to cell and organ dysfunction throughout the body. The glycosaminoglycans normally degraded by β-glucuronidase are long unbranched chains of hydrophobic polysaccharides that occupy a large volume relative to their mass. The chains are made up of disaccharide repeat units, which are distinguished by their sulfate groups, sugar residues, and the linkages between sugar units. In total, there are seven glycosaminoglycan forms: hyaluronic acid, chondroitin 4-

sulphate, chondroitin 6-sulphate, dermatan sulfate, heparin, heparan sulfate, and keratan sulfate (Watts and Gibbs 1986).

1.4.6.5 Incidence

The incidence of MPS VII is rare, occurring in less than 1 in 216,000 live births (Neufeld and Muenzer 1995). The combined incidence of the mucopolysaccharidoses is approximately 1 in 22,000 to 25,000 births (Poorthuis *et al.* 1999), while the combined incidence of all the lysosomal storage diseases is approximately 1 in 7700 life births (Meikle *et al.* 1999). Although individually rare, together the lysosomal storage diseases represent a substantial burden of genetic disease. Although MPS VII is rare, this disease closely resembles other mucopolysaccharidoses and lysosomal storage diseases with its characteristic neurodegeneration and organomegaly. Due to the well-characterized mouse model, MPS VII provides an excellent model upon which to develop novel therapies for all lysosomal storage diseases and neurodegeneration.

Figure 1.4.2 Degradation of glycosaminoglycans by β-glucuronidase.

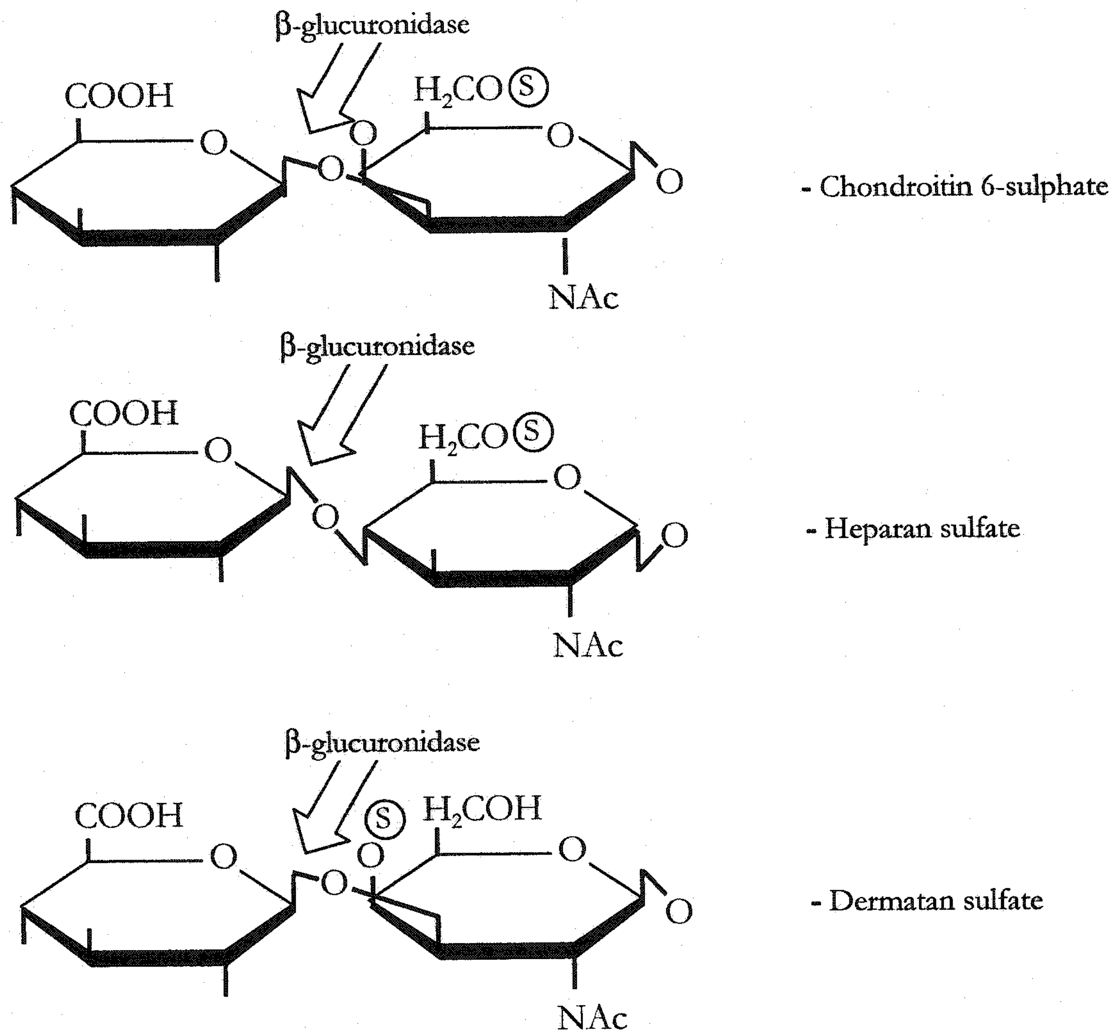


Figure 1.4.2 Degradation of glycosaminoglycans by β-glucuronidase.

β-glucuronidase is an exoglycosidase that hydrolyzes the cleavage of glucuronic acid residues from the terminal ends of chondroitin sulfate, heparan sulfate and dermatan sulfate.

1.4.6.6 MPS VII Animal Models

Birkenmeier and co-workers discovered the MPS VII mouse model in 1989. The MPS VII mouse has been well characterized and closely resembles the human disease with characteristic dysmorphic facial features, corneal clouding, abnormal gait, auditory deficits, dwarfism, skeletal deformities, joint abnormalities, severe hepatosplenomegaly, and a six-fold reduction of life span, averaging 5.5 months (Birkenmeier *et al.* 1989; Vogler *et al.* 1990b; Sands and Birkenmeier 1993; Sands *et al.* 1995). Furthermore, the neurodegeneration in MPS VII mice leads to cognitive and neurological deficits in the CNS (Vogler *et al.* 1990a; Chang *et al.* 1993a; Bastedo *et al.* 1994; Levy *et al.* 1996). The MPS VII mouse carries a single base pair (cytosine) deletion in exon 10 of the murine β-glucuronidase gene resulting in a frameshift mutation and early stop codon (Sands and Birkenmeier 1993). The MPS VII mouse has more than a 200-fold decrease in β-glucuronidase mRNA, and virtually no β-glucuronidase enzyme activity. The murine β-glucuronidase gene is made up of 12 exons over 20 kb on mouse chromosome 5. Transgenic studies have confirmed that the MPS VII mouse is solely affected by a deficiency of β-glucuronidase, because transgenic mutant MPS VII mice with a normal copy of the human β-glucuronidase gene exhibit a normal phenotype (Kyle *et al.* 1990).

Overall, the MPS VII mouse provides an excellent, well-characterized model of a relatively simple, monogenic neurodegenerative lysosomal storage disease. All the current experimental protocols and gene therapy vectors currently available have been tested in the MPS VII mouse, making this model extremely valuable for the direct comparison of different treatment strategies. Moreover, larger canine and feline models of MPS VII exist, enabling direct scale-up to a large animal model of the same disease. As a result, the MPS VII mouse provides an exceptional starting point upon which to develop and compare novel therapies for lysosomal storage diseases.

1.4.7 Treatment

1.4.7.1 Rationale

The treatment of lysosomal storage diseases by means of supplying the missing lysosomal enzyme is especially feasible because of the M6P-signal on lysosomal enzymes (Sly *et al.* 1978). The M6P-signal targets lysosomal enzymes to lysosomes via the M6P-receptor (Lobel *et al.* 1988; Pohlmann *et al.* 1995; Munier-Lehmann *et al.* 1996). Since M6P-receptors on cell surfaces are also capable of lysosomal enzyme capture and transport to the lysosomes (Sly *et al.* 1978), an extracellular source of enzyme, either infused intravenously or produced directly

within the patient, provides a strategy to overcome the enzyme deficiency. In addition, the lysosomal storage diseases provide an excellent model upon which to develop novel therapeutic strategies because these recessive diseases generally require low levels of therapeutic enzyme. In fact, many so-called pseudo-deficient patients with less than 10% of normal enzyme levels have been identified as being phenotypically normal (Thomas 1994), suggesting that low levels of enzymes are sufficient to limit or stop the onset of the disease (Chabas *et al.* 1991; Brooks *et al.* 1991). Thus, many treatments for the lysosomal storage diseases have been investigated (Table 1.4.2).

The feasibility of treating a lysosomal storage disease was first demonstrated *in vitro* by the cross-correction of cells from patients with different lysosomal storage diseases (MPS I and MPS II) with secreted corrective lysosomal enzymes (Fratantoni *et al.* 1968; Wiesmann and Neufeld 1970). This was subsequently shown in the cells of an MPS VII patient with the addition of β-glucuronidase to the cell-culture media(Sly *et al.* 1973). The affected phenotype of cells has also been corrected with enzyme replacement or gene transfer for most of the lysosomal storage diseases, including MPS I (Anson *et al.* 1992a; Unger *et al.* 1994; Fairbairn *et al.* 1996), MPS VI (Anson *et al.* 1992b), and MPS VII (Wolfe *et al.* 1990; Taylor and Wolfe 1994; Taylor and Wolfe 1997b).

1.4.7.2 Plasma Infusion

Twenty years before the first gene therapy trial, plasma from a normal individual was infused into MPS I patients to replenish the patient's supply of α-L-iduronidase. These plasma infusion trials claimed both clinical and chemical improvements in MPS I patients (Di Ferrante *et al.* 1971). However, further plasma infusion trials were disappointing (Di Ferrante *et al.* 1971).

1.4.7.3 Fibroblast Transplantation

In order to provide a higher dose of lysosomal enzyme to patients, fibroblasts from normal individuals were transplanted into patients with MPS I, MPS II, and MPS IIIB along with immunosuppression. It was hoped the fibroblasts would survive and produce the normally deficient enzymes, thereby maintaining a continuous source of therapeutic enzyme directly within the patients. However, the therapeutic enzymes were not detected and the clinical course of the disease was not altered in any patients following fibroblast transplantation (Gibbs *et al.* 1983).

Table 1.4.2 Treatment of lysosomal storage diseases.

Storage Disease	*Therapy*	*Recipient*	*Reference*
I. Sphingolipidoses			
Niemann-Pick Type A and B	BMT	Human	Hsu *et al.*, 1999
	BMT	Human	Vellodi *et al.*, 1987
	BMT	Mouse	Miranda *et al.*, 1998
Fabry disease	ER	Human	Eng *et al*, 2001
	ER	Human	Schiffmann *et al*, 2000
	BMT	Mouse	Oshima *et al.*, 1999
	Adeno	Mouse	Ziegler *et al.*, 1999
Gaucher's disease	ER	Human	Hoppe, 2000
	ER	Human	Mackenzie *et al.*, 1998
	BMT	Human	Rappeport, 1984
	ER	Human	Grabowski, 1993
	Rv	Human	Nimgaonkar, 1994
	ER	Rat	Zirzow *et al*, 1999
	Rv	Mouse	Correll *et al*, 1989
GM1-Gangliosidosis (Landing's)	BMT	Canine	O'Brien *et al.*, 1990
GM2-Gangliosidosis (Tay Sachs)	Adeno	Mouse	Guidotti *et al.*, 1999
	CNS-P	Mouse	Lacorazza *et al*, 1996
Krabbe's Disease	BMT	Mouse	Suzuki, 1988
	BMT	Mouse	Hoogerbrugge, 1988
	BMT	Mouse	Hoogerbrugge, 1989
Metachromatic leukodystrophy	BMT	Human	Kapaun *et al.*, 1999
	BMT	Human	Malm *et al.*, 1996
	BMT	Human	Imaizumi *et al.*, 1994
	BMT	Mouse	Gieselmann *et al*,1998
Sandhoff Disease	BMT	Mouse	Norflus *et al*, 1998
II. Glycoproteinoses			
Aspartyl-glucosaminuria	Ad	Mouse	Peltola *et al.*, 1998
	BMT	Human	Laitinen *et al.*, 1997
Fucosidosis	BMT	Human	Vellodi *et al.*, 1995
	Rv-BMT	Canine	Ferrara *et al.*, 1997
	BMT	Canine	Taylor *et al.*, 1986-92
α-Mannosidosis	BMT	Human	Wall *et al.*, 1998
	BMT	Human	Will *et al.*, 1987
	BMT	Cat	Walkley *et al.*, 1994
β-Mannosidosis	Liver Cells	Goat	Pearce *et al.*, 1990
Sialidosis	Corneal transplant	Human	Bohnke *et al.* 1999

Table 1.4.2 Treatment of lysosomal storage diseases (cont'd)

Storage Disease	*Therapy*	*Recipient*	*Reference*
III. Glycogen Storage			
Pompe's Disease	ER	Quail	Van Hove, 1998
IV. Mucopolysaccharidoses			
MPS I (Hurler)	ER	Human	Kakkis *et al*, 2001
	BMT	Human	Peters *et al.*, 1996
	Rv	Human	Salvetti *et al.*, 1994
	BMT	Human	Hopwood *et al.*, 1993
	BMT	Human	Aramburu *et al.*, 1990
	BMT	Human	Krivit, Whitley, 1987
	Rv-BMT	Canine	Lutzko *et al.*, 1999
	Rv-BMT	Canine	Lutzko *et al.*, 1999b
	BMT	Canine	Breider *et al.*, 1989
	DNA	Canine	Shull *et al.*, 1996
	Rv	Canine	Shull *et al.*, 1996
	ER	Canine	Shull *et al.*, 1994
	Rv	Mouse	Salvetti *et al.*, 1995
MPS I (Scheie)	BMT	Human	Navarro *et al.* 1991
	BMT	Human	Costa *et al.*, 1993
MPS II (Hunter)	Rv-BMT	Human	Stroncek *et al.*, 1999
	BMT	Human	Imaizumi *et al.*, 1994
	BMT	Human	Bergstrom *et al.*, 1994
	BMT	Human	Resnick *et al.*, 1992
MPS IIIA (Sanfillipo)	BMT	Human	Resnick *et al.*, 1992
MPS IIIB (Sanfillipo)	BMT	Human	Vellodi *et al.*, 1992
MPS VI (Maroteaux-Lamy)	BMT	Human	Imaizumi *et al.*, 1994
	BMT	Human	Krivit *et al.*, 1984
	Rv-BMT	Feline	Simonaro *et al.*, 1999
	Rv-BMT	Feline	Yogalingam *et al* 1999
	ER	Feline	Bielicki *et al.*, 1999
	ER	Feline	Byers *et al.* 1997
	ER	Feline	Brookes *et al.*, 1997
	BMT	Feline	Dial *et al.*, 1997
	ER	Feline	Crawley *et al.*, 1996
	BMT	Feline	Norrdin *et al.* 1995
	BMT	Feline	Gasper *et al.*, 1984
	BMT	Rat	Simonaro *et al.*, 1997
MPS VII (Sly)	BMT	Human	Yamada *et al.*, 1998
	Rv	Canine	Haskins *et al.*, 2001
	ER	Mouse	(Table 1.4.3)
	BMT	Mouse	(Table 1.4.3)
	Rv-BMT	Mouse	(Table 1.4.3)
	Rv	Mouse	(Table 1.4.3)
	CNS-P	Mouse	(Table 1.4.4)
	Herpes	Mouse	(Table 1.4.4)
	AAV	Mouse	(Table 1.4.3, 1.4.4)
	Adeno	Mouse	(Table 1.4.3, 1.4.4)
	Imm-Iso	Mouse	(Table 1.4.3, 1.4.4)

Table 1.4.2 Treatment of lysosomal storage diseases (cont'd)

Storage Disease	*Therapy*	*Recipient*	*Reference*
V. Disorders of Multiple Storage Products			
Mucolipidosis II	BMT	Human	Imaizumi *et al.* 1994
(I-cell)	BMT	Human	Yamaguchi *et al.* 1989
	BMT	Human	Kurobane *et al.* 1986

Legend: Abbreviations: BMT: Bone Marrow Transplant; Rv-: Retroviral modified; Rv: Retroviral vector; ER: Enzyme Replacement; DNA: naked plasmid DNA; Adeno: ΔE1/E3 adenoviral vector; AAV: adeno-associated viral vector; Herpes: herpesvirus vector; CNS-P: Retroviral modified CNS progenitor cells; Imm-Iso: Immuno-isolation gene therapy. (Partially adapted from Neufeld and Muenzer 1995; Ross and Chang 1999)

1.4.7.4 Bone Marrow Transplantation

Bone marrow transplantation (BMT) for the treatment of lysosomal storage diseases was investigated as an alternative to plasma infusion and fibroblast transplantation because bone marrow is enriched with long-lived stem cells. The results of BMT have been variable, but BMT has been effective for some lysosomal storage disease symptoms if diagnosed and treated early. BMT in the feline and canine models of α-mannosidosis and fucosidosis slowed the progression of neurological disease; however, treatment had to be initiated at a very early age (Taylor *et al.* 1986a; Taylor *et al.* 1986b; Taylor *et al.* 1987; Taylor *et al.* 1988; Taylor *et al.* 1989a; Taylor *et al.* 1989b; Taylor *et al.* 1992; Walkley *et al.* 1994). Following successful HLA-matched BMTs, patients with I-cell disease and the mucopolysaccharidoses (MPS I, MPS IIIB, MPS VI, and MPS VII) have shown some peripheral improvements, such as reduced dermal storage, improved facial features, and peripheral enzyme activity (Kurobane *et al.* 1986; Yamaguchi *et al.* 1989; Aramburu *et al.* 1990; Navarro *et al.* 1991; Vellodi *et al.* 1992; Hopwood *et al.* 1993; Costa *et al.* 1993; Imaizumi *et al.* 1994; Vellodi *et al.* 1995; Yamada *et al.* 1998; Hsu *et al.* 1999; Herskhovitz *et al.* 1999). Although complicated by moderately severe graft-versus-host disease, BMT for a young 8-month-old fucosidosis patient resulted in less developmental delay and improved MRI scans compared to an untreated sibling (Vellodi *et al.* 1995)

However, BMT has also been unsatisfactory for the treatment of several other disease manifestations, such as neurological and skeletal deterioration. Compared to the previous success of BMT for MPS III patients, a separate study revealed a diminished capacity to correct the hepatocyte storage lesions (Resnick *et al.* 1992). BMTs for MPS VI in rats, felines, and humans improved some peripheral features, but the skeletal manifestations of the disease worsened (Simonaro *et al.* 1997; Dial *et al.* 1997; Ohshima *et al.* 1999; Herskhovitz *et al.* 1999). BMT for Fabry's disease similarly improved peripheral features, but the neurological manifestations did not improve (Norrdin *et al.* 1995; Simonaro *et al.* 1997; Takenaka *et al.* 1999; Takenaka *et al.* 2000; Qin *et al.* 2001). BMT in the Sandhoff mouse corrected biochemical deficiencies in somatic tissues, but the treatment was unable to reduce storage pathology in the brain, although treatment improved longevity and neurological manifestations (Norflus *et al.* 1998). Although, BMT in a 3-month old canine model of GM1-gangliosidosis resulted in normalized enzyme activity in blood, the study concluded that BMT was an ineffective treatment because neurological deterioration continued and lysosomal storage in the CNS was undiminished (O'Brien *et al.* 1990). Similarly, BMT for Niemann-

Pick type C disease in a child could not halt the neurological deterioration (Hsu *et al.* 1999). BMT for metachromatic leukodystrophy slowed the disease progression in one patient (Kapaun *et al.* 1999), but did not stop the progressive neurodegeneration in two other trials (Imaizumi *et al.* 1994; Malm *et al.* 1996). BMT for a child with severe α-mannosidosis resulted in a cognitive stabilization (Wall *et al.* 1998), although in a separate patient autopsy 18 weeks post-BMT the authors concluded that the treatment did not reduce lysosomal storage within the brain (Will *et al.* 1987). Therefore, it has become apparent that BMT is not suitable for some lysosomal storage diseases that manifest neurodegeneration (Gieselmann and von Figura 1990).

For the treatment of MPS VII, BMT corrected the excessive peripheral organ lysosomal storage in mice, but did not correct the CNS pathology and behavioral manifestations. After bone marrow ablation with up to 10 Gy of radiation, BMT from syngeneic normal animals resulted in detectable β-glucuronidase activity, reduced lysosomal storage in spleen, liver, and cornea, and a three-fold increase in life-span, (Birkenmeier *et al.* 1991; Sands *et al.* 1993; Poorthuis *et al.* 1994; Sands *et al.* 1995). Although β-glucuronidase was detected at low levels in the CNS, there were no CNS improvements except for a partial correction of lysosomal storage in the meninges and perivascular cells (Birkenmeier *et al.* 1991; Sands *et al.* 1993; Bastedo *et al.* 1994; Poorthuis *et al.* 1994). In addition, radiation-induced toxicity was a significant problem in both normal and treated mutant mice. Low doses of radiation (2 Gy) resulted in decreased bone length, retinal disruption, and cerebellar disruption. Higher doses of radiation (4 Gy) also produced gross reductions in cerebellar mass, disorganization of the cortical layer, and loss of Purkinje and granule cells, and at the highest dose of radiation, ataxia (6 Gy) (Sands *et al.* 1993). Moreover, the behavioral deficits of the mutant mice were not corrected with BMT, and even with a low dose of radiation (2 Gy) both normal and mutant mice showed significant radiation-induced toxicity with severely impaired behavior (Bastedo *et al.* 1994).

BMT is the only therapy that has been applied to human MPS VII. An HLA-identical BMT in a young MPS VII patient restored low levels of enzyme activity and produced peripheral clinical improvements, including reduced bouts of recurrent infection and improved motor function(Yamada *et al.* 1998). However, the neurological damage could not be reversed (Yamada *et al.* 1998). The results of this human study echoed the murine BMT studies, demonstrating the potential to treat peripheral symptoms in humans. However, the

deleterious effects of radiation, the inability to halt neurodegeneration, and the scarcity of HLA-matched donors has strongly pushed the search for alternative treatments for MPS VII and other lysosomal storage diseases.

Until recently, BMT has been the only treatment available for lysosomal storage diseases. Although long-term improvements have occurred in some HLA-matched BMT, few patients have HLA-identical siblings or donors and most (70%) cannot find compatible donors (Parkman 1986). Moreover, not all matched BMTs are successful. BMT presents a difficult dilemma between a high dose of radiation potentially causing death, versus a low dose of radiation allowing graft rejection (Vellodi *et al.* 1992; Simonaro *et al.* 1997). HLA-matched BMTs for lysosomal storage diseases carry a 10% chance of acute mortality (20-25% unmatched), a 30-37.5% chance of graft-versus-host disease, and a 49% two-year survival rate (Parkman 1986; Peters *et al.* 1996). Due to the difficulty in locating donors, for most affected families the only available medical support is palliative care until death occurs, and prenatal diagnosis. Although BMT can achieve a favorable outcome in some patients, future protocols must address the high risk of graft failure and the impact of graft-versus-host disease, and the lack of neurological improvements.

1.4.7.5 Enzyme Replacement

As an alternative to finding HLA-matched BMT donors, infusions of purified recombinant enzyme has been used to treat lysosomal storage diseases. Enzyme replacement has been investigated in several animal models of lysosomal storage diseases. The dog model of MPS I treated with purified recombinant human α-L-iduronidase (25,000 U (0.1 mg)/kg/week, IV) demonstrated low levels of α-iduronidase and histologic improvement in the peripheral organs (Shull *et al.* 1994; Kakkis *et al.* 1996). Similar results were also observed in the feline model of MPS I (Kakkis *et al.* 2001b). However, histologic improvement was not observed in the CNS and antibodies to the human α-L-iduronidase were induced in all treated dogs and more than 80% of the cats. Moreover, in a higher dose (125,000 U/kg/week), all the treated cats developed antibodies (Kakkis *et al.* 2001b). Since MPS VI generally lacks CNS involvement, it was thought this disease could be more amenable to enzyme replacement. MPS VI cats that received infusions of recombinant arylsulfatase B showed reduced progression of the disease. Although skeletal morphology remained affected at 1.5 mg/kg, a 5 mg/kg dose partially improved skeletal morphology, and a 5 mg/kg dose initiated from birth nearly normalized skeletal pathology, except for corneal keratocytes and cartilage chondrocytes (Crawley *et al.* 1996; Byers *et al.* 1997; Brooks *et al.* 1997; Bielicki *et al.* 1999). Enzyme

replacement has also corrected the pathology and symptoms of Pompe's disease affected Quail, and is being examined in an animal model of fucosidosis (Van Hove 1998; Bielicki *et al.* 2000).

For the treatment of MPS VII, recombinant β-glucuronidase administered intravenously to newborn mice provided enzyme to peripheral tissues and significantly reduced lysosomal storage during the first 6 wk of life (Sands *et al.* 1994). Although the brain had detectable β-glucuronidase at 6 weeks with some mild improvements in perivascular cells, the meninges, and cerebral and hippocampal neurons, the disease pathology was not improved in Purkinje cells, glial cells, or chondrocytes. The CNS improvements resulted from enzyme entry into the CNS during the first two weeks of life. During these first two weeks, prior to development of a functional BBB, infused enzyme could enter the CNS and temporarily delay the onset of lysosomal storage. This was well-characterized in a series of staggered enzyme injections, beginning 0 to 35 days after birth, where a positive response to treatment in the CNS was found only when treatment was initiated in mice younger than 14 days (Vogler *et al.* 1999) corresponding with the development of the BBB in rodents at 10-14 days (Stewart and Hayakawa 1987). After 12 weeks of weekly enzyme infusions, β-glucuronidase was undetectable in the CNS and lysosomal storage became evident in neurons (O'Connor *et al.* 1998). Similarly, if enzyme infusions given weekly for 6 weeks after birth were stopped, enzyme levels dropped and lysosomal storage returned in the brain (within 12-52 weeks), bone (1-4 weeks), liver (4-12 weeks), and spleen (4-12 weeks)(Vogler *et al.* 1996). Thus, enzyme replacement is not an effective treatment for neurological lysosomal storage diseases such as MPS VII.

In humans, Fabry disease was the first lysosomal storage disease treated with enzyme replacement. However, no observable benefits were observed and the enzyme was rapidly cleared from circulation because insufficient doses were used, and the enzyme lacked appropriate endocytosis signals (Bergsma *et al.* 1973; Desnick *et al.* 1979; Brady *et al.* 1982). In spite of the early failures in humans, recent refinements have produced remarkable clinical improvements in patients with a mild form of Gaucher's disease. For this non-neuropathic form of type 1 Gaucher's, administration of glucocerebrosidase (Cerezyme®, Genzyme, MA), has produced clinical improvements including reduction in bone pain and organomegaly, and a gradual normalization of blood counts (Beutler *et al.* 1995). This therapy is now an accepted form of treatment in the U.S. Financial assistance to pay for Cerezyme® in Canada varies by province (MacKenzie *et al.* 1998). Under the Orphan Drug protection law with no

competitive markets, the cost for Cerezyme® is $21,000 per biweekly infusion, costing an annual $505,000 per 70 kg patient (1998 US dollars) (Food and Drug Administration 1991; Whittington and Goa 1995; MacKenzie *et al.* 1998) Although the response to a reduced (50%) dose has been successful in some patients (Beutler *et al.* 1995), the dose-response effects are variable depending on the disease manifestations of each patient (Hollak *et al.* 1997). Cerezyme® is ineffective in severe cases of Gaucher's disease (Takahashi *et al.* 1998) and antibodies to Cerezyme® are generated in 13-40% of Gaucher patients (Grabowski *et al.* 1995; Whittington and Goa 1995; Rosenberg *et al.* 1999; Lusher 2000).

Recent phase I clinical trials for the treatment of Fabry disease with α-galactosidase enzyme replacement demonstrated that the infusions were safe and biochemically active with significant reductions of the previously accumulated substrate (Schiffmann *et al.* 2000; Eng *et al.* 2001). However, the observed tissue half-life of this enzyme was roughly one-half that of Cerezyme® (α-galactosidase tissue half-life: 24 hours, plasma half-life: 2-5 minutes) so a higher cost and frequency of infusions would be required and the enzyme does not cross into the brain, making it unlikely that CNS manifestations of the disease will be improved (Mistry *et al.* 1996; Ioannou *et al.* 2001). Recently, enzyme replacement for ten MPS I patients with recombinant human α-L-iduronidase (125,000 U/kg/week, IV) demonstrated a reduction in hepatosplenomegaly in all patients, an increased rate of growth in young patients, and a reduction in urinary GAG excretion (Kakkis *et al.* 2001a). Forty percent of the patients developed antibodies against α-L-iduronidase; however, the antibodies did not neutralize α-L-iduronidase activity. In addition, 50% of the patients developed transient urticaria (hives) on the trunk, face, arms, and legs during enzyme infusions given after week 3. Notably, the patients that developed hives did not develop anti-α-iduronidase antibodies (Kakkis *et al.* 2001a). All 10 patients developed antibodies to Chinese-hamster-ovary cell proteins that were present as trace impurities in the enzyme preparation, but clinically important adverse events were not correlated with the presence or titer of these antibodies.

Healthcare resources consumed by Cerezyme® therapy (>$500,000/yr US per patient), represent a large cost taken from other therapeutic areas. The possible cost savings due to increased patient productivity and reduced palliative treatments remain unresolved (Whittington and Goa 1995). Although some patients may obtain increased benefit from Cerezyme® treatment, the cost is very high, and may preclude general use for many

patients. Overall, the high costs and inability to treat neurodegeneration using enzyme replacement reveal the need for alternate therapeutic strategies. Since the genes for many lysosomal enzymes have been cloned, gene therapy for lysosomal storage diseases may offer the ultimate solution to this serious health care problem (Beutler 1993).

1.4.7.6 Gene Therapy for Lysosomal Storage Diseases

The introduction of corrective genetic material into patients using gene therapy has been examined as a potential treatment for many lysosomal storage diseases. As an alternative to BMT, gene therapy could treat patients without the need for finding HLA-matched donors. Gene therapy would eliminate the high costs of protein purification required for enzyme replacement therapies. A continuous supply of therapeutic enzyme via gene therapy may be a more effective and long-term treatment alternative. Since many genes encoding lysosomal enzymes have been identified and cloned, once an effective gene therapy has been developed for one lysosomal storage disease, this protocol may be applied to the treatment of many lysosomal storage diseases, using different, disease-specific, therapeutic transgenes. For lysosomal storage diseases, a variety of gene therapies have been investigated in animal models and in a small number of human patients.

1.4.7.6.1 Ex Vivo

The use of *ex vivo* genetically-modified bone marrow re-implantation could offer BMT to patients without the need for HLA-matched donors. However, re-implantation of retroviral-modified bone marrow expressing α-iduronidase in the canine model of MPS I was abrogated by humoral and cellular immune responses against the α-iduronidase, transduced cells, and serum components of the culture media. Despite evidence for proviral marked cell engraftment, and in the absence of the immune responses with *in utero* or immunosuppressive treatments, there was no evidence of clinical improvement, and the therapeutic α-L-iduronidase was not detected (Lutzko *et al.* 1999a; Lutzko *et al.* 1999b). Similarly for MPS VI, despite the long-term (> 2 year) persistence of transduced cells in the MPS VI cat, the level of enzyme activity in the re-implanted animals was too low for clinical improvements (Simonaro *et al.* 1999; Yogalingam *et al.* 1999). Although previous BMT therapies for canine fucosidosis have shown clinical improvements, total graft failure occurred in all transplants with retroviral-transduced bone marrow resulting in no clinical improvements (Ferrara *et al.* 1997).

MPS I and MPS II patients are currently being treated in a phase I/II clinical trial of retroviral-modified autologous peripheral blood lymphocyte re-infusion. Although the therapeutic effects remain to be determined, initial results have shown that less than 2.5% of the re-introduced cells were transduced (Salvetti *et al.* 1994; Whitley *et al.* 1996; Stroncek *et al.* 1999a). The initial results from a Gaucher's disease trial, for which 4% of the re-introduced cells were transduced, were too low for any clinical benefit, enzyme activity did not increase in any patient, and the longest detection of marked cells was for 1 month (Dunbar and Kohn 1996; Schuening *et al.* 1997; Barranger *et al.* 1997; Dunbar *et al.* 1998). Overall, the *ex vivo* cell modification approach to therapy is currently limited by low engraftment and low levels of *in vivo* expressed enzyme

For the treatment of MPS VII, *ex vivo* gene therapy has been widely studied (Table 1.4.3). Re-implantation of *ex vivo* retroviral-modified, β-glucuronidase-expressing bone marrow, macrophages, or fibroblasts to MPS VII mice have shown peripheral histological improvements with diminished lysosomal storage in the liver and spleen, and low levels of peripheral β-glucuronidase activity (0.6 - 2%) (Wolfe *et al.* 1992b; Marechal *et al.* 1993; Moullier *et al.* 1993a; Moullier *et al.* 1993b; Freeman *et al.* 1999). Re-implanted, retroviral-modified, primary myoblasts showed genetic modification of up to 60% of the recipient's myofibrils after intramuscular injection, resulting in low levels of β-glucuronidase in the liver and spleen for up to 8 months (Naffakh *et al.* 1993; Naffakh *et al.* 1994; Naffakh *et al.* 1995; Naffakh *et al.* 1996). The first experimental treatment for canine MPS VII was conducted using intraperitoneal transplantation of retroviral-corrected autologous fibroblasts embedded in Gore-Tex fibres and collagen, resulting in low levels of β-glucuronidase taken up by the liver (Wolfe *et al.* 2000). Lysosomal storage in the liver was reduced by roughly 50% with β-glucuronidase levels in the range of 0.1 - 0.3% of normal, and slightly higher levels of enzyme (2.5%) could almost normalize lysosomal storage, suggesting that these low levels of β-glucuronidase may be therapeutically effective in human MPS VII patients (Wolfe *et al.* 2000).

1.4.7.6.2 In Vivo

Various viral vectors have been investigated for the direct *in vivo* transfer of the β-glucuronidase gene for the treatment of MPS VII (Table 1.4.3). Retrovirus was the first viral vector used, although β-glucuronidase expression lasted only 4 days (Lau *et al.* 1995). Recently, an intravenously injected retroviral vector, co-administered with an adenovirus expressing human growth factor to stimulate hepatocyte replication, resulted in

Table 1.4.3 Peripheral therapies for MPS VII.

Therapy	*Site/Notes*	*Enzyme Activity (%)* *Liver*	*Spleen*	*CNS*	*Duration*	*Improved Pathology* *Periph.*	*CNS*[a]	*References*
Bone Marrow Transplantation								
(Radiation)								
(0.5-10 Gy)	Adult	14	70	6	55 wk	+++	-	Birkenmeier *et al*,1991
(8.5 Gy)	Adult	10	99	6	28 wk	+++	-[b]	Porthuis *et al.* 1994
(2-8 Gy)	Neonate	18	78	7	10 wk	+++	+[b]	Sands *et al.*, 1993
(2 Gy)	Neonate	6	55	2	20 wk	+++	-	Bastedo *et al.*, 1994
(2 Gy)	Neonate	1	8	2	11 wk	+++	-	Sands *et al.*, 1995
Enzyme Replacement								
(Dose)								
(16k U weekly)	Adult	25	5	0	6 wk	+++	-	Vogler *et al.* 1999
(28k U/wk to wk 6)	Adult	0	0	0	52 wk	-	-	Vogler *et al.* 1996
(28k U weekly)	Neonate	28	4	7	6 wk	+++	+[c]	Sands *et al.*, 1994
(28k U, 2 Gy)	Neonate	5	20	1	52 wk	+	+[c]	Sands *et al.*, 1996
(28k U weekly)	Neonate	18	3	0	52 wk	+	+[c]	Sands *et al.*, 1997
(28k U weekly)	Neonate	28	4	0	12 wk	+++	+[c]	O'Connor *et al.*, 1998
(16k U weekly)	Neonate	25	5	<1	6 wk	+++	+[c]	Vogler *et al.* 1999
***Ex Vivo* Gene Therapy**								
Rv-BMT *(2 Gy)*	IV	1	2.0	0	16 wk	+++	-	Wolfe *et al.*, 1992
Rv-BMT *(4 Gy)*	IV	0.6	2.0	0.5	20 wk	+++	-	Marachel *et al.*, 1993
Rv-BMT *(4 Gy)*	IV	0.3	0.4	nd	8 wk	+++	-	Marachel *et al.*, 1993
Rv-Fibroblast	IP	1	0.4	0.1	22 wk	+++	-	Moulier *et al.*, 1993
Rv-Fibroblast	IP	2	0.3	0.1	8 wk	+++	-	Moulier *et al.*, 1993
Rv-Myoblast	IM	0.9	0.3	nd	4 wk	+++	-	Naffakh *et al.*, 1996
Rv-Macrophages	IV	3	0.0	0	4 wk	+++	nd	Freeman *et al.* 1999
Rv-Fibroblasts	IV	0.1-2	nd	nd	1-7 mo.	+	nd	Wolfe *et al.* 2000
***In Vivo* Viral Gene Therapy**								
Retrovirus	Intestine	nd	nd	nd	0.6 wk	nd	nd	Lau *et al.*, 1995
Adeno	Eye	nd	nd	nd	3 wk	+ eye	nd	Li & Davidson, 1995
Adeno	IV	85	nd	nd	16 wk	+++	-	Stein *et al.*, 1999
Adeno	IV	1	1	0	4 wk	+++	-	Ohashi *et al.*, 1997
AAV	IV	low	low	0	16 wk	+	-	Watson *et al.*, 1998
AAV	IM	low	low	0	16 wk	+	-	Watson *et al.*, 1998
Neonatal AAV	IM	1	0.2	0	16 wk	+	-	Daly *et al.*, 1999
Neonatal AAV	IV	1	0.3	10	16 wk	+[d]	+[c,d]	Daly *et al.*, 1999
Retrovirus	IV	1	0.4	nd	14 wk	+	nd	Gao *et al.*, 2000
Lenti	IV	38	5.7	nd	3 wk	+	nd	Stein *et al.*, 2001

Legend: (a) Does not include peripheral (perivascular, meningeal) improvement (b) Radiation-induced toxicity in CNS. (c) If initiated within 0-14 days of birth (d) Liver tumours in 60% of animals after 1 to 1.5 years. Enzyme activity: % of normal or very low to high as described by authors; Histopathology: unimproved (-), partially improved (+), improved (+++). Abbreviations: Rv: Retroviral; Adeno: ΔE1/E3 adenovirus; AAV: adeno-associated virus; Lenti: Lentivirus; IV: Intravenous; IM: Intramuscular; IP: Intraperitoneal; SC: subcutaneous; "Periph": peripheral organs (kidney, liver, spleen, muscle).

1% of normal liver β-glucuronidase activity with a reduction in lysosomal storage in some peripheral organs of MPS VII mice (Gao *et al.* 2000a). Adenovirus-mediated gene transfer of human β-glucuronidase from intravitreal inoculation in the eye corrected the ocular storage granule pathology in the retinal pigment epithelium (Li and Davidson 1995). High dose intravenous adenovirus (2 x 10^9 PFU) resulted in low levels of β-glucuronidase (1.3% of normal) in the peripheral organs for up to 4 weeks (Ohashi *et al.* 1997). In a separate study, higher levels of β-glucuronidase (85% of normal) were detected for up to 16 weeks (Stein *et al.* 1999). The authors conceded that these results were surprising with respect to the previous study; however, a different promoter may have improved the β-glucuronidase expression. AAV mediated delivery of β-glucuronidase to newborn MPS VII mice (5.4 x 10^6 PFU, IV or 1.7 x 10^6 PFU, IM) resulted in low levels of β-glucuronidase (1%) for up to 16 weeks, sufficient to reduce storage in the liver, but not in the spleen (Watson *et al.* 1998; Daly *et al.* 1999a; Daly *et al.* 1999b). Although secretion from intramuscular injection sites was inefficient due to highly localized enzyme, intravenous injections showed that the treatment could deliver enzyme very early in life. However, a 100-fold decline in β-glucuronidase expression in organs such as the liver and spleen during the first 12 weeks limited the long-term therapeutic value of the treatment. Recently, lentivirus administered intravenously to adult MPS VII mice resulted in high levels of enzyme in peripheral tissues after 3 weeks (1 to 38% of normal) with partial reductions in storage pathology, however, antibodies against β-glucuronidase developed in all of the treated mice (Stein *et al.* 2001).

In vivo gene therapy has also been studied for the treatment of MPS I, Fabry, and Tay-Sachs disease. In MPS I dogs, intramuscular injections of naked DNA encoding α-iduronidase resulted in no detectable enzyme and progressively decreasing PCR detection of α-iduronidase DNA (Shull *et al.* 1996). Gene therapy for Fabry disease using adenoviral vectors encoding human α-galactosidase injected intravenously into Fabry knockout mice produced normal levels of enzyme which declined rapidly, such that by 12 weeks, less than 10% of the original activity remained (Ziegler *et al.* 1999). Adenoviral-mediated gene transfer in the pseudo-Tay-Sachs mouse model has also shown short-term, high levels of enzyme (Guidotti *et al.* 1999).

However, none of these studies were able to correct the neurodegenerative manifestations in adult animals. Therefore, a therapeutic approach directed to the CNS that results in persistent β-glucuronidase activity and a reduction in lysosomal storage in the brain would be required.

1.4.7.6.3 Ex Vivo Gene Therapy in the CNS

Although the brain is severely affected in many lysosomal storage disorders, the BBB restricts the entry of most lysosomal enzymes into the CNS. Thus, the feasibility of delivering therapeutic lysosomal enzymes directly to the brain has been studied. *Ex vivo* gene therapy was the first therapeutic strategy to deliver lysosomal enzymes directly into the brain to target neurodegeneration (Table 1.4.4). For the treatment of MPS VII, retroviral-modified neural progenitor cells transplanted into the CNS of neonatal mice integrated and reduced lysosomal storage in neurons and glial cells near the graft (Snyder *et al.* 1995). Similarly, *ex vivo* retroviral-modified autologous fibroblasts secreting β-glucuronidase were grafted into the brains of adult MPS VII mice. The cells engrafted and expressed low levels of β-glucuronidase, reaching 4.7% of normal levels immediately adjacent to the graft site after 3 days, and declined to 1-2% by 8 weeks. Lysosomal storage was improved in neurons and glia within 2 mm of the graft (Taylor and Wolfe 1997a). Recently, adenoviral vector-transduced primary adult human astrocytes transplanted into the striatum of athymic nude mice produced high levels of β-glucuronidase (Serguera *et al.* 2001). However, even using immunodeficient animals, the transduced astrocytes were eliminated within 8 weeks, reducing β-glucuronidase to baseline levels.

Ex vivo gene therapy in the CNS is also being investigated for the treatment of Krabbe and Tay-Sachs diseases. Retroviral-modified neural progenitor cells expressing β-hexosaminidase A injected into the CNS of newborn normal mice produced enzyme in the CNS at potentially therapeutic levels for Tay-Sachs disease (Lacorazza *et al.* 1996), and neural progenitor cells expressing galactocerebrosidase have been created for the treatment of Krabbe disease (Torchiana *et al.* 1998).

1.4.7.6.4 In Vivo Gene Therapy in the CNS

In vivo gene therapy administered directly to the CNS has also been used to target the neurodegeneration of lysosomal storage diseases. MPS VII was the first lysosomal storage disease treated with *in vivo* gene transfer in the CNS (Table 1.4.4). Herpes vector-mediated gene transfer resulted in extremely low levels of β-glucuronidase

Table 1.4.4 CNS-directed gene therapies for MPS VII.

Therapy	*Age*	*Site*	*Enzyme Activity (%)*	*CNS Pathology Correction*	*Duration (weeks)*	*References*
***Ex Vivo* Gene Therapy**						
Rv-CNS-P	Neonatal	CNS	2	nd	8	Snyder *et al.*, 1995
Rv-CNS-P	Neonatal	CNS	9	+	24	Snyder *et al.*, 1995
Rv-CNS-P	Neonatal	CNS	4	+	3	Snyder *et al.*, 1995
Rv-Fibroblasts	Adult	CNS	1.2	+	8	Taylor & Wolfe, 1997
Rv-Fibroblasts	Adult	CNS	1.1	+	4	Taylor & Wolfe, 1997
Rv-Fibroblasts	Adult	CNS	2.7	+	2	Taylor & Wolfe, 1997
Ad-Astrocytes*	Adult	CNS	high	N/A*	4	Serguere *et al.*, 2001
***In Vivo* Gene Therapy**						
Herpes	Adult	CNS	v. low	-	16	Wolfe *et al.*, 1992
Adeno	Adult	CNS	v. low	-	2	Ohashi *et al.*, 1997
Adeno	Adult	CNS	high	+	12	Ghodsi *et al.*, 1998
Adeno	Adult	CNS	high	+++	3	Ghodsi *et al.*, 1999
Adeno	Adult	CNS	high	+++	16	Stein *et al.*, 1999
AAV	Neonatal	IV	10	+	16	Daly *et al.*, 1999
AAV	Adult	CNS	low	+	12	Elliger *et al.*, 1999
AAV	Adult	CNS	12	+++	20	Skorupa *et al.*, 1999
Herpes	Adult	CNS	10	nd	4	Zhu *et al.*, 2000
AAV	Neonatal	CNS	high	+	18	Frisella *et al.*, 2001

Legend: Enzyme activity as a % of normal or very low, low, or high as described by authors. Histopathology: unimproved (-), partially improved (+), or improved (+++). (*) Nude mice, not MPS VII mice. Abbreviations: Rv-: Retroviral modified; CNS-P: CNS progenitor cells; Herpes: Herpesvirus vector; Adeno: ΔE1/E3 adenoviral vector; AAV: Adeno-associated viral vector; Ad-Astrocytes: Adenoviral-modified primary human astrocytes in nude mice; IV: Intravenous.

expression in the trigeminal ganglia and brainstems of animals for up to four months (Wolfe *et al.* 1992a). However, the level of β-glucuronidase was sub-therapeutic and the herpes vectors were acutely pathogenic in the MPS VII mice at doses that were non-pathogenic in normal littermates (Wolfe *et al.* 1996). A modified version of this herpes vector with a restored latency promoter splice-mechanism and further deletions in the vector backbone reduced the vector pathogenicity and significantly improved β-glucuronidase delivery. The highest levels of β-glucuronidase adjacent to the injection site activity reached 10% of normal levels after 4 weeks, however, a reduction in lysosomal storage pathology was not reported (Zhu *et al.* 2000).

Adenoviral vectors administered to the ventricles of MPS VII mice transduced ependymal cells, resulting in low levels of β-glucuronidase localized to the periventricular area (Ohashi *et al.* 1997). Intrastriatal injection of a similar recombinant adenovirus produced intense enzyme expression near the injection site for up to 12 weeks, which spread from a relatively small number of transduced cells to reach up to 40% of the CNS with a concurrent reduction in storage pathology (Ghodsi *et al.* 1998; Stein *et al.* 1999). Moreover, mannitol could be co-administered during intraventricular administration to further expand vector penetration into the subependymal region and increase β-glucuronidase spread throughout the brain (Ghodsi *et al.* 1999).

An AAV vector intravenously administered to newborn mice could reach the CNS to reduce lysosomal storage; however, unlike mice, the BBB in humans is functional prior to birth. The tight junctions of the BBB are present from very early in development to exclude proteins from the brain (Saunders *et al.* 1999; Saunders *et al.* 2000; Dziegielewska *et al.* 2000; Frisella *et al.* 2001). As expected, intravenous administration of AAV in adults was unable to transduce cells of the CNS. Thus, intrathecal injection of the vector into cerebrospinal fluid was used to improve delivery to the brain, resulting in low levels of β-glucuronidase in the CNS and a partial reduction of storage pathology (Elliger *et al.* 1999).

Aspartylglucosaminuria and metachromatic leukodystrophy have also been treated in the CNS with viral vectors. An adenoviral vector expressing aspartylglucosaminidase injected intraventricularily led to transgene expression in the ependymal cells lining the ventricles, and a partial correction of lysosomal storage in tissues surrounding the ventricles in aspartylglucosaminuria mice (Peltola *et al.* 1998). Recently, lentiviral vectors were used to deliver arylsulfatase A to the brain of metachromatic leukodystrophy mice and showed a reduction of

neuropathology (Consiglio *et al.* 2001). Overall, however, toxicity, transient expression, and low levels of enzyme currently limit *in vivo* gene therapy for lysosomal storage diseases.

1.4.7.6.5 Immuno-Isolation Gene Therapy for Lysosomal Storage Diseases

The *in vitro* feasibility of an immuno-isolation gene therapy for a lysosomal storage disease was first demonstrated with the delivery of β-glucuronidase to cell culture medium from alginate microencapsulated cells (Awrey *et al.* 1996). The lysosomal storage diseases provide a particularly good model to test the efficacy of immuno-isolation gene therapy. The diseases are devastating and in need of effective therapy. Unlike growth hormone or insulin, the required therapeutic product does not require tight regulation, and only low levels are necessary to produce therapeutic effects.

1.5 Goals of Thesis

The aim of this research was to develop a treatment for a lysosomal storage disease using immuno-isolation gene therapy. The over 50 recessive, single-gene, lysosomal storage diseases carry a significant burden of disease. Together, the lysosomal storage diseases affect over 1 in 7700 people (Meikle *et al.* 1999).

The lysosomal storage diseases provide an excellent, relatively simple, model upon which to develop novel treatment strategies. These diseases require only low levels of therapeutic enzyme (Chabas *et al.* 1991), high levels of enzyme are not detrimental (Kyle *et al.* 1990; Brooks *et al.* 1991; Thomas 1994), and secreted enzymes can be taken up by affected cells (Brooks *et al.* 1991; Thomas 1994). In contrast, the development of elaborate gene therapies for complex infectious, dominant, or multifactorial diseases such as cancer will be difficult. As yet, the ability to control the level, duration, and location of any gene has not been achieved with gene therapy. On the other hand, gene therapies for the recessive lysosomal storage diseases are not complicated by the need for exact regulated levels of transgene expression, the affected genes are known, the molecular mechanisms are understood, and well-characterized animal models exist. Therefore, the development of a gene therapy for lysosomal storage diseases may provide a clear picture of the obstacles to be overcome by gene therapy. This knowledge may eventually be applied to the treatment of more complicated diseases.

In spite of the high prevalence and devastating consequences, there are no clinically accepted treatments for all but the mildest form of one lysosomal storage disease. BMT is unsuitable for most lysosomal storage diseases because of the inability to reverse neurodegeneration and the low availability of compatible donors. Enzyme replacement has reduced disease manifestations of non-neuropathic Gaucher's disease; however, the high costs and inability to treat neurodegeneration demonstrate the need for alternative therapeutic strategies. Current gene therapies for lysosomal storage diseases are also insufficient. *Ex vivo* strategies are limited by low therapeutic efficacy, the need for radiation induced myeloablation, or the high costs of constructing and safety monitoring individual vector-corrected cell lines. *In vivo* gene therapies have also been limited by short duration, low efficacy, inability to re-administer, and up to 85% of the population carry pre-existing inhibitory antibodies against certain viral vectors, such as AAV, due to previous exposure to wild-type virus. AAV-mediated gene transfer to the

peripheral organs of mice was unable to reduce lysosomal storage in all of the organs due to low levels of enzyme expression. Adenoviral vectors are abrogated by strong immune responses, transient expression, and systemic toxicity. In addition, *in vivo* gene therapies carry potential risks of insertional mutagenesis, germ-line transmission, irreversibility, and limited capacity for regulatory sequences. Currently, a clinically relevant, long-term, and safe delivery of a lysosomal enzyme to both the peripheral organs and the CNS has not been accomplished.

Immuno-isolation gene therapy offers several advantages as a viable alternative to current *in vivo* and *ex vivo* gene therapy approaches. Immuno-isolation gene therapy is a simple, rapid approach, amenable to scale-up with the efficient use of a single universal cell line and carries a practically unlimited cloning capacity for the incorporation of safety and regulatory elements. In addition, immuno-isolation gene therapy offers a potentially reversible treatment with removal of the encapsulated cells, compared to the irreversible infection of viral vectors or integration of progenitor cells.

We hypothesized that an immuno-isolation-based gene therapy strategy could be used to provide a continuous source of a normally deficient lysosomal enzyme from microencapsulated recombinant cells to overcome a lysosomal storage disease. Previously, immuno-isolation gene therapy using alginate-microencapsulated recombinant cells had been shown *in vitro* to supply recombinant mouse β-glucuronidase to cell culture media, and *in vivo* to deliver low levels of human growth hormone and factor IX to mice (Chang *et al.* 1993b; Hortelano *et al.* 1996). The clinical efficacy of this approach had only been described in a murine model of growth hormone deficiency, for which the treatment increased body weight and length (Al-Hendy *et al.* 1995). The goal of this research, therefore, was to test this hypothesis in the well-understood MPS VII animal model. Experiments were carried out to develop a novel immuno-isolation gene therapy to deliver β-glucuronidase to MPS VII mice.

(Chapter 3.1) An optimal microcapsule formulation was determined for a recombinant cell line that secreted high levels of β-glucuronidase. These results were also used to characterize two novel microcapsule formulations.

(Chapter 3.2, part I) Based on these results, mutant MPS VII mice were treated with microencapsulated cells secreting β-glucuronidase. The results of this experiment revealed three primary obstacles to be overcome: 1) Although the microcapsules were able to deliver high levels of β-glucuronidase to the mice to reduce disease

pathology, all of the mice developed a strong immune response against the re-supplied mouse β-glucuronidase, thereby abrogating the treatment efficacy. 2) This treatment did not address the early onset effects of the disease. 3) The β-glucuronidase was unable to cross the BBB into the CNS, again limiting the treatment efficacy because the disease manifests progressive neurodegeneration.

(Chapter 3.2, part II) To address the first problem, experiments were designed to overcome the anti-β-glucuronidase immune response using immunosuppression. This approach was successful in both increasing and prolonging the treatment effectiveness.

(Chapter 3.3) To address early onset effects of the disease, MPS VII mice were implanted within 0 to 5 days after birth. This resulted in a significant delay in the disease onset; however, the eventual development of antibodies limited the treatment. Tolerance to β-glucuronidase through neonatal administration was not achieved.

(Chapter 3.4) To address the third problem of gene product delivery to the CNS, microcapsules were implanted directly into the CNS. Alginate microcapsules had not previously been implanted into the CNS, and the size of microcapsules was too large for the mouse CNS; therefore, smaller alginate microcapsules were developed and tested *in vitro*.

(Chapter 3.5) Using the optimized small microcapsules, a marker gene was used to test and optimize the intraventricular implantation of small alginate microcapsules into the CNS using normal mice. These results showed that the recombinant marker gene product could indeed be expressed for at least 16 weeks (longest time point) in the mouse CNS.

(Chapter 3.6) Subsequently, microcapsules secreting β-glucuronidase were implanted into the CNS of MPS VII mice. The treated mice exhibited a significant reduction in many biochemical and histological parameters of disease, as well as an improvement in behavior as assessed by circadian rhythm analyses.

(Chapter 3.7) Finally, a combined approach of peripheral and CNS implantations of β-glucuronidase secreting microcapsules was performed in MPS VII mice. Treated animals showed both reduced peripheral and reduced CNS lysosomal storage pathology, an achievement not attained by any other treatment in adult MPS VII mice.

2.0 MATERIALS AND METHODS

2.1 Cell lines

Cells were maintained in 10 cm^2 tissue culture dishes (#353003, Applied Scientific, San Francisco, CA) at 37°C in a 5% CO_2 water jacket Isotemp® incubator in 100% humidity (Fisher Scientific International). Unless otherwise noted, all cells were grown in tissue culture medium containing Dulbecco's Modified Eagle's media (Gibco, BRL), 10% fetal bovine serum (Gibco, BRL), penicillin (100 U/ml), and streptomycin (100 μg/ml) (Gibco, BRL). Cells were harvested by aspirating off the old media, washing the dish with 10 ml PBS, adding 0.5 ml of 0.125% trypsin for 2 minutes to remove the cells from the dish, and resuspending the cells in serum free media. Cells were split by adding a portion of the harvested cells to a new dish of media.

2.1.1 Fibroblasts Expressing β-glucuronidase

Mouse 2A50 fibroblasts (Gift from W.S.Sly, Saint Louis University School of Medicine, St. Louis, MO) were transfected to express mouse β-glucuronidase at 522 ± 40 nmol activity/10^6 cells/hr. The 2A50 cells were deficient in the mannose 6-phosphate receptor due to a naturally occurring mutation in the MPR300 gene to permit increased lysosomal enzyme secretion. The plasmid used for 2A-50 transfection, pMSXND-MβG, encoded the cDNA for mouse β-glucuronidase downstream from the metallothionein mMTI promoter, and upstream from the cDNA encoding dihydrofolate reductase which allowed amplification of gene expression by selection in 3.2 μM methotrexate (Grubb *et al.* 1993). The 2A-50 cells were grown in regular tissue culture medium supplemented with 0.1 g/L sodium pyruvate, 2.2 g/L sodium bicarbonate, 1.2 mM L-glutamine (Gibco, BRL), and 3.2 μM methotrexate (Sigma, St. Louis, MO).

2.1.2 Fibroblasts Deficient in β-glucuronidase

β-glucuronidase deficient mouse fibroblast 3521 cells were used as a β-glucuronidase deficient control cell line (Gift from M. Sands, Washington University School of Medicine, St. Louis, MO). 3521 cells were maintained in regular tissue culture media supplemented with 2 mM L-glutamine (Gibco, BRL).

2.1.3 Myoblasts Expressing hGH

C2C12 cells were an untransfected mouse myoblast cell line (#CRL1772, ATCC, Rockville, MD). C41 myoblasts were C2C12 cells transfected with pNMG3 vector with the hGH cDNA under the control of the mouse metallothionein promoter mMTI and contained the phosphotransferase gene for G418 selection (Chang *et al.* 1990). The C41 cells secreted 8 ng hGH/10^6 cells/hr.

2.1.4 Myoblasts Expressing hFIX

The D6 myoblasts were mouse C2C12 myoblast cells transfected with a vector encoding human factor IX under the control of the human ß-actin promoter and the *Neo*^{R} gene conferring resistance to G418. This clone was isolated after selection in G418 (Hortelano *et al.* 1996).

2.1.5 Epithelial Cells Expressing hGH

MDCK cells, a canine renal epithelial cell line derived from a normal adult female cocker spaniel (#CCL-34, ATCC, Rockville, MD), were kindly provided by Dr. Frank Graham, McMaster University, Hamilton, Canada. The H8 MDCK cells were transfected through calcium phosphate-mediated DNA precipitation (Graham and van der Eb 1973) with the pNMG3 plasmid. This plasmid contained the human growth hormone gene (hGH) under the control of the mouse metallothionein promoter mMTI with an SV40 sequence upstream of the hGH gene and polyadenylation signal downstream of the gene and contained the phosphotransferase gene for G418 selection (Chang *et al.* 1990). MDCK cells were cultured in α–minimal essential medium (Gibco, BRL) supplemented with 10% fetal bovine serum (Gibco, BRL), penicillin (100 U/ml)-streptomycin (100 μg/ml) and 2mM L-glutamine (Gibco, BRL). The H8 MDCK cells secreted 19 ± 1.2 ng of hGH per million cells per hour.

2.2 Microcapsules

2.2.1 Cell Microencapsulation

Cells were harvested from tissue culture and encapsulated within alginate, a polysaccharide polymer composed of alternating blocks of D-mannuronic and L-guluronic acid (Sun 1988; Tse *et al.* 1996), as modified by Liu et al. (Liu *et al.* 1993). Alginate was sterilized by filtration through a 0.2μm syringe filter (#4192, Gelman Sciences, Ann Arbor, MI). Encapsulation of cells was carried out at 4-10°C under sterile conditions. For one ml of microcapsules, two million cells were harvested before reaching confluence and resuspended in a total volume of

0.5 ml cold saline, and mixed with one ml of 1.5% filtered potassium alginate (Keltone LV, Lot No. 17703A, kindly provided by Kelco, San Diego, CA). The mixture was drawn up into a 20 ml syringe which was placed in a modified Orion Sage pump (model M362) and extruded through a 27 gauge blunt end needle (#7400, Popper & Sons, New York, NY) at a rate of 99.9cm^3/hr x 1/100. A concentric air stream (4 litres/min) delivered to the tip of the needle sheared off small droplets. The droplets fell into a cold bath of a cross-linking agent, $CaCl_2$ or $BaCl_2$. In this solution, the alginate polysaccharide polymers ionically cross-linked to form solid spherical hydrogel beads containing embedded cells. Using different cross-linking agents and coatings, microcapsules with different properties were generated (Table 2.2.1).

2.2.1.1 *Hollow Alginate-Poly-L-lysine-Alginate Capsules (APA)*

To make hollow alginate-poly-L-lysine alginate (APA) microcapsules, the small droplets were cross-linked in a bath of cold 10 mM $CaCl_2$ (Table 2.2.1). The beads were transferred to a sterile 50 ml conical polypropylene tube and washed successively with 5 mM $CaCl_2$ in 0.9% NaCl, 2.5 mM $CaCl_2$ in 0.9% NaCl, 0.9% NaCl, 5 mM CHES (2-[N-Cyclohexylamino]ethan-sulfonic acid), and 10 mM $CaCl_2$. The permeability of the gelled beads was controlled by further laminating with 0.05% Poly-L-lysine (PLL, MW 15,000 - 30,000) (Sigma, St. Louis, MO) for 6 min. The capsules were then washed with 5 mM CHES, 10 mM $CaCl_2$, and 0.9% NaCl. The capsules were coated with another layer of 0.03% alginate for 4 min to confer a biocompatible surface. This treatment was followed with another wash in 0.9% NaCl and then the unpolymerized polysaccharide in the core of the capsule was solubilized with a chelating agent, 0.55 mM sodium citrate, for 6 min. After two more rinses in 0.9% NaCl the microcapsules were either immediately injected into mice, placed in serum free media until injection into mice, or rinsed in serum free media before being placed in complete medium and maintained under normal tissue culture conditions. Microcapsules of ~500 μm in diameter were used for intraperitoneal implantation and *in vitro* analysis.

2.2.1.2 *Small APA microcapsules*

Small APA microcapsules were developed for implantation into the ventricular space of mice. Cell encapsulation was carried out as normal for APA microcapsules, except the cell number was increased 2.5-fold to five million cells/ml alginate, the air flow was increased from 4 up to 6 L/min, and all wash times were shortened to compensate for a brief centrifugation (Sorval RT6000B, 30 sec, 20g, 300 rpm) following each wash to ensure the small microcapsules had settled prior to the next wash (Table 2.2.1). The small size of microcapsules was

Table 2.2.1 Composition and protocols for alginate microcapsule formulations.

	Microcapsule Formulations				
Capsule Type:	**APA**	**Small APA**	**Solid APA**	**BA**	**BPA**
Alginate %:	*1.5% alginate*	*1.5% alginate*	*1.5% alginate*	*2% alginate*	*2% alginate*
Air Flow:	4 L/min.	6 L/min.	4 L/min.	4 L/min.	4 L/min.
Diameter:	500 μm	150 μm	300 μm	500 μm	500 μm
	washes (min)	*washes (min)*	*washes (min)*	*washes (min)*	*washes (min)*
$CaCl_2$ (10 mM)	2	1 + spin	2	1.5 *(20 mM $BaCl_2$)*	1.5 *(20 mM $BaCl_2$)*
$CaCl_2$ (5 mM)	2	1 + spin	2	-	-
$CaCl_2$ (2.5 mM)	2	1 + spin	2	-	-
CHES (5mM)	3	2 + spin	3	-	-
$CaCl_2$ (10 mM)	2	1 + spin	2	-	-
PLL (0.05%)	6	4 + spin	6	-	6
CHES (5mM)	2	1 + spin	2	-	-
$CaCl_2$ (10 mM)	2	1 + spin	2	-	-
NaCl (154 mM)	2	1 + spin	2	2	-
Alginate (0.03%)	4	3 + spin	4	-	4
NaCl (154 mM)	2	1 + spin	2	2	-
NaCitrate (55 mM)	6	4 + spin	-	-	-
NaCl (154 mM)	2	1 + spin	2	2	2
NaCl (154 mM)	2	1 + spin	2	2	2
Dulbecco's MEM	2	1 + spin	2	2	2
Maintenance media	Fbs/Dmem	Fbs/Dmem	Fbs/Dmem	Fbs/Dmem	Fbs/Dmem
Implantation Media	Dmem	Dmem	Dmem	n/a	n/a

controlled using the airflow speed. Moreover, the heterogeneous mixture of small (50-200 μm) and medium (200-500 μm) microcapsules was subsequently enriched for small microcapsules using the microcapsule settling time gradient. The large (500-1000 μm) and medium microcapsules settled within 1 minute, while small microcapsules settled more slowly for up to 30 minutes. Thus, a fraction enriched for small microcapsules was isolated by allowing the microcapsules to settle for 2 minutes before removing the supernatant of unsettled small microcapsules. This fractionation was repeated if necessary. Microcapsules of ~150 μm in diameter were used for intraventricular implantation and *in vitro* analysis.

2.2.1.3 Solid APA microcapsules

Solid APA microcapsules were generated for subcutaneous implantations. Cell encapsulation was carried out as normal for hollow APA microcapsules, except the microcapsule core was not dissolved with 0.55 mM sodium citrate (Table 2.2.1). Microcapsules of ~300 μm in diameter were used for subcutaneous implantation and *in vitro* analysis.

2.2.1.4 Solid Barium Alginate Capsules (BA)

Solid barium alginate microcapsules were generated by extrusion of a 2% alginate-cell suspension into a cold bath of 20mM $BaCl_2$ to make solid barium alginate (BA) microcapsules (Table 2.2.1). The alginate was cross-linked with the barium for 90 seconds. After 90 seconds, the capsules were poured into 300 ml of 0.9% NaCl, and a fresh beaker of $BaCl_2$ was used to collect capsules for another 90 sec. Once the extrusion had finished, the capsules were rinsed in 0.9% NaCl, followed by serum free medium, and then maintained under normal tissue culture conditions in complete medium. Microcapsules of ~500 µm in diameter were used for *in vitro* analysis.

2.2.1.5 Solid Barium Alginate-Poly-L-lysine Alginate Capsules (BAPA):

The solid barium alginate-poly-L-lysine-alginate (BAPA) microcapsules were prepared as the BA microcapsules, but after cross-linking in the $BaCl_2$ solution for 90 seconds, the capsules were then treated with 0.05% PLL for 6 minutes while on a stirring platform and then rinsed with saline (Table 2.2.1). The capsules were then coated with another layer of 0.04% alginate for 4 minutes while on a stirring platform. Finally, the capsules were rinsed in 0.9% NaCl, followed by serum free medium, and then maintained under normal tissue culture conditions in complete medium. Microcapsules of ~500 µm in diameter were used for *in vitro* analysis.

2.2.2 *In Vitro* Characterization of Microencapsulated Cells

2.2.2.1 Cell Number

Cell number per microcapsule was determined by visually counting the capsules in 100 µl prior to gently crushing the capsules with a small pestle (#7495200000, Baxter International Inc.) into 50 µl trypsin (Gibco, BRL) and 50 µl trypan blue (Gibco, BRL). The trypsin helped break up clusters of cells and the trypan blue was used to determine cell viability. A 15 µl aliquot of the resulting suspension (15 µl) was transferred to a hemocytometer for counting. The average and standard deviation were calculated from at least five determinations. The BA and BAPA capsules were treated similarly, except 100 µl of 0.5M EDTA was used to weaken the microcapsules before rupturing with the pestle. Cell number per capsule and cell number per volume of microcapsules were calculated.

2.2.2.2 Cell Viability

Cell viability was measured using 0.5% trypan blue exclusion staining (#15250-061, Gibco) and counting of stained dead versus unstained viable cells. Cell viabilities were based on more than 3 counts of 200 cells per sample.

2.2.2.3 Transgene Product Secretion Rate

Cells and microencapsulated cells were assayed for transgene product secretion from time course assays of cell culture media samples. Samples were drawn from the media at 0, 1, 2, and 4 hours and replaced with an equivalent volume of equilibrated media. Samples were then measured for specific enzyme activity or mass assays with an ELISA.

2.2.2.4 Microcapsules Diameter

Microcapsule diameter was determined by measuring the size of the microscope field of view with a ruler and dividing the number of microcapsules that fit across the field of view by the size of the field of view.

2.2.3 *In Vitro* Characterization of Microcapsules

2.2.3.1 Microcapsule Shearing

Capsules suspended in medium were subjected to controlled fluid shear in a cone-and-plate flow device (Skarja *et al.* 2001) in order to assess their resistance to this type of mechanical stress. In this device the fluid suspension is contained in a circular well of diameter 1". The bottom of the well serves as the plate and a plexiglass cone with an angle of 4° is lowered into the well so that the tip just touches the plate. The cone is then rotated at a constant speed, thereby producing a uniform shear field across the diameter of the apparatus. At low cone angles and under laminar flow conditions, the shear rate γ is given by: $\gamma = \frac{\Omega}{\theta}$ where Ω is the rotational speed and θ is the cone angle. In the present experiments the rotational speed was 333 rpm giving a shear rate of 500 sec^{-1}. Times of exposure to shear were 15, 30 and 45 min. The microcapsule samples were removed from the wells at each time point and photographed using a Sony CDD colour video camera attached to a Zeiss light microscope using Vidas 21 software. The percentage of capsules remaining intact as scored by counting the total number of intact capsules in the field and dividing by the total number of capsules captured in the field. The

resulting number was expressed in terms of the percentage of capsules remaining intact with increasing duration of shear.

2.2.3.2 Microcapsule Osmotic Pressure Test

Microcapsules (100 µl) were equilibrated in 500 µl of serum free Dulbecco's Modified Eagle's Media and stained with trypan blue. After equilibration, the solution surrounding the microcapsules was removed by aspiration. At this point, 14 ml of hypotonic solution (distilled water, 0.78% media, or 3.2% media) was added to the microcapsules. The microcapsules were equilibrated with the new solution for 3 hours before the capsules were transferred to culture dishes for counting the number of broken and intact capsules. The samples were compared based on the percentage of intact capsules remaining (Van Raamsdonk and Chang 2001).

2.3 Animals

MPS VII mutant mice were bred from heterozygous MPS VII mice (#002086 C57BL/6J-*gus*^{mps} Tg (*Gus*^{sx}), Jackson Laboratories, Bar Harbour, MA) and genotyped by PCR (Gibco Taq polymerase) and restriction digestion. PCR products encompassing the mutation site (Sands and Birkenmeier 1993) were amplified with primers GUS-5'-1k (5'-CTA AAT TAA GGA CCA GGA GAT GTA-3') and GUS-3'-1k (5'-CCA GAG GCT AAG GGA GAT TGT-3'). The resulting 977 bp product was digested with Eco0191I (#R0503L, New England BioLabs, Beverly, MA) to generate 526, 251, and 200 bp fragments from the normal allele and 777 and 200 bp fragments from the mutant allele. Both mutant and normal alleles generate a 200 bp fragment for an internal restriction digest control.

2.3.1 Microcapsule Implantation

Mice were anesthetized with a combination of 1.4 units isofluorane (1-chloro 2,2,2-trifluorethyl difluoromethyl ether) (Anaquest, Madison, WI), oxygen (0.4 L/min), and nitrogen oxide (0.7 L/min) (Vapomatic, Med-Vet Anesthetic Systems, Inc., Canada) and prepared for surgery by cleaning, shaving, and swabbing the surgery site with iodine. Anesthetized mice were implanted intraperitoneally with at least 3 ml of microcapsules through a 16-gauge catheter unless otherwise noted. Neonatal mice, 0 to 5 days old, were injected subcutaneously with 25 µl of microcapsules through a 23-gauge needle. For subcutaneous microcapsule injections of adult mice, anesthetized mice were injected subcutaneously with 1 ml of microcapsules through a 23-gauge

needle. Intraventricular implantation of microcapsules into the CNS was performed by slowly infusing microcapsules into the lateral ventricle under stereotaxic guidance (Ralph *et al.* 1990).

2.3.2 Monitoring

Weekly 100 μl blood samples were collected under anesthesia from the tail vein or retro-orbitally through heparinized capillary tubes (Chase Instruments, Rockwood, TN), stored on ice, and the plasma was collected after centrifugation for 6 minutes at 4°C, 8600 rpm.

2.3.3 Immunosuppression

Mice were transiently immunosuppressed with purified anti-CD4 monoclonal antibody (Pharmingen, Canada) using a single treatment protocol of 250 μg injected IP on days -3, 0, and 3 (Potter *et al.* 1998).

2.3.4 Behavioral Analyses

Animals were housed individually with unlimited access to food and water in cages with computerized wheel running activity recording (Ralph *et al.* 1990) in compliance with the Canadian Council on Animal Care guidelines and International Animal Care Committee approval. After acclimatization for up to 10 days, mice were placed in constant darkness for 7-10 days to characterize the endogenous rhythm. Following implantation with microcapsules containing therapeutic β-glucuronidase producing cells or control β-glucuronidase deficient cells, the animals were allowed to recover for up to 3 days and then the activity of the mutant mice was recorded for approximately 30 days in constant darkness to monitor changes in wheel running activity and circadian rhythms.

2.4 Molecular and Biochemical Analyses

2.4.1 Assays for Secreted Gene Products

Dissected tissue samples were stored at -70°C or immediately homogenized. Homogenates were sonicated on ice for 20 seconds with 10 μl of homogenization buffer (10 mM β-mercaptoethanol, 20 mM Tris-HCl (pH 7.5), 140 mM NaCl, and 0.25% saponin) per mg tissue and centrifuged at 16000g for 30 minutes. Tissue homogenate supernatants were collected for enzyme and protein assays and stored at -70°C.

2.4.1.1 *Lysosomal Enzyme Activities*

β-Glucuronidase, β-hexosaminidase, and α-galactosidase activities were determined from the rate of hydrolysis of the non-fluorescent substrates 4-methylumbelliferyl-β-glucuronide-4-methylumbelliferyl-N-acetyl-β-D-glucosamide and 4-methylumbelliferyl-α-D-galactoside, respectively (#M9130, #M2133, #M7633, Sigma, St. Louis, MO,) (Glaser and Sly 1973) to produce fluorescent 4-methylumbelliferone (4-MU) (#69580, Sigma, St. Louis, MO). The substrate solutions were prepared in a working concentration of 10 mM 4-MU-X substrate in 37.5 mM sodium acetate and 37.5 mM glacial acetic acid and stored at -70°C for up to a year.

All samples were assayed in triplicate. For each assay, 10 μl of sample was thoroughly mixed with substrate in the well of a black, clear bottom, 96 well plate (Sigma, St. Louis, MO). The plate was incubated at 37°C for one hour, after which 100 μl of glycine stop buffer (pH 10) was added to stop the reaction. Fluorescence was measured with a 96 well fluorescence plate reader and correlated to a 1 to 500 μM 4-MU standard curve to quantify samples. Since the background values for each of the 4-MU-X substrates were different, the background reading for each substrate was determined separately in more than three blank wells. One unit of enzyme activity corresponds to 1 nmol 4-MU substrate hydrolyzed per hour at 37°C.

Since hemolysis could change plasma colouration and alter the β-glucuronidase assay genuine background levels, plasma β-glucuronidase measurements were individually zeroed with sample-specific blanks. One unit of enzyme activity corresponds to 1 nmol 4-MU substrate hydrolyzed per hour. Proteins were determined with the method according to Lowry (Lowry *et al.* 1951).

2.4.1.2 *Human Growth Hormone*

Human growth hormone (hGH) mass was measured with a UBI-Magiwell quantitative hGH solid phase enzyme linked immunosorbant assay (ELISA) kit (#HP-901, United Biotech Inc., CA) according to the supplier's instructions.

2.4.1.3 *Human Factor IX*

Human factor IX was detected by ELISA as described (Hortelano *et al.* 1996).

2.4.1.4 Protein Determination

Total protein content in samples was determined by the method of Lowry (Lowry *et al.* 1951). Absorbances were correlated to protein concentrations with a standard curve made using serial dilutions of bovine serum albumin (BSA).

2.4.2 Detection of Anti-β-glucuronidase Antibodies

An immunoprecipitation assay was used to measure anti-β-glucuronidase antibodies. Mouse plasma (0.1 - 5 μl) was diluted in 0.04 M Tris-HCl, pH 8.0 to a final volume of 20 μl and mixed with 27.1 units of β-glucuronidase enzyme (30 μl) collected from 2A-50 cell tissue culture media, and incubated at room temperature for 1h. This was followed by a 1h incubation at room temperature with 20 μl of Pansorbin® protein A (200 mg/ml) (#507861, Calbiochem, La Jolla, CA) and centrifugation to pellet any β-glucuronidase bound to the Pansorbin® in the presence of anti-β-glucuronidase antibodies. The supernatant, containing any unbound β-glucuronidase, was assayed for β-glucuronidase activity as a measure of anti-β-glucuronidase antibodies in the plasma sample and expressed as units of β-glucuronidase immunoprecipitated/ml plasma. Plasma from normal mice without anti-β-glucuronidase antibodies were assayed as controls for non-specific immunoprecipitation.

2.4.3 Antibody Neutralization Assay

A solution of 27.1 units of β-glucuronidase was incubated for one hour at RT with or without serial dilutions (1 to 1/10,000) of serum samples from four different mice that contained high titres of anti-β-glucuronidase antibodies. β-glucuronidase activities were compared to determine if the antibodies neutralized β-glucuronidase activity.

2.4.4 Glycosaminoglycan Determination

Urine GAG/Creatin was determined by mixing 2 μl urine with 600 μl of 20 μM 1,9-dimethylene blue (Aldrich) and absorbance read at 540 nm after 20 minutes (Whitley *et al.* 1989; Moullier *et al.* 1993a) and compared to a chondroitin sulfate C standard (Sigma, St. Louis, MO). To determine creatin levels, 1 μl of urine in 9 μl dH_2O was mixed with 50 μl of 0.2 M NaOH and 50 μl saturated picric acid (Sigma, St. Louis, MO) and absorbance read at 490 nm after 20 minutes and compared to a creatin standard (Sigma, St. Louis, MO)

2.5 Histology and Histochemistry

2.5.1 Toluidine blue sections

Dissected tissues were fixed in glutaraldehyde, embedded in Spurr resin (#SPRLV, Sigma, St. Louis, MO), cut into 0.5 μm sections, stained for 1 minute in 0.1% toluidine blue (#89640, Sigma, St. Louis, MO), and examined and photographed with a Zeiss Axioskop microscope.

2.5.2 H &E Stains

Cryostat sections were stained for Hematoxylin and Eosin (H&E) using standard protocols by the McMaster University Medical Centre pathology lab, and examined and photographed with a Zeiss Axioskop microscope.

2.5.3 β-glucuronidase Histochemistry

Cryostat sections were stained histochemically with AS-BI-β-D-glucuronide to detect β-glucuronidase activity (Hayashi *et al.* 1963; Sands and Birkenmeier 1993). A fresh substrate solution (pH 4.5) of 13% AS-BI-β-D-glucuronide (#70493, Sigma, St. Louis, MO) in 0.1 N HCl and 0.2 M sodium acetate was pre-incubated with 10-20 μm cut cryosections for 4 hours. Cryosections were then incubated for 2 hours with 10 ml of fresh substrate solution (pH 5.2) supplemented with 20 μl of freshly prepared 1:1 mix of 4% pararosaniline chloride in 2N HCl and 4% sodium nitrite in dH_20. Sections were washed in pH 5.2 substrate solution, sealed in Permount® mounting medium (#SP15-100, Fisher Scientific International), and examined and photographed with a Zeiss Axioskop microscope. The brains of untreated homozygous MPS VII mice deficient in β-glucuronidase activity and wild-type mice with normal β-glucuronidase activity were used for negative and positive controls, respectively, for AS-BI-β-D-glucuronide histochemistry.

2.6 Immunohistochemistry

Immunohistochemistry (IHC) was used to visualize the presence of the hGH reporter gene product and implanted myoblasts. Mice were sacrificed with CO_2 and perfused with saline and 4% paraformaldehyde. Brains were removed and placed in 4% paraformaldehyde for 30 min. and then in 20% sucrose for 16 h. at 4°C. Brains were embedded in OCT Tissue-Tek® (Miles Scientific, Naperville, IL) and rapidly frozen in a slurry of dry ice and acetone or liquid nitrogen and stored at -70°C. Frozen brains were cryostat sectioned to 6-15 μm. Sections were

air dried for 2 hours and fixed for 30 min. in 4% paraformaldehyde, permeabilized for 3 seconds in acetone, and then washed in phosphate buffered saline (PBS). Sections were incubated overnight with primary antibodies, 1:100 rabbit anti-hGH (Monosan, (Cederlane), Hornby, Ont., Canada) or 1:100 rabbit anti-desmin (Sigma, St. Louis, MO), and 3% goat serum and 6% normal brain homogenate blocking solution in PBS. Sections were washed with PBS and then incubated with the secondary antibody 1:50 goat anti-rabbit IgG (H+L) conjugated to Texas Red® or FITC (Jackson Immunoresearch Laboratories, Inc., West Grove, PA). Controls for antibody specificity included sections from the brains of treated mice stained without the primary antibody and sections from the brains of mice that were mock-treated with encapsulated C2C12 cells that did not produce hGH. Sections were washed with PBS and dH_2O, air-dried, mounted, and viewed and photographed on a Zeiss Axioskop fluorescence microscope.

2.7 Statistical Analyses

Statistical analyses for *in vitro* microcapsule analysis were carried out using Minitab statistical software using a one-way ANOVA. Pre- versus post-treatment statistical comparisons of circadian running activities were performed using paired *t*-tests. Statistical comparisons of circadian running activities between genotypes were made using Students *t*-tests, assuming separate variances.

3.0 RESULTS

3.1 Encapsulation of various recombinant mammalian cell types in different alginate microcapsules.

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3.1.1 Rationale

Prior to this experiment, one type of alginate microcapsule had been optimized for immuno-isolation of recombinant cells. However, to improve this microcapsule formulation, methods to increase microcapsule strength were investigated. This experiment was performed in collaboration with Michael Peirone and Gonzalo Hortelano in order to compare the effect of encapsulation of three different cell types in the three different microcapsules types. Two of these microcapsule types were newly developed microcapsules, which were subsequently found to have improved microcapsule strength.

In relation to this thesis, this research was a critical first step to determine the optimal microcapsule formulation to encapsulate a specific fibroblast cell line (2A-50) that overexpressed and secreted high levels of β-glucuronidase. This optimal formulation was used in subsequent experiments to deliver β-glucuronidase to MPS VII mice.

3.1.2 Contributions

This research was accomplished by an equal collaboration between Colin Ross, Gonzalo Hortelano, and Michael Peirone and the support of Dr. Patricia L. Chang. The collaborators simultaneously monitored the characteristics of one cell line encapsulated in three different microcapsule formulations. Michael performed the microcapsule shearing experiments to compare the strength of the microcapsule formulations. The paper was written by Colin and Michael, and submitted and edited for publication by Colin and Dr. Patricia Chang..

Encapsulation of various recombinant mammalian cell types in different alginate microcapsules.

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Abstract: Microencapsulation of recombinant "universal" cells with immunoprotective membranes is an alternate approach to somatic gene therapy. Therapeutic gene products secreted by these cells can be delivered to different patients without immunosuppression or genetic modification of the host's cells. The encapsulation of different mammalian cell types (epithelial cells, fibroblasts, and myoblasts) is compared among three alginate-based microcapsules: (1) calcium-linked alginate microcapsules with a solubilized core and a poly-L-lysine–alginate-laminated surface; (2) barium-linked alginate beads with a gelled core; and (3) a hybrid formulation of barium-linked alginate beads with a poly-L-lysine–alginate-laminated surface. The mechanical stability of the different microcapsule types, as measured with a cone-and-plate shearing apparatus, was superior in the two barium-linked alginate beads. All cell types maintained high viability (65–90%) in culture after encapsulation. The recombinant gene products secreted by these cells (human growth hormone MW = 22,000, human factor IX MW = 57,000, and murine β-glucuronidase MW = 300,000) were able to traverse the three microcapsule types at similar rates. Cell numbers within the microcapsules increased twofold to > 20-fold over 4 weeks, depending on the cell type. Epithelial and myoblast cell numbers were not affected by microcapsule formulation; however, fibroblasts proliferated the most in the calcium-linked alginate spheres. These results show that for culturing fibroblasts in a mechanically stable environment the classical calcium-linked microcapsules are adequate. However, where mechanical stability is a more critical requirement, the solid barium-linked gelled beads are more appropriate choices. © 1998 John Wiley & Sons, Inc. J Biomed Mater Res, 42, 587–596, 1998.

Key words: gene therapy; immunoisolation; human growth hormone; β-glucuronidase; factor IX

INTRODUCTION

Microencapsulation has been proven to be an effective method of immunoprotecting biomaterials for implantation into unmatched recipients without the need for immunosuppression. Materials such as cells, enzymes, hormones, and bioadsorbants can be protected from the extracellular environment while bidirectional exchange of small molecules, such as oxygen, nutrients, wastes, and cell secretions, can occur.[1] Microcapsules made of alginate first were used to enclose pancreatic islet cells for the treatment of diabetes.[2] Since then, alginate microcapsules have been used for various applications, particularly for the encapsulation of pancreatic islets[3–6] and the encapsulation of recombinant cells for the delivery of therapeutic gene products, such as human[7] or murine[8] growth hormone and human clotting factor IX.[9,10]

Alginate is a polysaccharide extracted from seaweed composed of homopolymeric regions of 1,4-linked β-D-mannuronic acid (M-blocks) and α-L-guluronic acid (G-blocks) residues in varying proportions and arrangements interspersed with MG-blocks.[11] Gels form due to interchain chelation of divalent cat-

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ions, such as Ca^{2+} and Ba, $^{2+}$ with G-blocks in the alginate. The resulting gel strength increases in proportion to the square of the alginate concentration, with higher proportions and lengths of G-blocks in the alginate resulting in further increased gel strength.[12] For a microcapsule to perform effectively, it requires a biocompatible semipermeable membrane that completely surrounds implanted cells with an optimal immunoisolating molecular cutoff while providing an environment compatible with cell growth. The optimal parameters for the production of strong, appropriately permeable and nonimmunogenic alginate microcapsules have been well characterized by many researchers since their first use.

A majority of the implantation work using microencapsulated cells as a delivery vehicle has employed capsules made from a 1.5% (w/v) alginate–cell solution crosslinked in a calcium chloride bath and coated with poly-L-lysine and another layer of alginate (APA capsules). The poly-L-lysine layer allows for the control of the molecular weight cutoff of the microcapsule membrane.[13] The capsule's alginate core is liquefied using sodium citrate, leaving cells to float freely in the center of the capsule. These APA capsules have been implanted into several animal models and successfully have delivered recombinant gene products for varying lengths of time *in vivo* in rodents.[2,7,8,10] Alternate types of microcapsules using higher alginate concentrations, different crosslinking reagents, omitting the poly-L-lysine and second alginate layers, or omitting core solubilization, also have been used *in vivo*.[4,14,15] These capsules were formulated to improve the mechanical stability without compromising the immunoprotective properties.

In this *in vitro* study of different types of microcapsules, we included a new type of capsule that is a hybrid of the two mentioned above. Barium-alginate-poly-L-lysine-alginate (BPA) microcapsules were made by extruding a 2% (w/v) alginate–cell solution into a barium–chloride bath, as in the BaAlginate capsules. These BPA capsules then were coated with a layer of poly-L-lysine and a second layer of alginate, as in the APA capsules. This hybrid capsule has the benefits of the increased strength (imparted by the higher alginate concentration and gelled alginate core) combined with the ability to control the permeability of the microcapsule membrane.

The behavior of encapsulated cells is compared among the three types of microcapsules described above in their ability to function as an implantable device for recombinant cells. Various cell types with the potential for use in gene therapy, that is, fibroblasts, epithelial cells, and myoblasts, are assessed as they all have the common properties of being explantable and proliferative, and they all are amenable to genetic modification. The *in vitro* properties of these implantable devices with the different entrapped mammalian cell types engineered to secrete recombinant gene products of different sizes are herewith reported.

MATERIALS AND METHODS

Large scale preparation of plasmid DNA

Bacteria were grown for 8 h in a 15 mL centrifuge tube containing 5 mL of luria broth (LB) medium supplemented with 50 μg/mL of ampicillin on a 37°C shaking platform. From this culture, 2.5 mL were transferred to a flask containing 250 mL of LB medium with 50 μg/mL of ampicillin for overnight growth. The plasmid DNA was extracted with the Maxi-prep protocol from a Qiagen DNA extraction kit.

Cell line

Unless otherwise stated, the cells were maintained in 75 cm^2 tissue culture flasks at 37°C in a 5% CO_2 water jacket incubator (Fisher Scientific, Isotemp Incubator). The culture medium was α-minimal essential medium supplemented with 10% fetal bovine serum, penicillin (100 U/mL)–streptomycin (100 μg/mL) and 2 m*M* of L-glutamine (Gibco, BRL). The untransfected MDCK cells, a canine renal epithelial cell line (derived from a normal adult female cocker spaniel in 1958 by Madin and Danby, ATCC, Rockville, Maryland, CCL-34) were kindly provided by Dr. Frank Graham, McMaster University, Hamilton, Canada. The 2A50 cells, kindly provided by Dr. W.S. Sly (Washington University, St. Louis, Missouri) were a mouse Rec^- fibroblast cell line deficient in the mannose 6-phosphate receptor and transfected to secrete murine β-glucuronidase at 522 ± 40 nmole/million cells/h. The plasmid used for transfection, pMSXND-MβG, encoded the cDNA for β-glucuronidase as well as dihydrofolated reductase, which allowed amplification of gene expression by methotrexate selection.[16] The D6 myoblasts were a mouse myoblast cell line C2C12 (ATCC#CRL1772) transfected with a vector encoding human factor IX under the control of the human β-actin promoter and the *Neo^R* gene, conferring resistance to G418. This clone was isolated after selection in G418 and secreted factor IX at 116 ± 16.24 ng/million cells/h.[10]

Transfection of MDCK cells

The MDCK cells were transfected through calcium phosphate-mediated DNA precipitation[17] with the plasmid pNMG3. This plasmid contained the human growth hormone gene (*hGH*) under the control of the mouse metallothionein promoter mMTI with an SV40 sequence upstream of the *hGH* gene and polyadenylation signal downstream of the gene.[18] Additionally, the *phosphotransferase* gene in pNMG3 allowed for the selection of transfected clones with

400 μg of G418/mL of medium. The surviving clones were assayed for *hGH* secretion. Secretion rates of the 24 highest producing clones were determined. The highest producing clone (H8) secreting 19 ± 1.2 ng/10^6 cells/h was chosen for subsequent experiments.

Encapsulation

All encapsulation procedures were carried out at 4°–10°C under sterile conditions. Cells were harvested before reaching confluence from 75 cm^2 tissue culture flasks using 0.125% trypsin, re-suspended in 500 μL of cold saline and mixed with the desired volume of 1.5% alginate (for APA capsules crosslinked with $CaCl_2$) or 2% alginate (for BaAlginate and BPA capsules crosslinked with $BaCl_2$; Keltone LV alginate Lot No. 17703A, kindly provided by Kelco, San Diego, California). Approximately 2 × 10^6 cells were added per mL of sterile, filtered (0.2 μm syringe filters, Gelman Sciences #4192) potassium alginate, and the mixture drawn up into a 20 cc syringe that had been placed in a syringe pump (Orion Sage pump, model #M362). This cell suspension was extruded through a 27 gauge blunt end needle (Popper & Sons, New York, Cat. #7400,) at a rate of 99.9 cm^3/h × 1/100 and dispersed with a concentric air stream 1 mm in diameter delivered at 4 L/min. The resulting capsules fell into 20 mL of cold 1.1% $CaCl_2$ or 20 m*M* of $BaCl_2$, which served to crosslink the polysaccharide polymers.

Capsule preparation

APA: Alginate-poly-L-lysine-alginate capsules

The capsules were fabricated according to the procedure of Sun,[19] as modified by Liu et al.[9] Briefly, after extrusion of the cell–alginate mixture into the crosslinking agent, the capsules were transferred to a sterile 50 mL conical polypropylene tube and washed successively with 0.55% and 0.28% $CaCl_2$ in saline, 0.85% saline, 0.1% CHES (2-[N-Cyclohexylamino]ethan-sulfonic acid), and 1.1% $CaCl_2$. The droplets were further crosslinked with 0.05% poly-L-lysine (PLL, MW 22,000) for 6 min, washed with 0.1% CHES, 1.1% $CaCl_2$, 0.85% saline and coated with another layer of 0.03% alginate for 4 min. This treatment was followed with another wash in 0.85% saline, and the unpolymerized polysaccharide in the core of the microcapsule was dissolved by washing for 6 min in 0.55 m*M* of sodium citrate. After two rinses in serum-free medium, the capsules were transferred to complete medium and kept under normal culture conditions.

BaAlginate: Barium alginate capsules

The cell–alginate suspension was extruded as droplets into a 20 m*M* $BaCl_2$ bath for 90 s while the alginate crosslinked with the barium. After 90 s, counted from the beginning of the extrusion, the capsules were poured into 300 mL of 0.9% NaCl solution, and a fresh beaker of $BaCl_2$ was used to collect capsules for another 90 s. Once extrusion had finished, the capsules were rinsed six–eight times in three capsule volumes of 0.9% NaCl solution and kept under normal tissue culture conditions.

BPA: Barium-poly-L-lysine alginate capsules

The capsules were prepared as above, but after crosslinking in the $BaCl_2$ solution for 90 s, the capsules were treated with 0.05% PLL for 6 min while on a stirring platform and rinsed with 0.9% NaCl. The capsules then were coated with another layer of 0.04% alginate for 4 min while on a stirring platform. Finally, the capsules were rinsed with two washes of three capsule volumes of 0.9% NaCl, followed by two rinses with three volumes of serum-free medium, and kept under normal tissue culture conditions.

Assay for secreted gene products

The *in vitro* determination of rate of secretion from microencapsulated or unencapsulated cells was performed on culture media sampled at specific time intervals. The medium was removed from the capsules or cells, which were washed with sterile PBS before being re-fed with a medium previously equilibrated in the incubator. Samples were drawn from the media at 0, 1, and 2 h and replaced with an equivalent volume of equilibrated medium. Human growth hormone (*hGH*) was measured quantitatively using an enzyme-linked immunosorbent assay (ELISA) kit (UBI-Magiwell hGH Quantitative kit, United Biotech Inc., California, Cat. # HP-901) according to the supplier's instructions. β-glucuronidase activity was assayed fluorometrically using 4-methylumbelliferyl-β-D-glucuronide[20] (Sigma Cat .#M9130; 1 unit β-glucuronidase activity = 1 nmol of 4-MU released/h). Human factor IX was detected by ELISA.[10]

Characterization of microencapsulated cells

Viability

Viability was determined by scoring the percentage of unstained cells after staining crushed microcapsules on a slide with 0.4% Trypan blue (Gibco, Cat. #15250-061).

Cell number per capsule

A capsule suspension (100 μL) containing a known number of APA capsules was transferred to an Eppendorf tube. The cells were released from the microcapsules by rupturing the capsules with a small pestle (Baxter, 749520-0000). An aliquot of the resulting suspension (15 μL) was transferred to a hemacytometer for counting. The average and standard

deviation were calculated from five determinations. The BaAlginate or BPA capsules were treated similarly, except 100 μL of 0.5M of EDTA were used to weaken the microcapsules before rupturing with the pestle.

Microcapsule shearing

Capsules suspended in medium were subjected to controlled fluid shear in a cone-and-plate flow device[21] in order to assess their resistance to this type of mechanical stress. In this device the fluid suspension is contained in a circular well 1 inch in diameter. The bottom of the well serves as the plate, and a Plexiglas cone with an angle of 4° is lowered into the well so that the tip just touches the plate. The cone then is rotated at a constant speed, thereby producing a uniform shear field across the diameter of the apparatus. At low cone angles and under laminar flow conditions, the shear rate γ is given as

$$\gamma = \frac{\Omega}{\theta}$$

where Ω is the rotational speed and Θ is the cone angle. In the present experiments the rotational speed was 333 rpm, giving a shear rate of 500 s^{-1}. Times of exposure to shear were 15, 30, and 45 min. The microcapsule samples were removed from the wells at each time point and photographed using a Sony CDD color video camera attached to a Zeiss light microscope using Vidas 21 software. The percentage of capsules remaining intact was scored by counting the total number of intact capsules in the field and dividing by the total number of capsules captured in the field. The resulting number was expressed in terms of the percentage of capsules remaining intact with increasing duration of shear.

Data analysis

Statistical analyses were carried out using Minitab statistical software. A one-way ANOVA was performed (P = 0.05).

RESULTS

Microcapsule morphology

When compared morphologically, each capsule type displayed a distinctive appearance that was characteristic of its individual formulation. In the APA microcapsules with the alginate core liquefied and enclosing the MDCK epithelial cells, within 24 h the cells aggregated and grew in a clump, often gravitating towards one side of the capsule wall (Fig. 1, APA). The surface of the capsule wall was clearly visible, presumably due to the presence of the poly-L-lysine and the alginate coats. Because the alginate core was liquefied, the microcapsules swelled from 150 to 200% of their original volume, resulting in microcapsules with a final diameter of 700–900 μm.

The BaAlginate capsules, having a solid alginate core and no poly-L-lysine or alginate coat, were distinguished from the APA capsules by the lack of a distinctive surface when examined with dark field microscopy (Fig. 1, BaAlginate). In addition, the enclosed cells remained distributed throughout the interior of the capsule, growing in discreet small clumps. Retention of the solid alginate core in this capsule type prevented swelling, resulting in a smaller microcapsule (500–700 μm) than the APA type had.

The BPA capsule, being a hybrid of the aforementioned types, shared qualities of both capsules (Fig. 1, BPA). The solid alginate core of this capsule type kept cells distributed throughout the interior of the capsule, where they grew in discreet clumps, as in the BaAlginate capsules. The presence of the poly-L-lysine layer and the alginate coat, however, allowed for the surface of the capsule wall to be easily visualized, as in the APA capsules. Retention of the solid alginate core, as in the BaAlginate type, prevented swelling, resulting in both Ba^{2+} capsule types being about the same volume.

Encapsulated cell viability

In general all three capsules were similarly compatible with the survival of various cell types (Fig. 2). Viability for the encapsulated MDCK epithelial cells remained high (> 90%) throughout the 4-week experiment [Fig. 2(a)]. There was no significant difference in cell viability among the APA, BaAlginate, and BPA microcapsules on all days except day 14, when the cell viability in the BaAlginate capsules was slightly lower that that of APA or BPA encapsulated cells. Similarly, the D6 myoblasts also showed no difference in viability among the three capsule types [Fig. 2(b)]. However, this cell type was a less robust cell line than the MDCK cells. Hence the viability after encapsulation was relatively low throughout the 4 weeks (40–70%). A similar trend was observed in a third cell type, the 2A50 fibroblasts [Fig. 2(c)]. These cells showed no difference in viability among the three capsule types. The cells in each type of capsule showed a similar decline after prolonged culture, from about 85% on day 21 to about 75% on day 28.

Encapsulated cell proliferation

Although all three capsule types supported growth for a variety of cell types, the extent of proliferation in

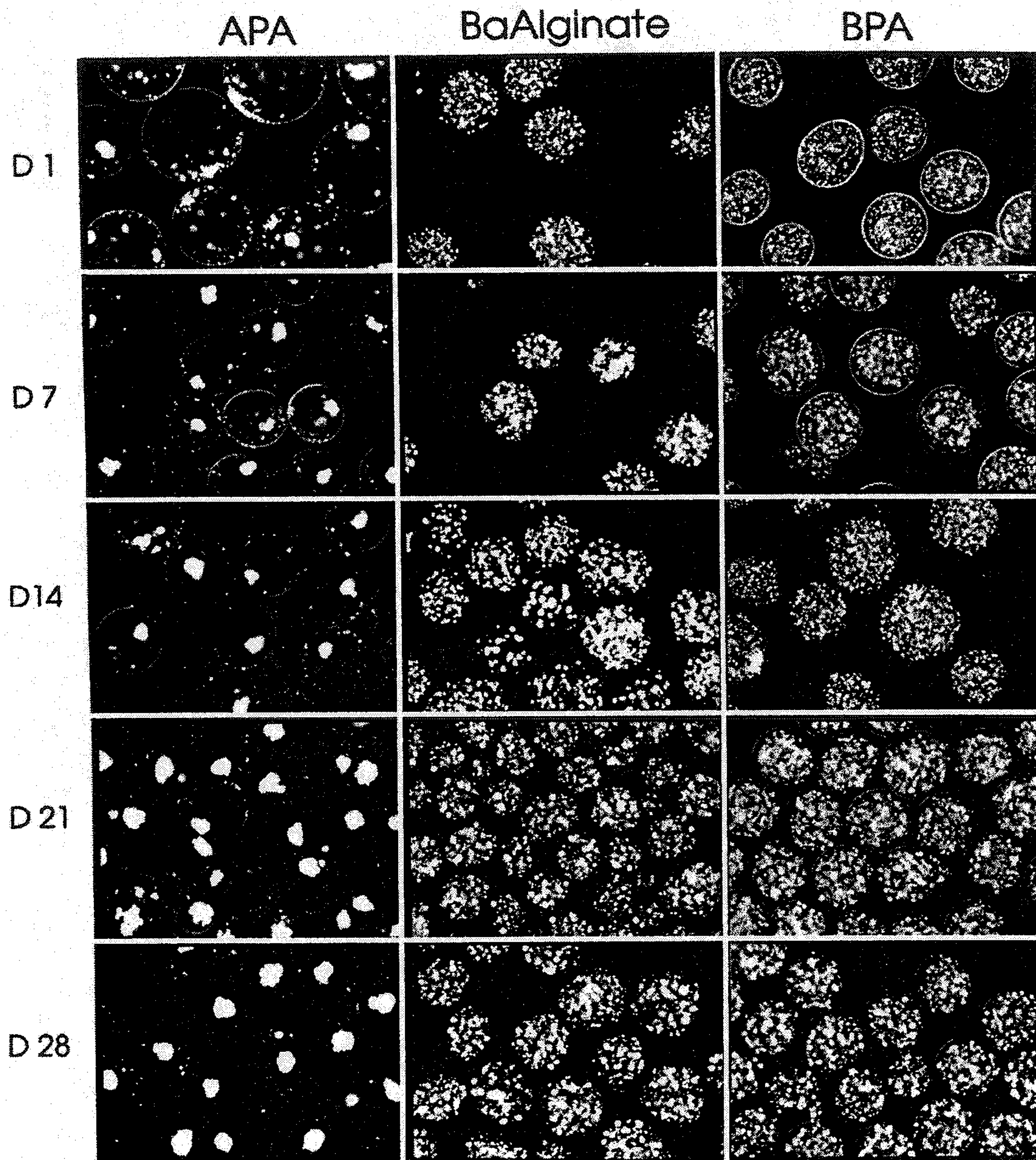

Figure 1. Morphology of the three types of microcapsules. The canine epithelial MDCK cells, encapsulated at an initial cell density of 2×10^6 cells/mL of alginate were photographed in each of the three microcapsule types (APA, BaAlginate, BPA) on various days postencapsulation (D1, D7, D14, D21, D28). The surface of the APA and BPA microcapsules visually can be distinguished from the BaAlginate capsules by the presence of the poly-L-lysine and second alginate layers. In the absence of this layer, the surface of the BaAlginate capsule was not visible with the dark field photomicroscopy. Original magnification = ~×1000.

each capsule type was dramatically different depending on the cell type (Fig. 3). For example, the encapsulated epithelial MDCK cells showed a similar profile of cell proliferation in all the capsule types [Fig. 3(a)]. From days 1 to 7 postencapsulation, the number of cells inside all the capsules increased by 50 to 100%. Cell numbers generally continued to increase consistently in all microcapsule types over the course of the 4-week experiment except that in the BPA capsules, the cell number reached a plateau earlier, by the 3rd

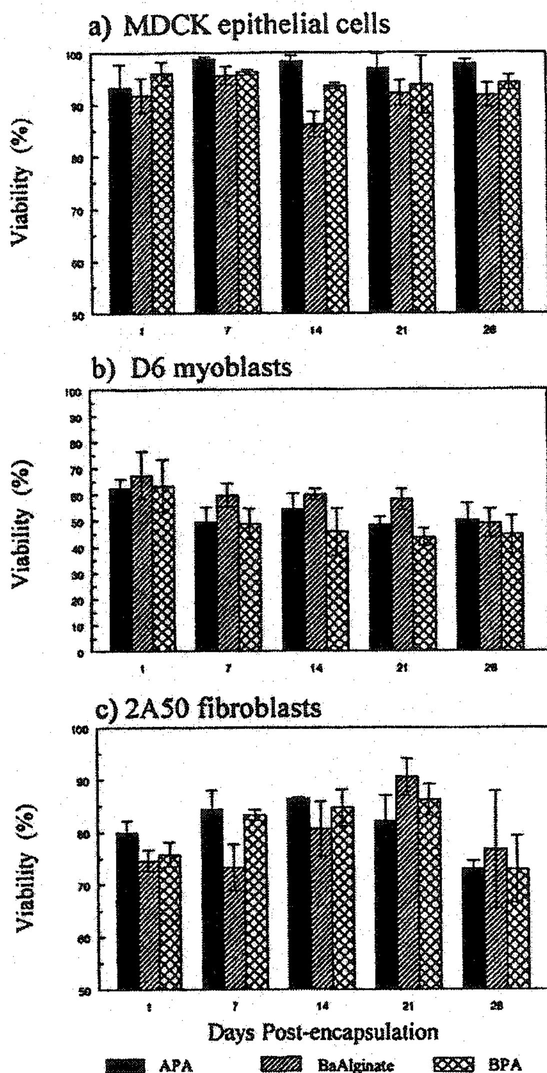

Figure 2. Viability of microencapsulated cells. The viability of various encapsulated cell types was measured weekly for 4 weeks by crushing the microcapsules and releasing the cells, which then were scored by trypan blue exclusion staining, as described in the Materials and Methods section: (a) MDCK epithelial cells; (b) D6 myoblasts; (c) 2A50 fibroblasts. Data represent the average of five measurements ± SD and statistical significance was estimated with ANOVA ($P = 0.05$).

week (D21). These results suggest that all the microcapsule types are similarly supportive of epithelial cell proliferation.

In the second cell type, the D6 myoblasts, there also was little difference in cell proliferation among the capsule types [Fig. 3(b)]. Consistent with their less robust state, compared to the MDCK epithelial cells and 2A50 fibroblasts, their cell numbers increased only about twofold after the 4-week period even though the increase was consistent and gradual throughout the experiment.

In contrast, for the 2A50 fibroblasts, the APA capsules supported a greater rate of proliferation over 28 days *in vitro* than the BaAlginate or BPA capsules [Fig. 3(c)]. In the APA capsules, there was an increase of > 300% in the number of cells between days 7 and 14 postencapsulation, reaching a plateau at day 14 and

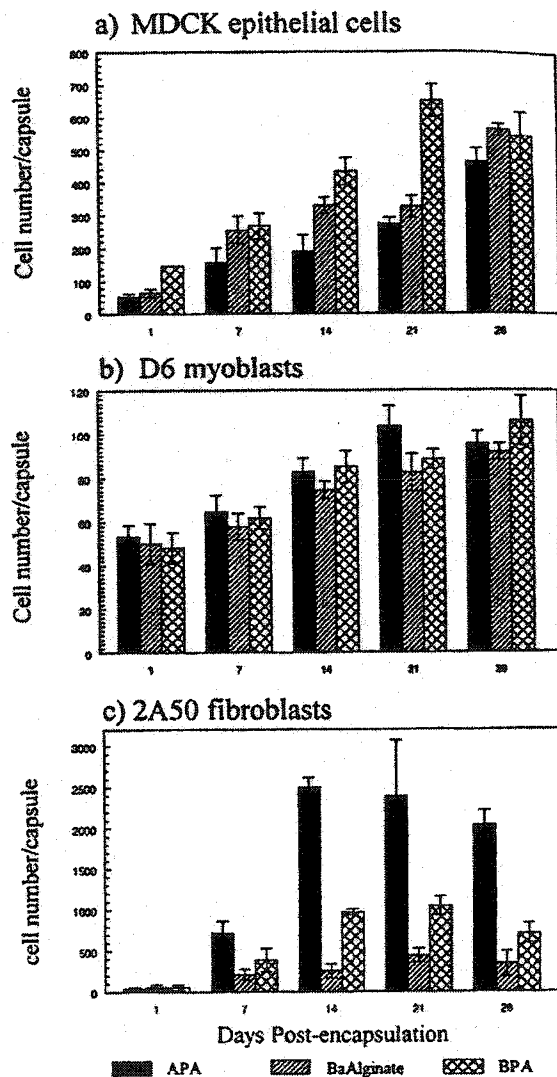

Figure 3. Proliferation of recombinant cells within microcapsules. The proliferation of cells inside each type of microcapsule was estimated weekly for 4 weeks by releasing cells from a known number of microcapsules and counting them using a hemacytometer, as described in the Materials and Methods section: (a) MDCK epithelial cells; (b) D6 myoblasts; (c) 2A50 fibroblasts. Data represent the average of five measurements ± SD.

then decreasing slightly until the end of the experiment on day 28. In contrast, the cell number in the BaAlginate capsules increased only slightly (by ~20%) between days 7 and 14 postencapsulation. However, the increase was sustained at a higher rate (by ~80%) at D21 before declining by 23% on day 28. The rate of cell proliferation in the BPA capsules was higher than that in the BaAlginate capsules, with cell numbers increasing by > 100% between days 7 and 14 before declining by 31% on day 28.

Secretion of recombinant gene products from microencapsulated cells

In general, regardless of the size of recombinant products, which ranged from 22–300 kDa, all three microcapsule types permitted their diffusion at all times studied (Fig. 4). Furthermore, the rates of secretion by the recombinant cells in most cases were similar among the three capsule types with the exception that the BPA capsules appeared slightly superior to the other two capsule types at times, particularly for the secretion of hGH. Thus the secretion of human factor IX [Fig. 4(b)] and mouse β-glucuronidase [Fig. 4(c)] was very similar among the three capsule types, with no significant differences ($P > 0.05$). In contrast, the rate of hGH secretion from cells inside the BPA capsules was greater than that from either APA or BaAlginate capsules throughout most of the time periods studied [Fig. 4(a)]. On days 7, 21, and 28 postencapsulation, hGH was secreted from the BPA capsules at a rate about twofold that of the other two microcapsule types. Overall, secretion of all recombinant products appeared to be the highest within the first week of encapsulation, declining steadily after that. This decrease was more dramatic in the capsules containing cells secreting hGH [Fig. 4(a)] and β-glucuronidase [Fig. 4(c)] and less so for factor IX [Fig. 4(b)]. Hence, while there was a slight difference in the secretion characteristics of the recombinant cells, the different types of microcapsules were capable of permitting diffusion of recombinant molecules over a wide range of molecular weights. The similarity in β-glucuronidase secretion profiles [Fig. 4(c)] among the different capsule types showed that a molecule the size of this enzyme (MW 300,000) could diffuse from all the microcapsule types.

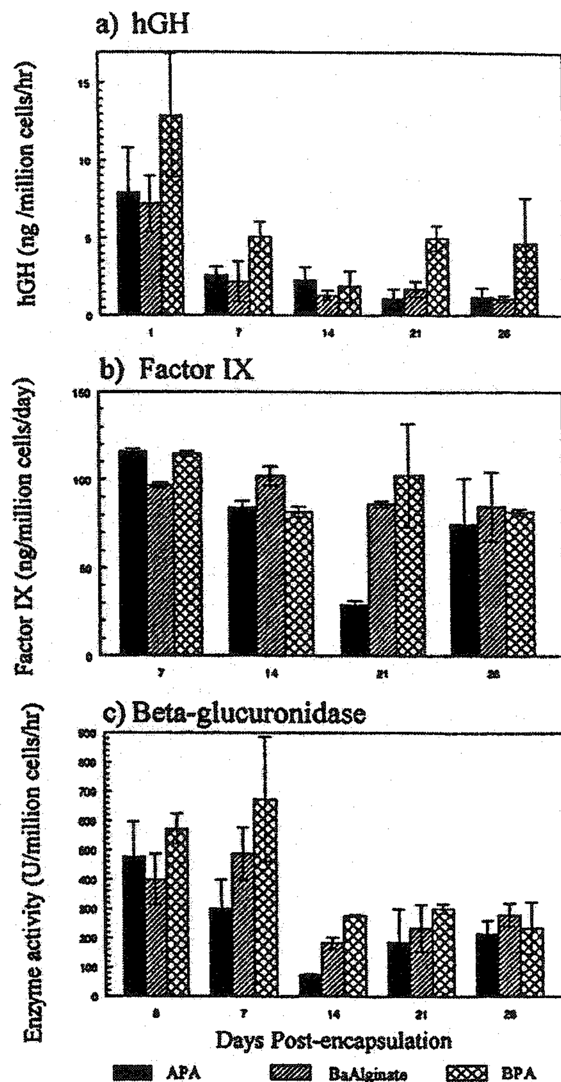

Figure 4. Secretion by microencapsulated recombinant cells. After the cells were encapsulated in the three different types of microcapsules, the rate of secretion of the recombinant product into the media was estimated at weekly intervals for 4 weeks: (a) MDCK cells secreting human growth hormone (MW 22,000); (b) D6 myoblasts secreting human clotting factor IX (MW 57,000); (c) 2A50 cells secreting murine β-glucuronidase (MW 300,000). Data represent the average of four measurements ± SD and statistical significance estimated with ANOVA ($P = 0.05$).

Mechanical strength of different microcapsules

To assess the effects of different alginate concentrations and coatings on the mechanical stability of the capsules, we subjected each capsule type to a defined fluid shear field. The shear stress generated by the cone-and-plate apparatus at a shear rate of 500 s^{-1} provoked distinctly different responses from the three types of microcapsules (Fig. 5). When the APA capsules were sheared for 15 min, 7.7% were broken, with the percentage broken increasing to 22.7% after 45 min of shearing. In contrast, following 15 min of shearing

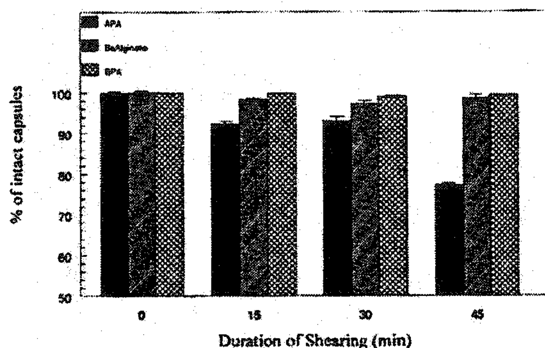

Figure 5. Response of microcapsules to the fluid shear ($500\ s^{-1}$ shear rate) using the cone-and-plate apparatus. The three types of microcapsules were fabricated and subjected to a uniform fluid shear field. A sample was removed at the various time points for morphometric analysis, as described in "Materials and Methods." Data represent an average of three measurements ± SD.

only 1.6% of the BPA and BaAlginate capsules were broken and hardly any of the BPA capsules were ruptured. Even after 45 min of shearing, neither barium capsule type sustained any further significant rupture except that the BaAlginate capsules appeared slightly more deformed than the BPA type (data not shown).

DISCUSSION

In these *in vitro* experiments, we showed that different alginate microcapsules demonstrate a great deal of similarity in their interactions with a variety of recombinant cell types secreting gene products of different sizes. However, distinct differences also were observed, such as their mechanical properties and support of cell-type specific proliferation. These differences will dictate the types of applications for which they are best suited.

In the APA capsules, the liquefied alginate core allowed the enclosed cells to grow together in a clump at the periphery of the capsule (Fig. 1). The clumping of the cells likely was due to the fact that these MDCK cells are adherent in growth. In the absence of a growing surface inside the capsule, the cells rely on one another for attachment. The phenomenon of the encapsulated cells having a peripheral localization inside the APA capsules may be related to the chelation and subsequent dechelation of the microcapsule after it is washed with $CaCl_2$ and then with the subsequent washing solutions.[22] It has been observed that during the production of APA microcapsules, when the K^+ in the alginate is exchanged for Ca^{2+}, the CaAlginate bead expands and the enclosed cells move to the periphery of the bead. Coating the CaAlginate bead in poly-L-lysine results in shrinkage due to the crosslinkage of the alginate, and subsequent liquefaction of the capsule core occurs, leaving the cells localized near the periphery of the capsule. This peripheral localization of cells was observed less often in the solid-core $BaCl_2$ crosslinked capsules, possibly because the cells remained embedded in the alginate gel. In the case of the BaAlginate and BPA capsules, the cells grew in small discreet clumps in the capsules, never coming together into one large cell clump as in the APA capsules (Fig. 1).

By liquefying the alginate core of the APA capsules, this capsule type increased by 50–100% of its original volume. The resulting capsule diameter ranged from 700–900 μm, as opposed to the 500–700 μm microcapsules obtained with the solid-core BaAlginate and BPA capsules. Since the same number of cells originally was mixed with the alginate to make the different microcapsule types, the resulting larger APA capsules would be less efficient at cell loading in terms of numbers of cells per volume of capsules. Hence, a larger volume of APA microcapsules would need to be implanted in a recipient in order to have the same number of cells as in a smaller volume of BaAlginate or BPA capsules, an important consideration for implantation therapy *in vivo*.

At various times throughout the experiment, the viability of the MDCK epithelial cells inside the APA capsules was slightly higher than that in either the BaAlginate or BPA capsules, but those of the myoblasts and fibroblasts did not vary significantly with the capsule types (Fig. 2). The reason for this may have been a temporary reaction to the toxicity of the $BaCl_2$,[23] to which the epithelial cells may be more sensitive than the other cell types. The long-term potential disadvantage of Ba^{2+} toxicity also needs to be addressed if this cation is released from coacervation with the alginate polymer. However, for both barium-linked capsule types, the viability of the enclosed MDCK epithelial cells still remained above 90% throughout the experiment. Furthermore, the viability of these epithelial cells was consistently higher than those of the myoblast and fibroblast cell lines, thus highlighting that the growth potential inherent to different cell lines is a more important determinant of survival than the environmental factors offered by the three capsule types.

The presence of a gelled core did not affect the proliferative capacity of the epithelial MDCK cells or myoblasts but appeared less favorable for the growth of fibroblasts (Fig. 3). While there was no significant difference among the three capsule types in supporting growth of epithelial cells and myoblasts, the 2A50 fibroblasts appeared to flourish best within APA capsules [Fig. 3(c)]. The absence of a gelled core in this capsule type might facilitate the spreading of fibro-

blastic processes that naturally occur in cultured fibroblasts.

In spite of their different physical conformations, all three capsule types have sufficiently large pore sizes to allow for the diffusion of nutrients into the capsules, as evidenced by the high cell viability, while allowing the diffusion of products ranging from the 22 kDa hGH [Fig. 4(a)] to even the 300 kDa β-glucuronidase [Fig. 4(c)]. This lack of permeability barrier for molecules above 150 kDa, the size of immunoglobulin IgG, now has been observed repeatedly in encapsulation with alginate.[24,25] Other types of immunoisolation devices, such as HEMA–MMA, seem to maintain such a permeability barrier.[26] The loss of a permeability threshold at 150 kDa did not seem to affect the *in vivo* immunoprotection of encapsulated nonautologous cells.[7,24] Hence it is likely that exclusion of even larger molecular species (greater than 10^6 Da; i.e., IgM, complement) and cellular immune mediators (i.e., CTL) play a more critical role in providing immunoprotection, particularly for allogeneic cellular implants.[27]

The most distinctive difference among the capsule types was their mechanical stability. They clearly differed in their ability to withstand fluid shear (Fig. 5). It has been documented that alginate beads made from the higher alginate concentration are more stable.[12] Additionally, the higher affinity of alginate for Ba^{2+} ions than for Ca^{2+} ions would increase the relative stability of the BaAlginate and BPA capsules.[15] This was verified by the increased durability of the BaAlginate and BPA capsules made from 2% alginate compared to the 1.5% alginate APA type under the same shear conditions (Fig. 5). Such mechanical stability is critical if the technology of immunoisolation for gene therapy is to be applied to large animals.[28] The APA capsules that were stable for > 7 months after implantation in mice[10] disintegrated in < 14 days after implanting into dogs.[28] However, when the BaAlginate or BPA capsules were used to deliver hGH from recombinant cells to dogs, the duration and levels of recombinant gene product were vastly improved, thus emphasizing the importance of biomaterial mechanical stability in the efficacy of this form of gene therapy.

CONCLUSIONS

Alginate microcapsules fabricated with or without the PLL–alginate laminated surface, crosslinked with Ca^{2+} or Ba^{2+}, and retaining a gelled or solubilized alginate core all supported growth of a variety of recombinant cell types. Secretion of recombinant gene products ranging in size from 22 kDa to 300 kDa occurred equally well in all three capsule types, and the presence of a poly-L-lysine alginate membrane did not affect the exit of these products. However, unlike the epithelial cells and myoblasts, the fibroblasts favored the Ca^{2+} linked spheres with a solubilized core. Thus this type of microcapsule is the preferred choice if growth from encapsulated fibroblasts in a mechanically stable environment is desired, such as *in vitro* production of recombinant gene products or *in vivo* implantation in rodents. In contrast, the microcapsules with a solid alginate core and crosslinked with Ba^{2+} appeared mechanically more stable than the Ca^{2+}-linked alginate spheres with a solubilized core. These microcapsules should be considered when immunoisolation devices are used for gene therapy in large-animal models, such as canines, where mechanical stability is a more important requirement and where recombinant cell lines other than fibroblasts can be used.[28]

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3.2 Treatment of a lysosomal storage disease, MPS VII, with microencapsulated recombinant cells.

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3.2.1 Rationale

This rationale for this experiment was to determine if a novel immuno-isolation gene therapy strategy could be applied to a metabolic genetic disease, using the murine lysosomal storage disease MPS VII as a model. Previously, the optimal microcapsule formulation to encapsulate the β-glucuronidase overexpressing 2A50 fibroblast cell line had been determined (Chapter 3.1). In this experiment, microencapsulated cells were used to deliver β-glucuronidase to MPS VII, β-glucuronidase deficient mice.

In a second part of this study, anti-CD4-mediated immunosuppression was investigated to circumvent the anti-β-glucuronidase immune response that resulted after implantation of β-glucuronidase secreting microencapsulated cells into MPS VII mice. MPS VII mice do not express β-glucuronidase, and responded to the re-supplied β-glucuronidase as a foreign antigen with a strong humoral immune response.

3.2.2 Contributions

This research was accomplished by Colin Ross with the support of Dr. Patricia L. Chang and the assistance of Laila Bastedo, Stephanie Maier, and Dr. Mark Sands (Washington University School of Medicine). Colin performed the animal surgeries, blood and tissue collection, microcapsule analyses, histology, enzyme assays and antibody assays for the data from week 0 to week 10, and Laila performed animal surgeries and collected samples and performed enzyme assays for week 2 and week 4. Mark Sands performed the tissue histochemistry analysis from tissue samples provided to him. Laila Bastedo performed the GAG assay. For the anti-CD4 immunosuppression experiments, Colin performed the β-glucuronidase and anti-β-glucuronidase antibody assays, and Stephanie assisted in animal surgeries and blood collection.

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Mary Ann Liebert, Inc.

Treatment of a Lysosomal Storage Disease, Mucopolysaccharidosis VII, with Microencapsulated Recombinant Cells

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ABSTRACT

Most lysosomal enzyme deficiencies are catastrophic illnesses with no generally available treatments. We have used the β-glucuronidase-deficient mouse model of mucopolysaccharidosis type VII (MPS VII) to develop an alternative approach to therapy. A "universal" cell line engineered to secrete the missing enzyme is implanted in all recipients requiring the same enzyme replacement. The cells, although nonautologous, are rendered immunologically tolerant by encapsulation in microcapsules that provide protection from immune mediators. Using this strategy, we injected β-glucuronidase-secreting fibroblasts enclosed in alginate microcapsules into mutant MPS VII mice. After 24 hr, β-glucuronidase activity was detected in the plasma, reaching 66% of physiological levels by 2 weeks postimplantation. Significant β-glucuronidase activity was detected in liver and spleen for the duration of the 8-week experiment. Concomitantly, the intralysosomal accumulation of undegraded glycosaminoglycans was dramatically reduced in liver and spleen tissue sections and urinary glycosaminoglycan content was reduced to normal levels. Elevated secondary lysosomal enzymes β-hexosaminidase and α-galactosidase were also reduced. However, implanted mutant MPS VII mice developed antibodies against the murine β-glucuronidase, demonstrating a potential obstacle in patients with a null mutation who react against the replaced enzyme as a foreign antigen. The antibody response was transiently circumvented with a single treatment of purified anti-CD4 antibody coadministered with the microcapsules. This resulted in increased levels and duration of β-glucuronidase delivery. Similarly, treated heterozygous mice maintained elevated levels of β-glucuronidase and did not develop antibodies. This novel cell-based therapy demonstrates a potentially cost-effective and nonviral treatment applicable to all lysosomal storage diseases.

OVERVIEW SUMMARY

We set out to explore the feasibility of delivering therapeutic recombinant gene products from an encapsulated universal cell line as a treatment for lysosomal enzyme deficiencies. Mice with the lysosomal storage disease mucopolysaccharidosis VII (MPS VII) received injections of nonautologous cells expressing murine β-glucuronidase. To prevent the eventual graft rejection these recombinant cells are immunologically protected with alginate microcapsules. Although a high level of expression was initially achieved with concomitant reductions in disease pathology, the implanted mutant mice developed antibodies against the β-glucuronidase. However, this antibody response could be inhibited by immunosuppression, namely T cell-mediated immune modulation, thereby prolonging the effective delivery of the replacement enzyme. We have thus demonstrated the potential of a long-term and potentially cost-effective cell-based therapy applicable to all lysosomal storage diseases, and the means to mitigate the inhibitor development in recipients who may develop an immune response against the therapeutic product.

INTRODUCTION

More than 300 human gene therapy clinical trials are in progress and more than 3000 patients worldwide have

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been enrolled in these studies (Marcel and Grausz, 1997; Anderson, 1998; Clinical Trials Update, 1999). Most are focused on common non-Mendelian disorders such as cancer, and only a minority (~10%) address classic Mendelian disorders.

Lysosomal storage diseases are a group of more than 40 separate disorders caused by a deficiency of one of the several enzymes responsible for macromolecule catabolism (Neufeld and Muenzer, 1989). Because of the lack of effective treatments for most of the lysosomal storage diseases, many novel gene-based techniques are being developed in a well-characterized mouse model of mucopolysaccharidosis VII (MPS VII, Sly syndrome). MPS VII is a representative lysosomal storage disease that results from deficiency of the lysosomal enzyme β-glucuronidase (Sly *et al.*, 1973). The incomplete breakdown and progressive accumulation of glycosaminoglycans lead to eventual cell and organ dysfunction. The murine model of MPS VII is caused by a single base pair deletion in the coding sequence of β-glucuronidase, generating a premature stop codon and no detectable mRNA (Sands and Birkenmeier, 1993). The affected MPS VII mouse closely reflects the human disease and has been well characterized (Birkenmeier *et al.*, 1989; Levy *et al.*, 1996) with severe peripheral organ abnormalities such as hepato- and splenomegaly due to lysosomal inclusions, skeletal deformities, cataracts, as well as auditory, cognitive, and neurological deficits in the central nervous system (Vogler *et al.*, 1990; Chang *et al.*, 1993; Sands *et al.*, 1995).

Currently, two approaches in gene delivery have been used to treat the MPS VII mice: *in vivo* (Wolfe *et al.*, 1992a; Lau *et al.*, 1995; Li and Davidson, 1995; Wolfe *et al.*, 1996; Ohashi *et al.*, 1997; Daly *et al.*, 1999a,b; Ghodsi *et al.*, 1999; Skorupa *et al.*, 1999; Stein *et al.*, 1999), and *ex vivo* (Wolfe *et al.*, 1992b; Marechal *et al.*, 1993; Moullier *et al.*, 1993; Naffakh *et al.*, 1996; Snyder and Wolfe, 1996; Taylor and Wolfe, 1997) gene therapy both requiring virally mediated gene modification of the recipient's own cells. We propose a third approach in which no direct genetic modification of the recipient is required. Instead, nonautologous cell lines that can be genetically modified to produce the therapeutic product are used. By enclosure within immunoisolation devices with controlled permeability, these cells can be protected from graft rejection. Influx of large immune mediators such as complement (MW > 10^6) or lymphocytes will be inhibited while smaller molecules including recombinant products with a molecular weight range from 22,000 to 300,000 (Peirone *et al.*, 1998a,b), nutrients, and metabolic waste can diffuse through the devices freely. Hence, a single, well-characterized "universal" cell line will serve the need for all patients requiring the same product replacement (Chang, 1995).

Previously, we have used alginate-poly-L-lysine-alginate (APA) microcapsules to provide immunoisolation for nonautologous cells and permit secretion of a recombinant human factor IX (Hortelano *et al.*, 1996) and human growth hormone (hGH) (Peirone *et al.*, 1998a,b; Potter *et al.*, 1998; Ross *et al.*, 1999). In this study, we report the efficacy of a therapeutic lysosomal enzyme delivery secreted from nonautologous, encapsulated recombinant cells implanted intraperitoneally into mutant MPS VII mice. The recombinant cells secrete high levels of murine β-glucuronidase for systemic distribution and mannose 6-phosphate receptor-mediated uptake into diseased tissues (Neufeld and Muenzer, 1989; Taylor and Wolfe, 1994). We also examine whether anti-CD4 immunosuppression could inhibit an anti-β-glucuronidase immune response.

MATERIALS AND METHODS

Mice

MPS VII mutants bred from heterozygous MPS VII mice were genotyped by polymerase chain reaction (PCR) and restriction digestion. PCR products encompassing the mutation site (Sands and Birkenmeier, 1993) were amplified with primers GUS-5′-1k (5′-CTA AAT TAA GGA CCA GGA GAT GTA-3′) and GUS-3′-1k (5′-CCA GAG GCT AAG GGA GAT TGT-3′). The resulting 977-bp product was digested with *Eco*0191I to generate 526-, 251-, and 200-bp fragments from the normal allele and 777- and 200-bp fragments from the mutant allele. Both mutant and normal alleles generate a 200-bp fragment for an internal restriction digest control.

Microcapsules

Mouse 2A-50 fibroblasts (gift from W.S. Sly, St. Louis, MO) expressed the amplicon including the mouse β-glucuronidase cDNA downstream from the metallothionein I promoter and upstream from the cDNA encoding dihydrofolate reductase and were selected with 3.2 μ*M* methotrexate (Grubb *et al.*, 1993). β-Glucuronidase expression from the 2A-50 cells was thus enhanced by amplification of the amplicon including the β-glucuronidase gene via methotrexate selection. Cells were encapsulated in alginate (Liu *et al.*, 1993), a polysaccharide polymer composed of alternating blocks of mannuronic and guluronic acid, by mixing them with the polymer, and then extruded through a syringe needle. The beads were sheared off by a concentric air flow as droplets, and ionically cross-linked with Ca^{2+} to form a hydrogel. The permeability of the gelled beads was controlled by laminating with poly-L-lysine (MW 22,000–35,000), coated with alginate to confer a biocompatible surface, and the core of the capsule was solubilized with a chelating agent citrate. Microcapsules of ~500 μm in diameter were used for intraperitoneal implantation. Encapsulation of cells was carried out at 4–10°C under sterile conditions.

Microcapsule implantation

Mice anesthetized with isofluorane were implanted intraperitoneally with at least 3 ml of microcapsules through a 16-gauge catheter. The mice were treated three times with anti-CD4 (PharMingen, San Diego, CA), at 250 μg every 3 days, as described by Potter *et al.* (1998).

Blood collection

Under anesthesia, 100–200 μl of blood was collected retroorbitally through heparinized capillary tubes (Chase Instruments, Rockwood, TN) and the plasma was collected after centrifugation.

Lysosomal enzyme assays

β-Glucuronidase, β-hexosaminidase, and α-galactosidase activities were assayed with the fluorogenic substrates 4-methy-

lumbelliferyl (4-MU)-β-glucuronide, 4-methylumbelliferyl-*N*-acetyl-β-D-glucosamide, and 4-methylumbelliferyl-α-D-galactoside (Sigma, St. Louis, MO), respectively (Glaser and Sly, 1973). One unit of enzyme activity corresponds to 1 nmol of 4-MU substrate hydrolyzed per hour. Proteins were determined by the method according to Lowry (Lowry *et al.*, 1951).

Glycosaminoglycan determination

Urine glycosaminoglycan (GAG)/creatine was determined by mixing 2 μl of urine with 600 μl of 20 μ*M* 1,9-dimethylene blue (Aldrich, Milwaukee, WI), and the absorbance was read at 540 nm after 20 min (Whitley *et al.*, 1989; Moullier *et al.*, 1993) and compared with a chondroitin sulfate C standard (Sigma). To determine creatine levels, 1 μl of urine in 9 μl of distilled H_2O was mixed with 50 μl of 0.2 *M* NaOH and 50 μl of saturated picric acid (Sigma) and absorbance was read at 490 nm after 20 min and compared with a creatine standard (Sigma).

Histology

Dissected tissues were fixed in glutaraldehyde, embedded in Spurr resin, cut into 0.5-μm sections, stained with toluidine blue, and examined with a Zeiss (Thornwood, NY) Axioskop. Frozen dissected tissues were cryosectioned and histochemically stained for β-glucuronidase activity with naphthol-AS-BI-β-D-glucuronide and *p*-rosaniline (Sands and Birkenmeier, 1993).

Assay for anti-β-glucuronidase antibodies

An immunoprecipitation assay was used to measure anti-β-glucuronidase antibodies. Mouse plasma (0.1–5 μl) was diluted in 0.04 *M* Tris-HCl, pH 8.0, to a final volume of 20 μl and mixed with 27.1 units of β-glucuronidase enzyme (30 μl) collected from 2A-50 cell tissue culture medium, and incubated at room temperature for 1 hr. This was followed by a 1-hr incubation at room temperature with 20 μl of Pansorbin protein A (200 mg/ml) (Pansorbin; Calbiochem) and centrifugation to pellet any β-glucuronidase bound to the Pansorbin in the presence of anti-β-glucuronidase antibodies. The supernatant, containing any unbound β-glucuronidase, was assayed for β-glucuronidase activity as a measure of anti-β-glucuronidase antibodies in the plasma sample and expressed as units of β-glucuronidase immunoprecipitated per milliliter of plasma.

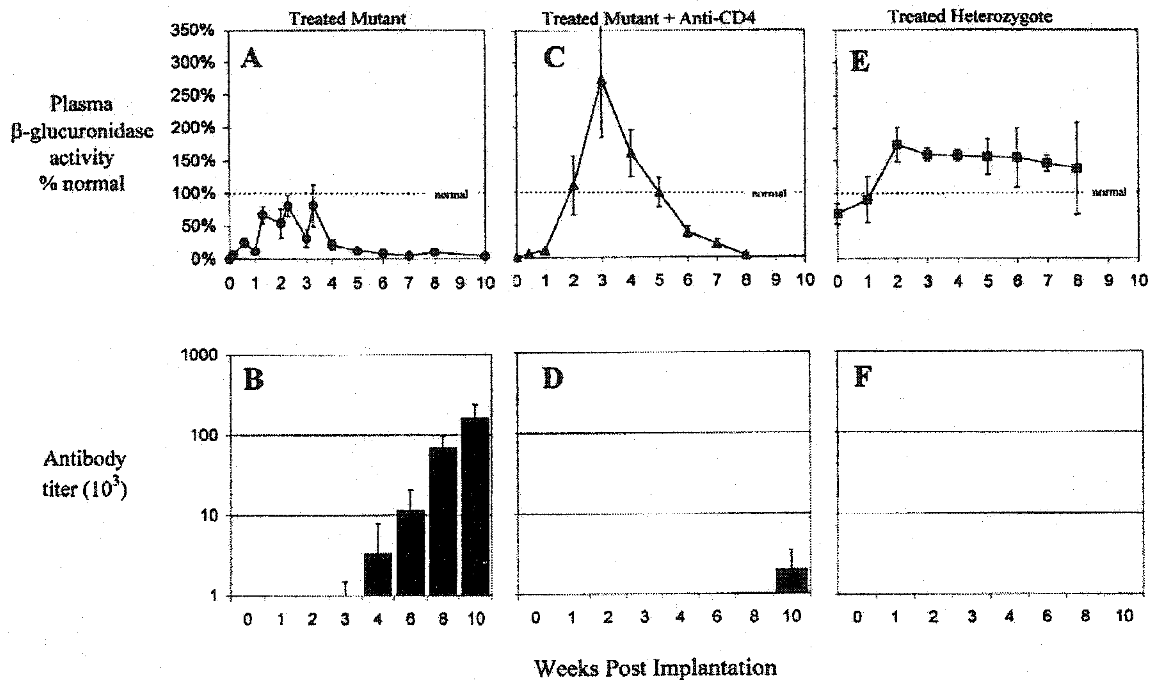

FIG. 1. Delivery of β-glucuronidase to the plasma of MPS VII mice and development of anti-β-glucuronidase antibodies after intraperitoneal implantation of microcapsules. (**A**) MPS VII mutant mice implanted with microencapsulated cells have β-glucuronidase levels reaching 66% of normal by day 9, followed by a decrease to background levels by week 4 when anti-β-glucuronidase (**B**) antibodies are detectable ($N = 13$, 8, and 2 on weeks 0–2, 3–8, and 9–10, respectively). Mutant mice implanted with microencapsulated cells and treated by anti-CD4 immunosuppression (**C**) have high levels of β-glucuronidase in the blood, up to 274% of normal by week 3, but decline to background levels by week 10 when anti-β-glucuronidase antibodies (**D**) become detectable ($N = 2$). Heterozygous mice implanted with microencapsulated cells maintain β-glucuronidase levels above normal wild-type levels for the duration of the experiment (**E**) and anti-β-glucuronidase antibodies are not generated (**F**). Day 0 represents initiation of treatment. Data represented ± SEM or range ($N < 3$) of percent normal enzyme activity per milliliter of plasma or antibody titers (units immunoprecipitated per milliliter of plasma).

RESULTS

Microcapsules of ~500 μm in diameter containing 2A-50 fibroblasts secreting 522 units of murine β-glucuronidase/10^6 cells per hour were implanted intraperitoneally in mutant and heterozygous mice, 6–8 weeks old. At 24 hr postimplantation, β-glucuronidase activity was detected in the plasma of treated animals, and gradually increased to greater than 50% of normal by week 2 (Fig. 1A). This peak level declined in the ensuing weeks, so that by weeks 6–10, when the experiment was terminated, plasma β-glucuronidase returned to background levels. However, concomitant with this decline of circulating β-glucuronidase, circulating anti-β-glucuronidase antibodies appeared. The titer of the antibodies rose exponentially after week 4 (Fig. 1B). We hypothesized that the apparent decline of β-glucuronidase in the plasma was related to clearance from the circulation by anti-β-glucuronidase antibodies. If this immune response could be suppressed, the level and duration of β-glucuronidase would be expected to improve. Hence, in a second experiment we administered anti-CD4 antibodies to the MPS VII mice at the time of implantation. This treatment indeed led to an increase in the peak circulating β-glucuronidase level by about 7-fold (Fig. 1C), as well as an inhibition of anti-β-glucuronidase antibodies (Fig. 1D). However, after a prolonged period in the absence of further anti-CD4 administration, an antigenic response to β-glucuronidase in mutant mice eventually developed by week 10. Two normal heterozygous littermates implanted with β-glucuronidase-producing microcapsules maintained elevated levels of β-glucuronidase in the plasma for up to 8 weeks postimplantation (Fig. 1E) without the development of anti-β-glucuronidase antibodies (Fig. 1F). Hence, these data confirmed that the apparent decline in β-glucuronidase delivery was associated with the host's antibody response to the delivered gene product, rather than a loss of efficacy of the microencapsulated cells, which continued to express β-glucuronidase activity after retrieval from the implantation (Table 1).

Within 2 weeks of implantation enzyme delivery reached 12, 19, and 54% of normal in the liver, spleen, and kidney of treated mutants and the secondary elevations of β-hexosaminidase and galactosidase were all significantly reduced (Fig. 2). The secondary elevation of these hydrolases was not reduced in the brain of treated mice, consistent with no significant increase of β-glucuronidase in the brain.

In the treated mutants, a loss of the lysosomal inclusions characteristic of the disease phenotype was observed in liver, spleen, and kidney by 2 weeks postimplantation (Fig. 3). By 4 weeks postimplantation, the histology of all the above-described organs was almost indistinguishable from that of the normal organs, with disappearance of the lysosomal vacuoles from the Kupffer cells and hepatocytes in the liver, the parenchymal and sinusoidal cells in the spleen, and the parenchymal and glomerular cells of the kidney. Although the level of enzyme delivery decreased to 0.1–0.7% of normal in the organs of mutants after 8 weeks of treatment, the histology of the liver and spleen remained normal. The kidney, in contrast, never acquired a completely normal phenotype even though the extent of lysosomal vacuolation was much reduced compared with that of the untreated mutants.

Histochemical staining of a treated mutant at 4 weeks revealed β-glucuronidase activity throughout the liver localized to the Kupffer cells and not the hepatocytes. β-Glucuronidase-positive cells in the spleen were found localized to the periphery of the white pulp. In the kidney, β-glucuronidase activity was localized to the kidney capsule (Fig. 4a–c). In the mutant controls, no β-glucuronidase activity was detected in any of these organs (Fig 4d–f). In the normal controls, a high level of enzyme activity was detected throughout the liver (Fig. 4g), the red pulp of the spleen (Fig. 4h), and the renal tubule cells of the kidney (Fig. 4i).

Prior to implantation, urinary GAG levels in the mutants were more than 2-fold greater than normal levels. By day 5 postimplantation, GAG levels had significantly decreased in the treated mice ($p < 0.05$) (Fig. 5). By day 18, GAG levels were not significantly different between normal and treated mutants. When compared with untreated mutants, urinary GAG levels had decreased by 41% after 25 days of therapy.

Since the β-glucuronidase transgene expression in the 2A-50 fibroblasts was maintained under methotrexate selection in the *in vitro* perpetuation of this cell line, it was important to verify whether the implanted cells could maintain β-glucuronidase expression in the absence of selection *in vivo* (Table 1). Within 15 days of selection removal, β-glucuronidase secretion decreased to 49% of its initial level. However, its expression appeared to reach a plateau, with no further decline after 1 month until at least day 56 postimplantation, at 31% of original expression levels. Similar results were observed *in vitro* from microcapsules maintained in tissue culture. The decline in β-glucuronidase expression over time was likely due to reduction of the amplicon copy number in the absence of the methotrexate selection.

TABLE 1. SECRETION OF β-GLUCURONIDASE FROM MICROCAPSULES[a]

	Percentage β-glucuronidase secretion		
	In vitro		
Day	*+MTX*	*−MTX*	In vivo
0	100 ± 8	100 ± 8	100 ± 8
15	116 ± 8	64 ± 4	49 ± 11
29	154 ± 17	49 ± 4	32 ± 8
56	114 ± 21	29 ± 1	31 ± 14

[a]Effect of removal of selection pressure on gene expression *in vitro* and *in vivo* is demonstrated. The 2A50 fibroblasts were transfected to express β-glucuronidase in an amplicon whose expression was maintained with methotrexate selection. Microcapsules containing 2A50 cells were implanted into mice while *in vitro* microcapsules were cultured in either the presence (+MTX) or the absence (−MTX) of 3.2 μM methotrexate. Secretion rates of β-glucuronidase were determined by time course assays of media collected from capsules maintained *in vitro* at 0, 15, 29, and 56 days and expressed as a percentage of the day 0 secretion rate (522 units/hr/10^6 cells). At 15, 29, and 56 days postimplantation, the microcapsules were retrieved from mice, washed in PBS, and analyzed for β-glucuronidase activity. Data represent means ± SD.

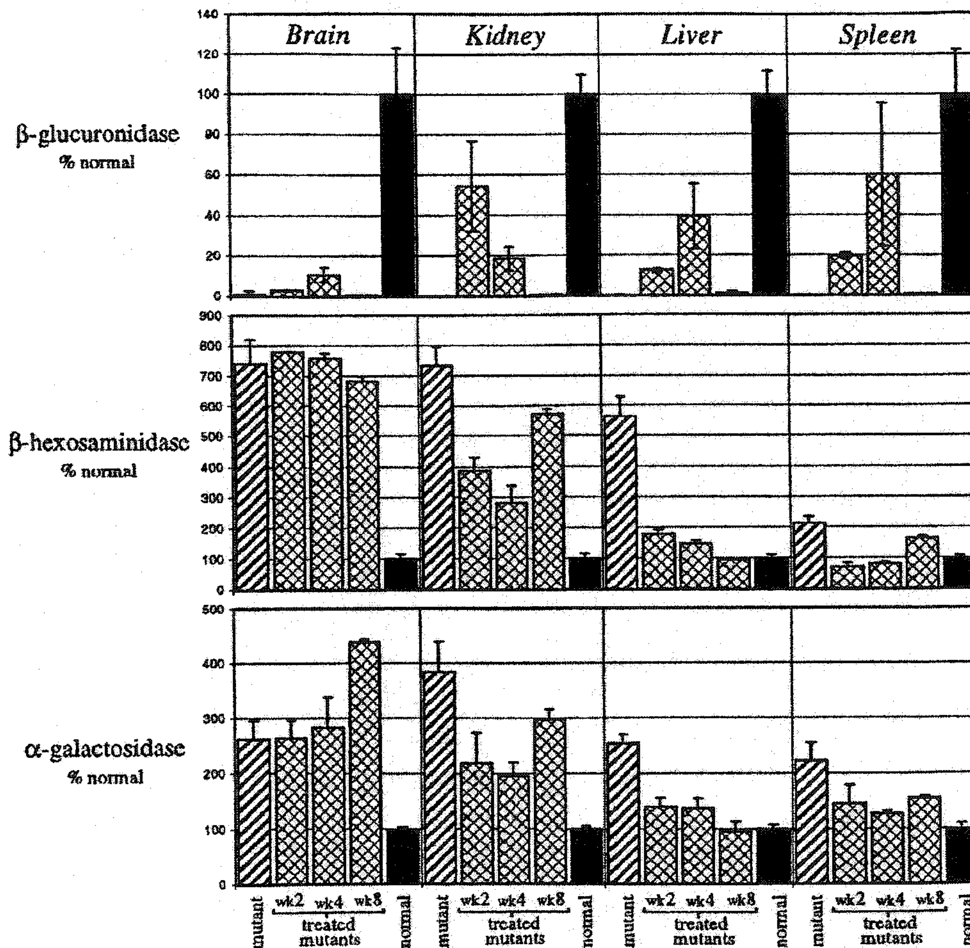

FIG. 2. Activity of lysosomal enzymes in the organs of mice implanted with microencapsulated cells. At various times postimplantation, the organs were removed from the treated mutants (N = 4, 4, and 2 at weeks 2, 4, and 8, respectively), and their lysosomal enzyme activities were compared with those of normal littermates (N = 3) and untreated mutants (N = 3). The enzyme activities of secondarily elevated lysosomal enzymes α-galactosidase and β-hexosaminidase were also determined. The α-galactosidase and β-hexosaminidase levels in the kidney, liver, and spleen were reduced in all treated organs except the brain. Each sample was assayed for enzyme activity and protein (units per milligram) in triplicate and averaged from the various animals. Error bars represent the SEM.

DISCUSSION

We report here the efficacy of a technology for the treatment of somatic deficits caused by a lysosomal storage disease, in which no genetic modification of the recipient is required. A nonautologous cell line genetically modified to secrete a therapeutic product was used to deliver β-glucuronidase in mutant MPS VII mice for at least 8 weeks. Cell microencapsulation was used to render the implanted cells immunologically protected from the host immune system mediators.

After microcapsule implantation into the peritoneal cavity, the early presence of β-glucuronidase in the plasma (24 hr) may be achieved via lymphatic drainage of the peritoneal fluid through the portal circulation and into the systemic circulation (Fig. 1). The β-glucuronidase secreted from the encapsulated cells was taken up by cells at distant sites in the kidney, liver, and spleen (Fig. 2), likely mediated by mannose 6-phosphate receptor endocytosis resulting in the breakdown of excess glycosaminoglycan storage, as indicated by the reduction in histological storage lesions (Fig. 3). In addition, this treatment reduced the elevated secondary lysosomal enzymes β-hexosaminidase and α-galactosidase (Fig. 2) and, as few as 5 days after implantation, urinary GAG levels had significantly declined in the treated mutants (Fig. 5). These surrogate measures of disease correction indicated that the β-glucuronidase secreted from the microcapsules was rapidly taken up by tissues, where it was functionally active. Microcapsules retrieved from mice 8 weeks postimplantation continued to secrete β-

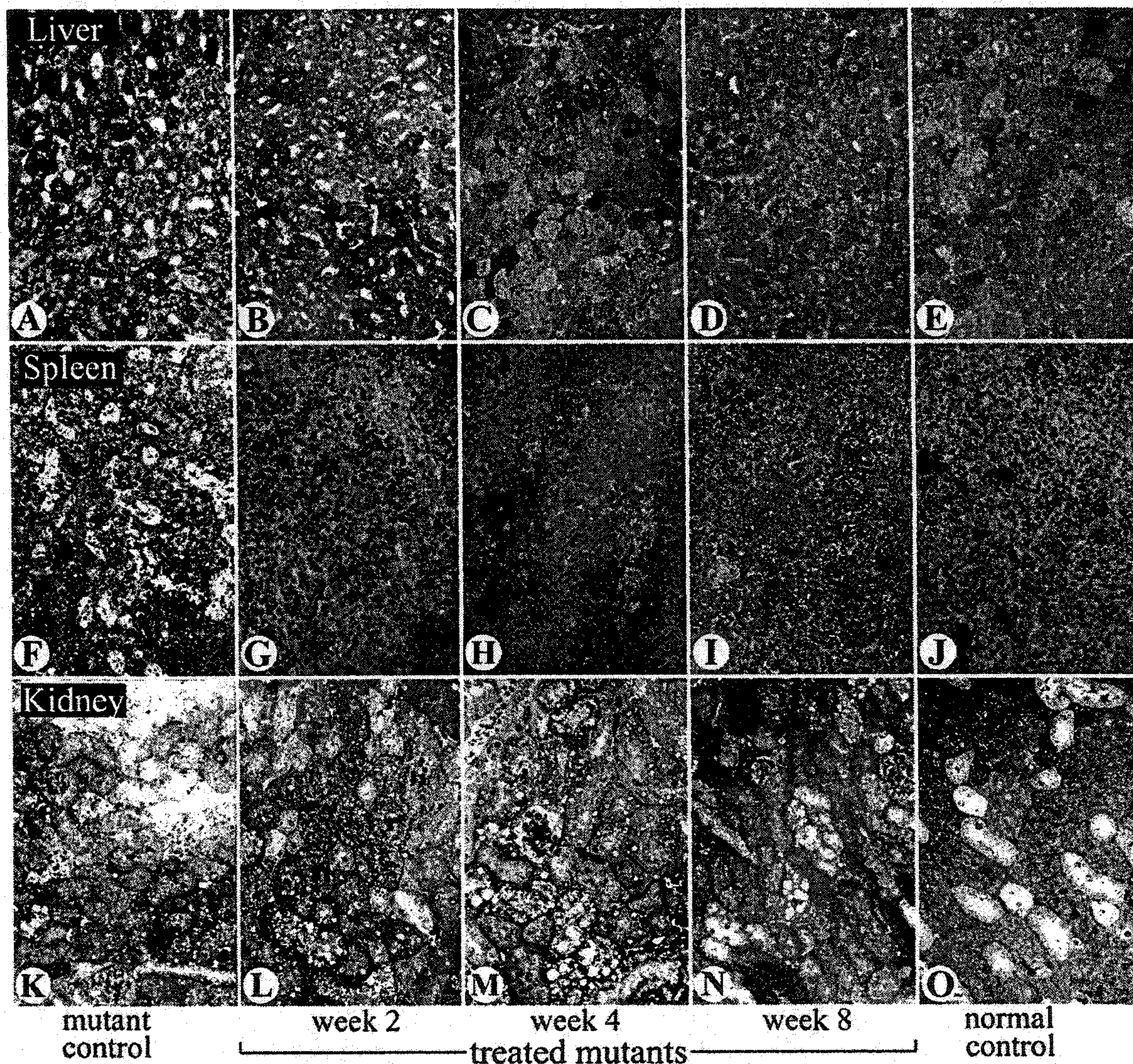

FIG. 3. Resolution of lysosomal storage granules from various organs after microcapsule implantation. Histological sections of liver, spleen, and kidney showed decreased lysosomal storage in treated mutants for up to 8 weeks postimplantation. Toluidine blue-stained thin sections were prepared from tissue samples from mutant controls. (**A, F,** and **K**), treated mutants at 2, 4, and 8 weeks postimplantation, and normal controls (**E, J,** and **O**). In the liver, the intralysosomal accumulation of GAGs apparent in untreated control mutant liver (**A**) was reduced at week 2 (**B**) and almost completely disappeared by weeks 4 (**C**) and 8 (**D**), appearing similar to that of the normal control (**E**). In the spleen, marked lysosomal distention in the splenic sinusoidal lining cells of the untreated mutants (**F**) was dramatically reduced in treated mutants from 2 weeks up to 8 weeks postimplantation (**G–I**) to resemble that of the normal spleen (**J**). In the kidney, untreated mutants (**K**) showed numerous distended lysosomes in renal tubular cells that were substantially reduced in treated mutants from weeks 2 to 8 postimplantation (**L–N**) but never disappeared completely to resemble the normal control (**O**). Original magnification: ×200.

glucuronidase, albeit at reduced levels. A similar reduction in expression was found with cells cultured *in vitro* in the absence of methotrexate, indicating that this reduction could be explained by the lack of methotrexate selection *in vivo* (Table 1).

After the development of antibodies, the decrease in β-glucuronidase levels and localization of the β-glucuronidase in the liver to the Kupffer cells and not the hepatocytes (Fig. 4) suggest a decrease in receptor-mediated endocytosis, replaced with an immune-mediated clearance of the enzyme from circulation.

In spite of the development of antibodies after 3 weeks, a dramatic reduction of storage lesions was observed until the end of the experiment at 8 weeks postimplantation. This may be due to small amounts of undetected β-glucuronidase that remain in

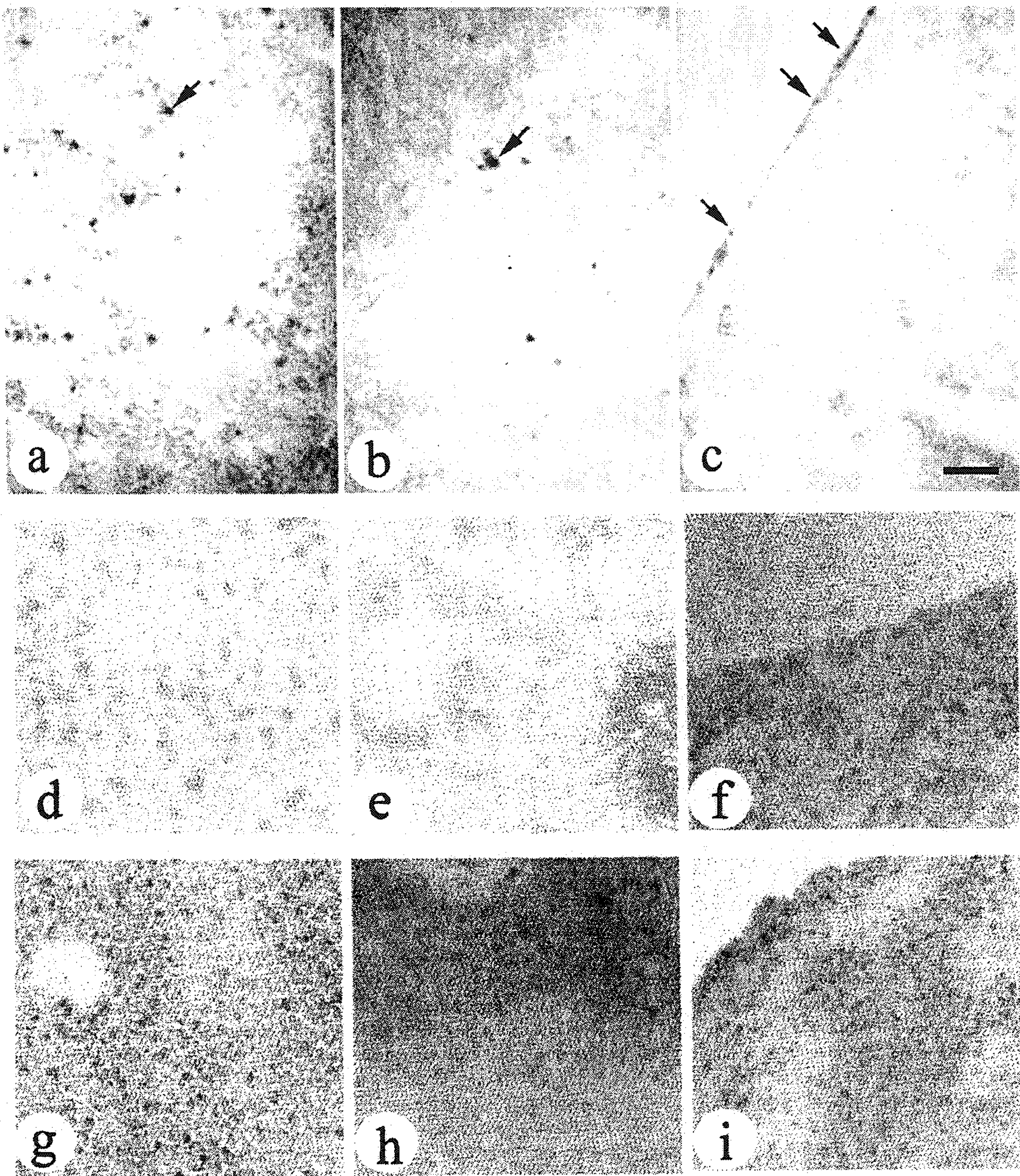

FIG. 4. Histochemical detection of the product of the introduced transgene, β-glucuronidase, in the liver, spleen, and kidney of treated mutants. β-Glucuronidase activity (arrow, red deposits) was detected in cryostat-sectioned organs of treated mice killed 4 weeks postimplantation. β-Glucuronidase activity was localized primarily to the Kupffer cells of the liver (**a**). β-Glucuronidase-positive cells in the spleen were found localized to the periphery of the white pulp (**b**). In the kidney, β-glucuronidase activity was localized to the kidney capsule (**c**). There was no β-glucuronidase activity detected in the liver (**d**), spleen (**e**), or kidney (**f**) of an untreated animal. However, β-glucuronidase activity was observed throughout the liver (**g**), and was concentrated in the red pulp of the spleen (**h**) and in the renal tubule cells of the kidney (**i**) in a normal mouse. Scale: 100 μm.

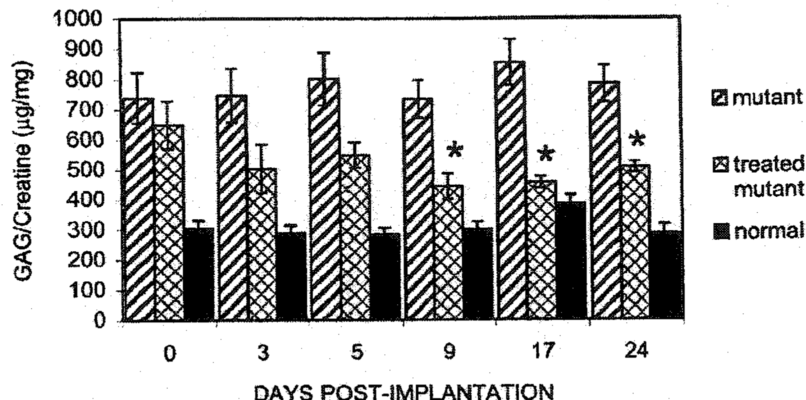

FIG. 5. Urinary glycosaminoglycans (GAGs) were significantly decreased in treated MPS VII mutant mice after implantation of microcapsules producing β-glucuronidase. Urinary GAG/creatine content in mutants treated with microcapsules was measured on different days for up to 24 days postimplantation ($N = 9$, 7, and 3 for days 9, 17, and 24, respectively), and in normal controls ($N = 13$) and mutant controls ($N = 9$). Glycosaminoglycan levels were significantly decreased in treated mutants at all time points examined after 5 days postimplantation ($*p < 0.05$). Day 0 represents the day of implantation. Data represent means ± SEM.

the hepatocytes. With the long half-life of β-glucuronidase in the liver (2–3 days) (Smith and Ganschow, 1978; Vogler *et al.*, 1993) the expected level of enzyme would decrease from 40%, as observed at week 4, to less than 0.1% by week 8 without any additional supply of enzyme. This expected level is in range with the actual level of 0.7% at week 8. It is also possible that intercellular GAG exchange (Yanagishita, 1992) between the hepatocytes and the corrected Kupffer cells resulted in the clearance of storage material in the hepatocytes. Finally, the reaccumulation of lysosomal storage is a gradual process that would not begin until after the supply of β-glucuronidase was fully exhausted. It was previously demonstrated that lysosomal storage did not reaccumulate in the liver and spleen until some time between 29 and 85 days after the last intravenous injection of recombinant enzyme (Vogler *et al.*, 1996).

The MPS VII mice have been used as a model to test many forms of therapy, including bone marrow transplantation with syngeneic marrow (Birkenmeier *et al.*, 1991; Wolfe *et al.*, 1992b), retrovirally transduced bone marrow, enzyme replacement therapy (Vogler *et al.*, 1993; Sands *et al.*, 1994), organoid implant containing genetically modified syngeneic fibroblasts (Moullier *et al.*, 1993), and direct injection of adeno-associated virus (AAV) encoding human β-glucuronidase (Daly *et al.*, 1999a). These studies provided much insight into the level of enzyme replacement needed to elicit therapeutic responses, the duration of responses, and the degree to which pathological corrections can be achieved. In general, less than 10% of normal activity appeared adequate to correct the histological pathology of the disease. A similar level of efficacy in visceral organs has thus been achieved with the microcapsule implantation (Figs. 1 and 2).

Although significant levels of β-glucuronidase activity in the brain, as well as reductions of lysosomal storage and associated improvements in cognitive functions, were observed in the MPS VII mouse after enzyme replacement therapy (Sands *et al.*, 1994; O'Connor *et al.*, 1998), no activity was detected in the brains after microcapsule implantation. This is not entirely unexpected since the enzyme replacement studies were performed in neonates at a time when the blood–brain barrier may not be completely formed. The studies described here were performed in young adult mice with an intact blood–brain barrier. It has been demonstrated previously that soluble β-glucuronidase cannot gain access to the brain, and there is no reduction in lysosomal stage if the therapy is initiated at 14 days of age or beyond (Vogler *et al.*, 1999).

In spite of the demonstrated clinical efficacy, several potential problems with this technology require resolution before human application. These include possible failure of the microcapsules due to breakdown of the membrane biomaterial, loss of cell viability, and loss of gene expression and escape of recombinant cells with tumorigenic potential. Conceptually, these problems can be addressed by repeat implantations of fresh microencapsulated cells, improved biomaterial chemistry, appropriate expression vector construction, and a judicious choice of nontransformed cell lines.

In our experience with treating animal models, an important concern is the development of antibodies against the therapeutic protein in recipients who are crossreacting material (CRM) negative. It appears that development of antibodies against the enzyme used for treatment can be a significant complication, as seen in the MPS I dog (Shull *et al.*, 1996) and now in the MPS VII mouse (Fig. 4), thus abrogating the clinical efficacy of any replacement therapy. However, by providing an immunosuppressive antibody such as anti-CD40 (Stein *et al.*, 1999), or as in this study with anti-CD4 (Potter *et al.*, 1998), the early development of antibodies could be prevented, and the level of β-glucuronidase was increased severalfold. Hence, the use of anti-CD4 antibody or similar strategies to prevent early development of the antibodies should be an effective adjunct strategy to resolve this immunological issue.

The advantages of using nonautologous cells for the implantation are the simplicity of the procedure and the potential cost-effectiveness. Currently, only bone marrow transplantation (Yamada *et al.*, 1998) and enzyme replacement (Beutler, 1993) have been shown to be clinically useful for treatment of somatic pathology in patients with lysosomal storage diseases. However, the serious side effects of bone marrow transplantation such as cognitive impairment and life span reduction (Birkenmeier *et al.*, 1991; Bastedo *et al.*, 1994), and high morbidity/mortality rates, and the high costs associated with both procedures (U.S. $200,000–1,000,000 for bone marrow transplantation; ~ U.S. $250,000/70 kg per year for enzyme replacement) clearly warrant a search for alternative forms of treatment. The success of *in vivo* gene therapy with AAV vectors both for hemophiliac dogs (Snyder *et al.*, 1999; Herzog *et al.*, 1999) and MPS VII mice (Daly *et al.*, 1999b) indicates the possibility of long-term delivery of replacement gene product via *in vivo* gene therapy. However, since scaling up this form of therapy for human application can be technically challenging, it is a potential obstacle to clinical application (Linden and Woo, 1999). The currently proposed approach of nonautologous cell implantation with immunoisolation devices offers many potential advantages that should be explored. First, it carries none of the morbidity/mortality risks of bone marrow transplantation. Given the similar level of efficacy achieved, the potential saving in cost and increase in safety are important assets. Second, compared with the clinically proven treatment by enzyme replacement therapy, such as regular infusion of ceredase to treat Gaucher disease, microencapsulated cells ideally need be administered only once in a lifetime, but realistically at infrequent intervals of months or years. Furthermore, the recombinant product is delivered *in vivo* and *in situ*, circumventing the costly industrial-scale protein/viral purification process. These production/delivery issues are serious deterrents for commercial development to treat the relatively rare single-gene disorders, no matter how effective the therapy may be. Finally, in recent studies we have achieved potentially excessive levels of enzyme delivery (Ross *et al.*, 2000), successfully demonstrating the capability of this approach, but also highlighting the need for reversible or regulated gene product delivery. The almost unlimited cloning capacity of recombinant encapsulated cells may be advantageous for the incorporation of large regulatory elements necessary for precise regulation (Hagihara *et al.*, 1999; Rivera *et al.*, 2000). Moreover, the cell encapsulation approach is reversible, with removal of the encapsulated cells from the host, compared with the uncontrolled dissemination and irreversible infection from virally mediated *in vivo* gene therapy. Hence, this form of cell-based gene therapeutics (Chang, 1995) provides an alternative platform that is technically feasible to scale up for different human disease applications, and permits an increased level of safety and quality assurance.

In conclusion, using a novel and potentially cost-effective approach of nonautologous somatic gene therapy, we have shown its efficacy in treating a lysosomal storage disease in a mouse model. The concomitant improvements in biochemical, histological, and histochemical outcomes in peripheral organs are supportive of further development of this technology towards human clinical application.

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3.3 Delivery of β-glucuronidase from microencapsulated cells to neonatal MPS VII mice.

3.3.1 Rationale

Immuno-isolation gene therapy successfully delivered β-glucuronidase to adult β-glucuronidase deficient MPS VII mice (Chapter 3.2). However, at the time of administration, the 6-8 week old MPS VII mice were already severely affected by the disease. Moreover, within weeks of treatment, the adult MPS VII mice developed a strong humoral immune response directed against β-glucuronidase, which abrogated the therapeutic efficacy of the treatment. Since the β-glucuronidase missense mutation in MPS VII mice does not produce normal β-glucuronidase, the MPS VII mice responded to mouse β-glucuronidase as a foreign antigen. Although immunosuppression was capable of inhibiting this immune response, the rationale for this experiment was to examine neonatal administration of β-glucuronidase secreting microcapsules in order to supply β-glucuronidase to newborn mice at a critical time in development, thereby reducing the early-onset effects of the disease. A second objective was to use neonatal administration to allow the newborn MPS VII mice to develop an immune tolerance to β-glucuronidase.

3.3.2 Contribution

This research was accomplished by Colin Ross with the support of Dr. Patricia L. Chang.

3.3.3 Abstract

Immuno-isolation gene therapy has been used to deliver mouse β-glucuronidase to adult mucopolysaccharidosis type VII (MPS VII) mice. However, at the time of administration, the 6-8 week old MPS VII mice are already severely affected by β-glucuronidase deficiency. Moreover, within weeks of treatment, the adult MPS VII mice develop a strong humoral immune response directed against β-glucuronidase, thereby abrogating the therapeutic effects of the treatment. MPS VII mice do not produce normal β-glucuronidase due to a missense mutation. Consequently, β-glucuronidase presents as a foreign antigen in these mice. In this study, we investigated neonatal administration of β-glucuronidase secreting microcapsules to supply β-glucuronidase to newborn mice at a critical time in development and reduce the early-onset effects of the disease. Neonatal administration was also investigated as an alternative to immunosuppression to allow the newborn MPS VII mice to develop an immune tolerance to β-glucuronidase. MPS VII mice were subcutaneously injected at 0 to 5 days after birth with 25 μl of β-glucuronidase secreting microencapsulated cells. From 1 to 28 days post-implantation, significant plasma β-glucuronidase activity was detected at 3.6 ± 0.3% of normal levels. At week 4, mice were re-treated with a second 1 ml dose. Low levels of β-glucuronidase in the peripheral organs transiently improved histological and biochemical disease parameters for 12 weeks. However, at week 4, anti-β-glucuronidase antibodies were detected in some animals. Within the next two weeks, anti-β-glucuronidase antibodies developed in all of the treated animals, reducing plasma β-glucuronidase to background levels. Even though microencapsulated cells retrieved at 6 months post-implantation continued to secrete β-glucuronidase at 15% of pre-implantation levels, lysosomal storage pathology became evident after 12 weeks as a result of the anti-β-glucuronidase antibodies. In summary, β-glucuronidase delivered to neonatal mice delayed the onset of disease, but a permanent immune tolerance was not achieved. This suggests that the delivery of β-glucuronidase to patients in early life may slow the progression of the disease; however, the immune system could impede therapy in null allele patients that react against the therapeutic gene product.

3.3.4 Introduction

Mucopolysaccharidosis type VII (MPS VII; Sly syndrome) is an autosomal recessive lysosomal storage disorder due to an inherited deficiency of β-glucuronidase (Sly *et al.* 1973). A naturally occurring mouse model for this disease was discovered at the Jackson Laboratory and shown to be due to a homozygous 1-bp deletion in exon 10 of the β-glucuronidase gene (Birkenmeier *et al.* 1989; Sands and Birkenmeier 1993). The incomplete break-down and progressive accumulation of glycosaminoglycans in MPS VII patients leads to eventual cell and organ dysfunction including hepato- and splenomegaly, skeletal deformities, cataracts, as well as auditory, cognitive, and neurological deficits in the central nervous system (Birkenmeier *et al.* 1989; Vogler *et al.* 1990a; Chang *et al.* 1993a; Sands *et al.* 1995; Levy *et al.* 1996). The murine model of MPS VII has been well characterized and used extensively to evaluate experimental therapies for lysosomal storage diseases, including bone marrow transplantation (Bastedo *et al.* 1994; Sands *et al.* 1995), enzyme replacement therapy (O'Connor *et al.* 1998; Vogler *et al.* 1999), and gene therapy (Wolfe *et al.* 1992a; Wolfe *et al.* 1992b; Marechal *et al.* 1993; Moullier *et al.* 1993a; Li and Davidson 1995; Lau *et al.* 1995; Wolfe *et al.* 1996; Naffakh *et al.* 1996; Snyder and Wolfe 1996; Ohashi *et al.* 1997; Taylor and Wolfe 1997a; Ghodsi *et al.* 1999; Stein *et al.* 1999; Skorupa *et al.* 1999; Daly *et al.* 1999a; Daly *et al.* 1999b).

We have examined the delivery of a therapeutic recombinant gene product from an encapsulated universal cell line for the treatment of MPS VII. To prevent the eventual graft rejection these recombinant cells are immunologically protected with alginate microcapsules. The recombinant cells secrete high levels of murine β-glucuronidase for systemic distribution and mannose-6-phosphate receptor-mediated uptake into diseased tissues (Taylor and Wolfe 1994). MPS VII mice receiving injections of non-autologous cells expressing murine β-glucuronidase initially attained high levels of β-glucuronidase with concomitant reductions in disease pathology (Ross *et al.* 2000a). However, at the time of administration, the adult MPS VII mice were already severely affected by the disease. Moreover, within weeks of treatment, the adult MPS VII mice develop a strong humoral immune response directed against β-glucuronidase, thereby abrogating the therapeutic effects of the treatment. This antibody response could be inhibited with immunosuppression to prolong the effective delivery of the replacement enzyme.

In this study, neonatal administration of β-glucuronidase secreting microcapsules was examined in order to supply the enzyme to newborn mice at a critical time in development to reduce the early-onset effects of the disease. Furthermore, neonatal administration was investigated as an alternative to immunosuppression as a means for the newborn MPS VII mice to develop an immune tolerance to β-glucuronidase.

3.3.5 Methods

3.3.5.1 Mice

MPS VII mice were used as described in Chapter 2, Methods.

3.3.5.2 Microcapsules

Mouse 2A-50 fibroblasts expressing high levels of mouse β-glucuronidase were encapsulated in small (100-300 μm diameter) solid alginate-poly-L-lysine-alginate (APA) microcapsules as described in Chapter 2, Methods.

3.3.5.3 Microcapsule Implantation

The entire newborn litters from heterozygous MPS VII breedings were subcutaneously injected with 25 μl of β-glucuronidase secreting microencapsulated cells through a 23-gauge needle into the scruff of the neck. Microcapsules were delivered subcutaneously because in large animal models this route of administration is more effective than intraperitoneal delivery (Stockley *et al.* 2000) During injections, newborn pups were not removed from their cage and were handled with cotton-covered sterile forceps. The treated pups were subsequently genotyped to identify MPS VII mice as described in Chapter 2, Methods. MPS VII pups were treated on day 0 (n = 6), day 1 (n = 3), day 3 (n = 3), and day 5 (n = 3) post-birth. The neonatal treated MPS VII mice were subsequently re-implanted with 1 ml of microcapsules after one month.

3.3.5.4 Weekly Monitoring

Treated mice were monitored with weekly 50-100 μl blood samples collected under anesthesia, beginning at week 4. Plasma samples were assayed for β-glucuronidase activity and anti-β-glucuronidase antibodies. Mice were sacrificed at days 1 and 4, and weeks 4, 8, 12, 16, 24, and 40 post-birth to obtain blood and organ samples for enzyme assay and histological analyses.

3.3.5.5 Lysosomal Enzyme Assays

β-Glucuronidase, β-hexosaminidase, and α-galactosidase activities and protein levels were assayed as described in Chapter 2, Methods. Biological significance was established at the 95% confidence level. Data are presented as mean ± SEM.

3.3.5.6 Assay for Anti-β-glucuronidase Antibodies

An immunoprecipitation assay was used to measure anti-β-glucuronidase antibodies as described in Chapter 2, Methods.

3.3.5.7 Antibody Neutralization Assay

Antibody neutralization was measured as described in Chapter 2, Methods.

3.3.5.8 Histology

Dissected tissues prepared for thin sections and stained with toluidine blue as described in Chapter 2, Methods.

3.3.6 Results

3.3.6.1 Neonatal Implantation

A 25 μl volume of 100-300 μm diameter APA microcapsules containing 4.1 x 10^5 2A-50 fibroblasts with 86.3 ± 1.8% viability secreting 41 ± 3 units of β-glucuronidase per hour were implanted subcutaneously into MPS VII mice between 0 and 5 days post-birth.

3.3.6.2 Initial delivery of β-glucuronidase

One day after microcapsule implantation, β-glucuronidase activity was detected in the plasma of treated animals (3.4 ± 0.5% of normal ± SD). β-glucuronidase activity was maintained at an average 3.6 ± 0.3 % of normal during the first four weeks (Fig. 1). In the peripheral organs, β-glucuronidase reached significant levels in the kidney, liver, and spleen within a week of treatment (0.2%, 0.2%, 0.1% of normal; $P < 0.01$) (Fig. 3). Furthermore, in muscle near the implantation site, β-glucuronidase reached high levels (33% of normal) ($P < 0.001$). After 4 weeks, β-glucuronidase in the peripheral organs declined, but remained significantly above background levels in the kidney, liver, spleen, and muscle (0.02, 0.01, 0.01, 0.4% of normal; $P < 0.01$). β-hexosaminidase is abnormally

elevated in the absence of β-glucuronidase in untreated MPS VII mice, and this secondary elevation of β-hexosaminidase was significantly reduced in the peripheral organs of treated mice at 4 weeks (P < 0.03), consistent with the presence of β-glucuronidase in the peripheral organs.

3.3.6.3 Anti-β-glucuronidase Antibodies

However, significant levels of circulating anti-β-glucuronidase antibodies were detected at week 4 in some mice (Fig. 2). There was no difference in antibody onset in mice treated on different days after birth. Mice treated on day 0, 1, 3, and 5 developed antibodies at 4-6, 4-7, 5-7, and 5 weeks of age, respectively. Antibody titres increased exponentially for at least 8 weeks after the initial onset. Consequently, plasma β-glucuronidase levels returned to background levels after week 6. The anti-β-glucuronidase antibodies appeared to be non-neutralizing because the sera from 4 treated MPS VII mice with high antibody titres did not inhibit the *in vitro* activity of β-glucuronidase.

3.3.6.4 Re-treatment at 4-Weeks

At 4 weeks of age, the mice were re-treated with a second subcutaneous 1 ml dose of microencapsulated cells. Plasma β-glucuronidase activity initially rose from 3.5% to 6% of normal (Fig. 1). However, as mentioned, anti-β-glucuronidase antibodies had begun to develop and plasma β-glucuronidase levels dropped to background levels within 3 weeks in all of the animals. In the muscle near the implantation site, β-glucuronidase levels remained significantly higher than background levels (3.3 - 13.7% of normal, P < 0.01) for the duration of the 40-week experiment, indicating the continued production and secretion of β-glucuronidase from the microencapsulated cells. β-glucuronidase levels in the spleen (0.2 - 1.0% of normal), liver (0.03 - 0.7%), and kidney (0.1 - 2.4%) remained low, but above background levels (P < 0.01) for the duration of the experiment.

In the muscle and spleen, the secondary elevation of β-hexosaminidase remained significantly reduced for up to 8 and 12 weeks respectively (P < 0.02); however, β-hexosaminidase levels were eventually elevated. After the peak β-hexosaminidase elevation at week 24, severe lysosomal pathology became histologically apparent in the muscle and spleen (Fig. 4). In the liver and kidney, the secondary elevation of β-hexosaminidase remained significantly reduced for up to 16 weeks (P < 0.02), after which β-hexosaminidase was elevated to untreated levels,

at which time severe distended lysosomal pathology became evident. By 40 weeks of age, significant disease pathology was apparent in all organs and β-hexosaminidase levels were highly elevated, even though β-glucuronidase was detected at low levels in the muscle (3.3%), kidney (2.4%) spleen (1.0%), and liver (0.7%).

At all time points from 4 days to 40 weeks post-implantation β-glucuronidase was not detected at significant levels in the CNS of neonatal treated MPS VII mice. β-hexosaminidase levels were not significantly reduced in the CNS; further indicating that β-glucuronidase did not cross the blood brain barrier (Fig. 3).

3.3.6.5 Microcapsules Retrieval

At six months post-implantation, microcapsules were removed from three animals for in vitro analyses. The retrieved microcapsules contained 1.1×10^5 viable cells (26% of pre-implantation levels), continued to secrete 6.2 ± 0.5 units of β-glucuronidase (15% of pre-implantation levels), and showed a 38 ± 19% cell viability (44% of pre-implantation levels).

3.3.7 Discussion

This study demonstrates the first administration of APA encapsulated cells to neonatal mice. Neonatal MPS VII mice were subcutaneously implanted with a small volume of microencapsulated cells secreting high levels of β-glucuronidase in order to overcome β-glucuronidase deficiency. In the plasma, significant therapeutic levels of β-glucuronidase were initially detected; however, after 4-7 weeks, anti-β-glucuronidase antibodies developed in all of the animals, indicating that an immune tolerance to β-glucuronidase was not achieved.

While untreated MPS VII mice normally exhibit histologic pathology within 2 weeks of birth (Daly *et al.* 1999a), histologic analyses indicated that the treated MPS VII mice were free of lysosomal storage disease pathology for up to 16 weeks. Thus, the treatment effectively delayed the onset of disease. However, elevated β-hexosaminidase and lysosomal disease pathology became apparent within 8 and 16 weeks respectively. Therefore, after β-glucuronidase release from the microcapsules, the presence of antibodies inhibited the ability of β-glucuronidase to reduce the lysosomal storage. Nonetheless, even in the presence of high anti-β-glucuronidase

antibodies, the survival was increased over 2-fold compared to the average survival of untreated MPS VII mice (5 months) for the last animal in the experiment to be sacrificed at 10 months of age.

The anti-β-glucuronidase antibodies appeared to be non-neutralizing because the sera from 4 treated MPS VII mice with high antibody titres did not inhibit the *in vitro* activity of β-glucuronidase. The reduced levels of β-glucuronidase in tissues and plasma could have resulted from increased β-glucuronidase turnover or degradation in the presence of anti-β-glucuronidase antibodies. Antibodies can lead to enzyme inactivation and increased enzyme degradation (Grabowski *et al.* 1995; Whittington and Goa 1995; Rosenberg *et al.* 1999; Lusher 2000).

The anti-β-glucuronidase antibodies did not hinder the long-term production of β-glucuronidase from the encapsulated cells. Microcapsules retrieved six months after implantation continued to produce β-glucuronidase and carry viable 2A-50 cells at 15% and 26% of pre-implantation levels, respectively. In addition, β-glucuronidase levels in the muscle near the implantation site rose to an average 9 ± 4% of normal (± SEM) for up to six months. These results demonstrate long-term continuous production and secretion of β-glucuronidase following a single administration of microencapsulated cells expressing β-glucuronidase. Lower levels of β-glucuronidase were also detected in the liver, spleen, and kidney for the duration of the 40-week experiment. These results support similar findings that have shown continued production of a recombinant transgene (human factor IX) from APA encapsulated cells for up to 213 days post-implantation (Hortelano *et al.* 1996; Gomex-Vargas *et al.* 2001).

Since neonatal tolerance was not attained in this animal model, alternative methods to control the immune response without systemic immunosuppression should be explored. For example, low level immunosuppression from transgenic grafts that secrete anti-CD4 antibody has been used to protect against graft rejection, while only affecting the CD4 population at the graft site (Zhan *et al.* 2000). Thus, regulated delivery of low levels of anti-CD4 may be an alternative means to achieve β-glucuronidase tolerance in both neonatal and adult MPS VII mice. An effective means of controlling the immune response will be an important factor in developing treatments for null allele patients with any genetic disease that is amenable to gene therapy. Recently, a new MPS VII murine model has been developed with a missense mutation in the active site, resulting in a model of MPS VII that does not produce antibodies against β-glucuronidase (Sly *et al.* 2001). Although this animal model may facilitate the

development of therapies for patients that do not develop antibodies against β-glucuronidase, this model does not address the serious problem in patients with null alleles that are likely to develop antibodies. In patients, the development of antibodies against a therapeutic gene product would not only block the effectiveness of the treatment, but would also block the effectiveness of any future gene therapy treatments for the patient.

The findings of the present study demonstrate that even though significant levels of β-glucuronidase were delivered to neonatal MPS VII mice, resulting in a delayed onset of disease and prolonged survival, neonatal tolerance was not achieved in any treated MPS VII mice. An alternative to neonatal tolerance and systemic immunosuppression is required for a safe and effective long-term gene therapy of lysosomal storage diseases.

Figure 3.3.1 β-glucuronidase in plasma of neonatal-treated MPS VII mice.

Within one day of treatment, neonatal-treated MPS VII mice showed low levels of plasma β-glucuronidase activity (3.4 ± 0.5% of normal). From 1 to 28 days post-implantation, plasma β-glucuronidase activity averaged 3.6 ± 0.3% of normal. At week 4, the mice were re-treated with a second dose and β-glucuronidase activity rose to 5.6 ± 3.5% and 6.0 ± 4.2% of normal in the following two weeks. However, anti-β-glucuronidase antibodies were detected from weeks 4 to 7 post-birth (Fig. 2), and the plasma β-glucuronidase activity levels correspondingly declined to background.

Figure 1

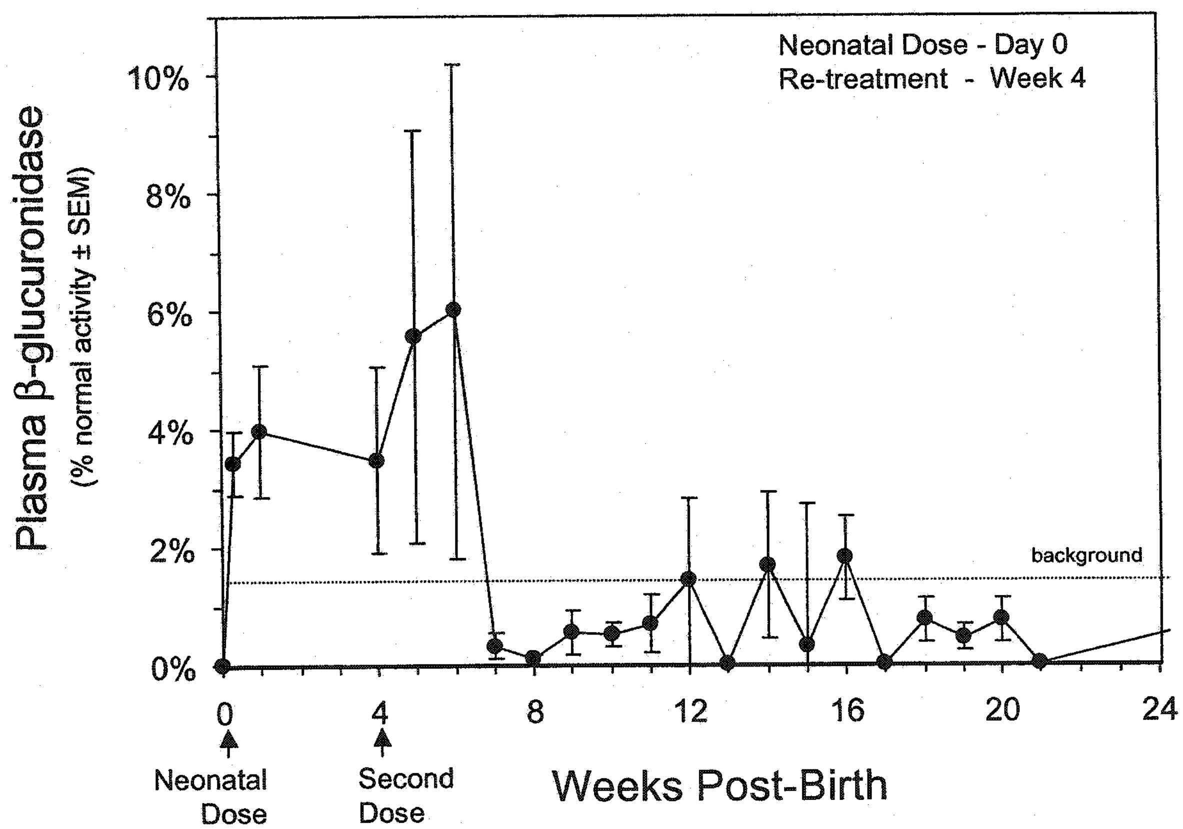


Figure 3.3.2 Anti-β-glucuronidase antibodies in neonatal-treated MPS VII mice.

Anti-β-glucuronidase antibodies were detected in neonatal-treated MPS VII mice within 4 weeks of treatment. There was no difference in whether the mice were treated on the day of birth or up to 5 days post-birth, all the animals eventually developed antibodies. The levels of antibodies increased exponentially for up to 8 weeks. The peak levels of anti-β-glucuronidase antibodies in one millilitre of plasma could immunoprecipitate the equivalent β-glucuronidase in 9.5 litres of wild-type plasma. Consequently, plasma β-glucuronidase activity declined after the development of antibodies .

Figure 2

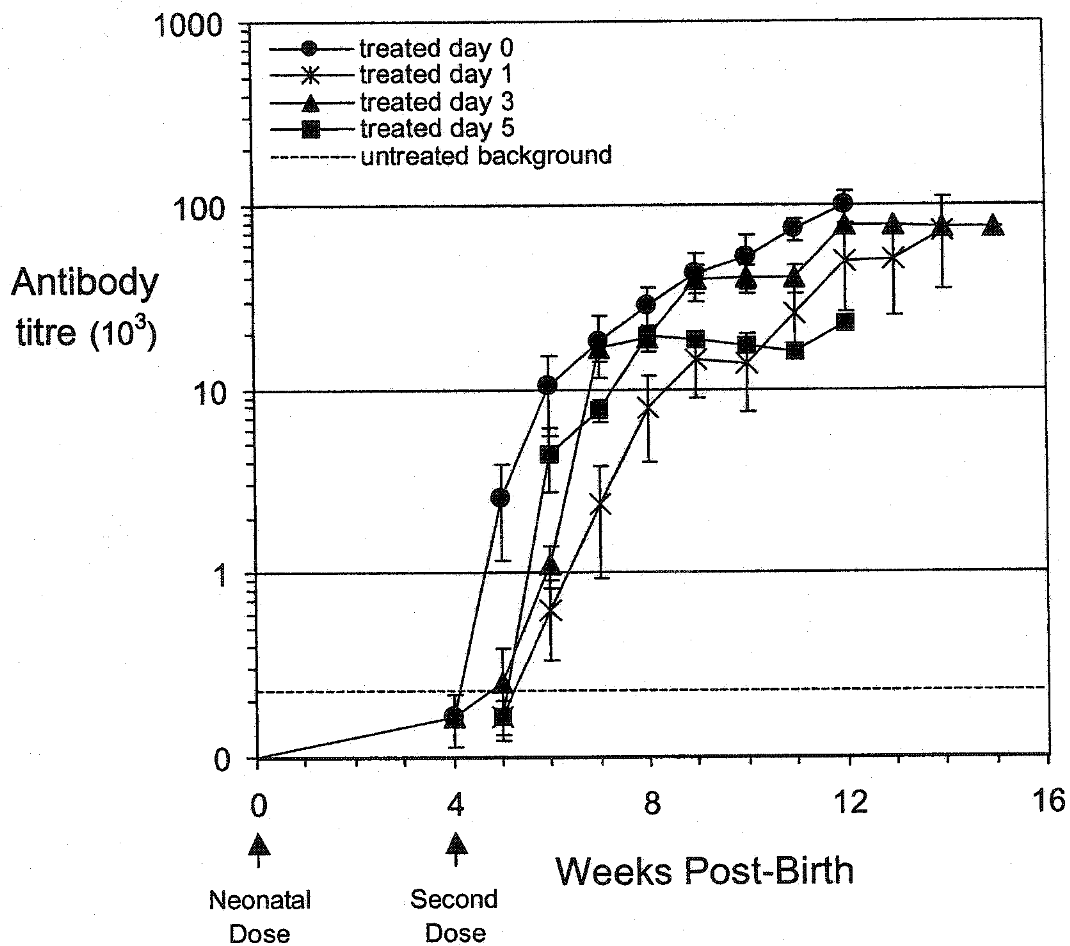


Figure 3.3.3 Lysosomal enzymes in the organs of neonatal-treated MPS VII mice.

At various times post-implantation, the lysosomal enzyme activities of organs were compared between treated MPS VII mice (n = 1 per time point) and untreated normal (n = 3) and mutant mice (n = 3). Within a week, low but significant levels of β-glucuronidase were detected in the muscle (33% normal, 5.5 ± 0.8 U/ml, $P < 0.001$), kidney (0.2% normal, 2.7 ± 0.2 U/ml, $P < 0.01$), and liver (0.2% normal, 3.3 ± 0.2 U/ml, $P < 0.001$). By week 4, β-glucuronidase declined, but was still significantly above background levels in all of the peripheral organs. After re-treatment at week 4, β-glucuronidase levels remained at very low levels, except muscle, which exhibited an average 7.6% of normal. After 40 weeks post-birth, β-glucuronidase was still detected at extremely low, nonetheless significant levels in the muscle (3.3% of normal, 1.8 ± 0.2 U/ml, $P < 0.01$), kidney (2.4 % normal, 8.6 ± 0.2 U/ml, $P < 0.001$), spleen (1.0% normal, 7.2 ± 0.1 U/ml, $P < 0.01$), and liver (0.7% normal, 5.3 ± 0.3 U/ml, $P < 0.01$). Significant β-glucuronidase was not detected in the CNS, and secondarily elevated β-hexosaminidase remained elevated. The secondary lysosomal enzyme β-hexosaminidase is abnormally elevated in MPS VII mice. In the peripheral organs of treated MPS VII mice, β-hexosaminidase activity was reduced for up to 8 weeks in muscle and spleen, and for up to 16 weeks in liver and kidney. However, β-hexosaminidase levels eventually returned to the elevated levels of untreated MPS VII mice. Each sample was assayed for enzyme activity and protein (units/mg) in triplicate and averaged ± S.D.

Figure 3.

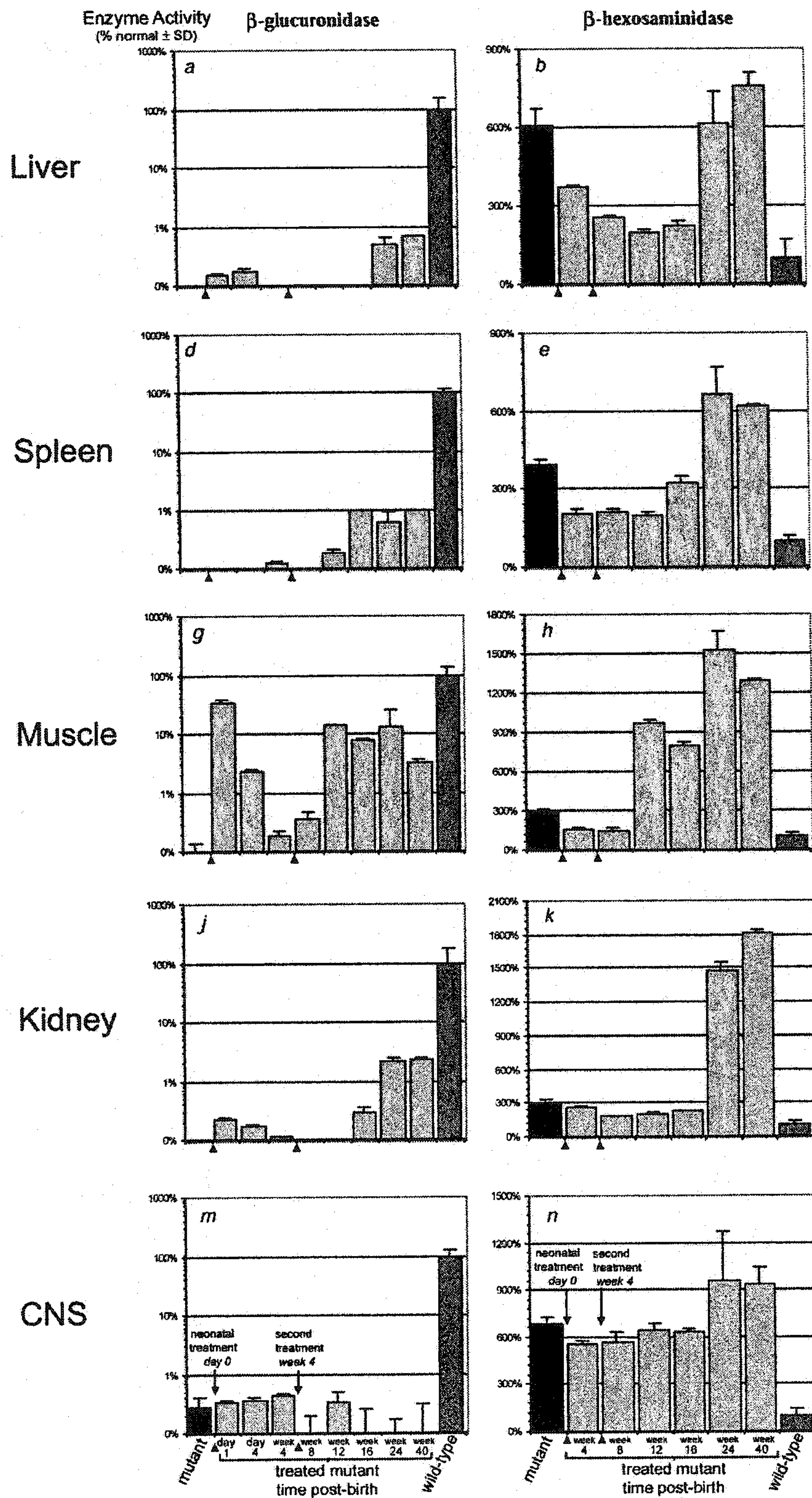
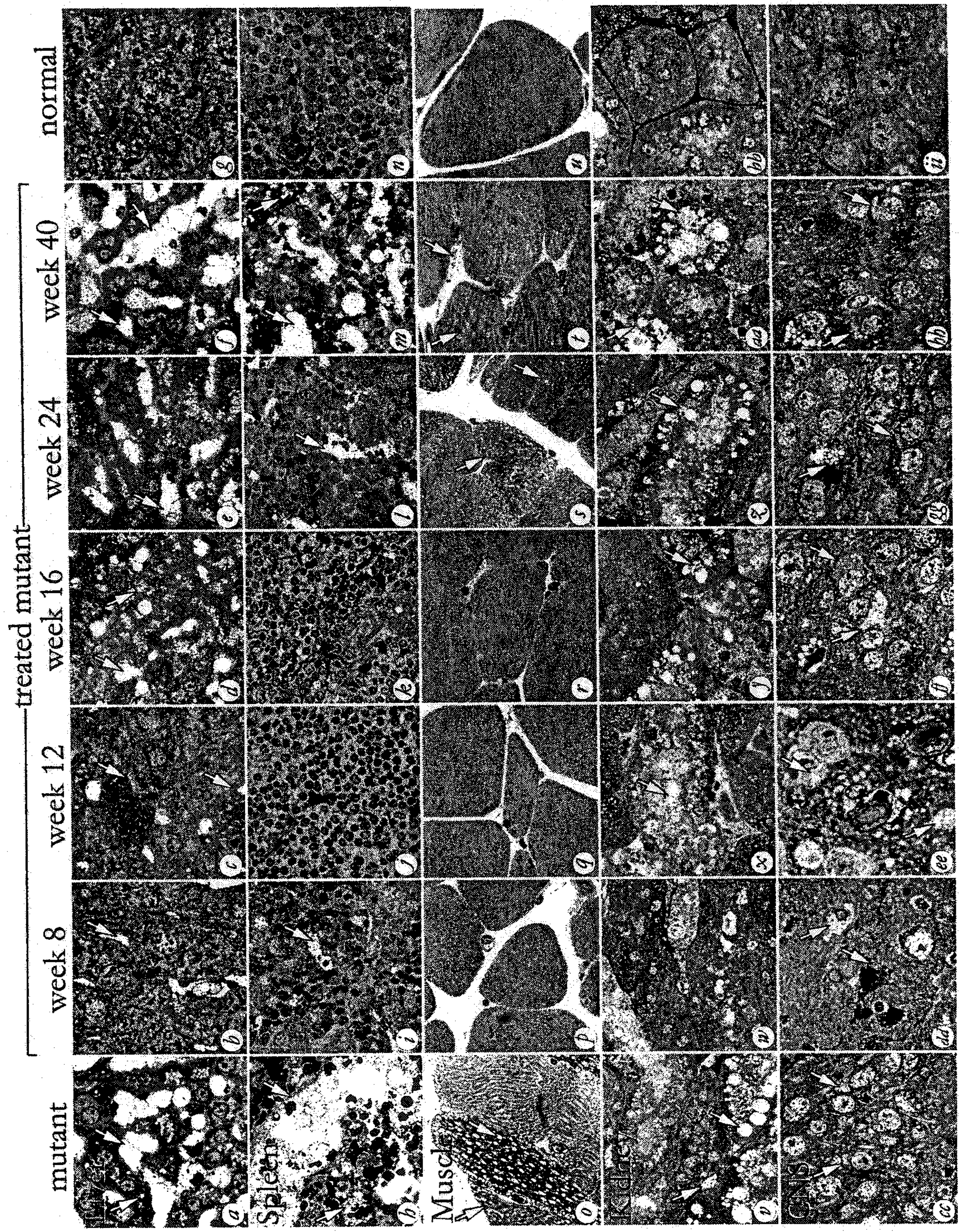


Figure 3.3.4 Histology of neonatal-treated MPS VII mice.

The lysosomal storage granules from the organs of MPS VII mice were evident in histological sections of liver, spleen, muscle, kidney, and CNS. Toluidine blue thin sections were prepared from tissue samples from mutant controls (a, h, o, v, cc), treated mutants at 8, 12, 16, 24, and 40 weeks post-implantation, and normal controls (g, n, u, bb, ii). In the liver (b-f) and kidney (v-aa) of treated MPS VII mice, the intralysosomal accumulations of GAGs was reduced to near normal levels until week 12, after which distended lysosomes in the liver and kidney renal tubular cells became evident. Similarly, the spleen (i-m) and muscle (p-t) showed decreased lysosomal storage for up to 16 weeks. However, marked lysosomal distention in the splenic sinusoidal lining and muscle fibres were evident by week 24. Original magnification 1000X.

Figure 4



3.4 Development of small alginate microcapsules for delivery to the rodent CNS.

3.4.1 Rationale

Previously, it was established that an intraperitoneal injection of microencapsulated recombinant cells could deliver β-glucuronidase to the peripheral tissues, and improve the clinical features of MPS VII mice (Chapter 3.2). However, the enzyme was unable to bypass the blood-brain barrier (BBB), and neither enzyme nor clinical improvements were observed in the CNS. As a result, the direct implantation of recombinant microencapsulated cells into the CNS has been considered. However, the normal microcapsule size (500-700 μm) was too large for safe implantation into the mouse CNS. The small mouse brain would be severely damaged using a large bore injection needle. In this experiment, we developed and characterized small microcapsules amenable to implantation into the mouse CNS.

3.4.2 Contribution

This research was accomplished by Colin Ross with the support of Dr. Patricia L. Chang.

3.4.3 Abstract

Immuno-isolation gene therapy using non-autologous recombinant cells immunologically protected with alginate-poly-L-lysine-alginate (APA) microcapsules has been successfully used to treat rodent genetic diseases, including the lysosomal storage disease, mucopolysaccharidosis VII (MPS VII). However, the therapeutic enzyme for MPS VII, β-glucuronidase, is unable to bypass the blood-brain-barrier (BBB). As a result, neither β-glucuronidase activity nor clinical improvements are observed in the CNS. We now report the development of smaller APA microcapsules for implantation into the mouse CNS, for the purpose of eventually treating neurodegenerative diseases with recombinant gene product delivery to the CNS. By varying the encapsulation protocol, mouse 2A50 fibroblasts expressing mouse β-glucuronidase were encapsulated in 5 to 2000 μm diameter APA microcapsules. The optimal size for implantation into the mouse CNS was determined to be 100-200 μm, based on the smallest, homogeneously sized, non-empty microcapsules that could satisfactorily fill the 500 μm inner-diameter of a CNS-implantation needle. Compared to medium sized (500-700 μm) microcapsules, the small microcapsules pack more tightly together, resulting in an increased number of cells and β-glucuronidase production per ml of microcapsules, even though there is a reduced number of cells per capsule. The small microcapsules also display an increased strength compared to large microcapsules. The excellent *in vitro* properties of small 100-200 μm microcapsules warrants further *in vivo* investigation into the feasibility of using immuno-isolation gene therapy to deliver recombinant gene products to the brain.

3.4.4 Introduction

Delivery of therapeutic gene products to the CNS may be beneficial for treatments of neurodegenerative diseases, such as lysosomal storage diseases (Neufeld and Muenzer 1989). However, the blood-brain barrier (BBB) impedes the passage of many substances into the brain, thereby limiting the application of most current gene transfer protocols aimed at delivering genes to peripheral organs. Attempts have been made at introducing foreign gene products directly into the CNS, bypassing the BBB. First proposed in 1987, grafting genetically-modified autologous cells in the CNS can produce recombinant marker gene expression for up to 8 weeks (Gage *et al.* 1987; Doering and Chang 1991). However, the CNS is not totally immunologically isolated and the need for expensive, patient-specific, genetically modified cells may limit the feasibility of such an approach. In contrast, immuno-isolation gene therapy could treat many patients that require the same recombinant gene product with a single "universal" recombinant cell line (Chang 1995). Polymer-encapsulated cells have been implanted in the brain to produce recombinant nerve growth factor (Date *et al.* 1997), and recombinant ciliary neurotrophic factor (Aebischer *et al.* 1996b; Emerich *et al.* 1997).

Most of the over 50 lysosomal storage diseases affect the CNS with severe neurodegenerative manifestations (Neufeld and Muenzer 1989; Gieselmann 1995a). Deficiency of β-glucuronidase causes the neurodegenerative lysosomal storage disease, mucopolysaccharidosis VII (MPS VII) (Sly *et al.* 1973). Immuno-isolation gene therapy using alginate-poly-L-lysine-alginate (APA) microcapsules reduces the peripheral disease in the mouse model of MPS VII. However, β-glucuronidase is unable to bypass the BBB, and neither β-glucuronidase activity nor clinical improvements are observed in the CNS. Potentially, APA microcapsules could be implanted into the brain to provide a source of β-glucuronidase for the CNS. However, APA microcapsules (500-1500 μm diameter) are currently too large for safe implantation into the rodent CNS.

Alginate microcapsule size depends upon the diameter of the alginate-extrusion needle, the airflow rate, and the outer diameter of the airflow nozzle (Wolters *et al.* 1991). The smallest alginate microcapsules that have previously been characterized and used in vivo were 315 μm in diameter (Leblond *et al.* 1999). We now report the development of smaller APA microcapsules (100-200 μm diameter) with properties that are ideal for implantation into the rodent CNS.

3.4.5 Methods

3.4.5.1 Cells

Mouse 2A50 fibroblasts were maintained as described in Chapter 2, Methods.

3.4.5.2 Small Alginate Microcapsules

Cells were encapsulated in standard APA microcapsules (Ross *et al.* 2000a), except the starting cell number and the airflow over the extruded alginate were increased for smaller microcapsules. Normal (500-700 μm) microcapsules were generated with a standard two million cells per millilitre of alginate, and a regular airflow (3 L/min) over the tip of the needle from which the alginate solution was extruded. Small (100-200 μm) microcapsules were generated with a 2.5-fold higher starting cell number and a higher airflow (6 L/min). Very small (<100 μm) microcapsules were also generated with a 2.5-fold higher starting cell number, and the highest airflow (7-8 L/min). Unlike standard microcapsules, the smaller microcapsules did not settle during washes, so all washes were shortened to leave time for centrifugation (30 seconds, 20g, Sorval RT6000B, 300 rpm). The short centrifugation allowed the supernatant of each wash solution to be removed before the next wash.

3.4.5.3 Analysis of Microencapsulated Cells

The number of microcapsules per millilitre, cell viability, cell number per microcapsule, and cell number per volume of microcapsules were calculated on 3 counts of 200 cells per sample as described in Chapter 2, Methods.

3.4.5.4 Recombinant Gene Product Secretion

The rate of β-Glucuronidase secretion from microencapsulated cells was determined from time-course assays of cell-culture media. After microcapsules were washed in PBS and placed in fresh equilibrated media, media samples were drawn at 0, 1, 2, and 4 hours and assayed for β-glucuronidase activity as described in Chapter 2, Methods.

3.4.5.5 Microcapsule Osmotic Pressure Test

Microcapsules were assayed with the osmotic pressure test as described in Chapter 2, Methods.

3.4.6 Results

3.4.6.1 Development of Small Alginate Microcapsules

Smaller alginate microcapsules were developed for implantation into the intraventricular space of mice. To reduce the size of alginate beads, the airflow was increased over the tip of the needle from which the alginate solution was extruded. The increased airflow broke off alginate beads more rapidly, producing smaller microcapsules. By varying the airflow, initial experiments produced microcapsules ranging from 5 to 3000 μm in diameter.

The high airflow (>7 L/min) necessary to produce very small microcapsules (<100 μm) produced a heterogeneous population of microcapsules, ranging widely from 5 to 1000 μm in diameter (Fig. 1*a*). Exploiting a size-dependent, microcapsule settling-time gradient, microcapsules of a desired size could be separated; however, this procedure was very labour-intensive. Alternatively, it was found that uniformly sized 100-200 μm microcapsules could be generated without further size selection (Fig. 1*b*). Thus 100-200 μm microcapsules were the smallest, homogenously sized, microcapsules that could be quickly and reliably generated.

However, in microcapsules less than 200 μm, empty microcapsules occurred in up to 50% of the resultant microcapsules. These small empty microcapsules could not be separated from cell-carrying microcapsules. Since a significant number of empty microcapsules would necessitate a larger microcapsule implantation volume, the starting number of cells to be encapsulated was increased 2.5-fold to reduce the number of empty microcapsules. This increased starting cell number was sufficient to reduce the number of empty microcapsules (100-200 μm) to less than 10%.

Finally, it was determined that microcapsules less than 200 μm were suitable for injection through a 500 μm inner diameter injection needle suitable for murine intraventricular implantations. Microcapsules of a larger size could not be efficiently loaded into the injection needle. Since 100 μm was the smallest microcapsule diameter that could be reliably generated, and microcapsules of less than 200 μm were suitable for *in vivo* injection, small 100-200 μm diameter microcapsules were optimal for rodent CNS implantation.

3.4.6.2 Microcapsule Packing Efficiency

The microcapsule size and empty space between microcapsules was significantly reduced in very small (<100 μm) and small (100-200 μm) microcapsules compared to larger microcapsules (Fig 1). As a result, the number of microcapsules per volume of packed microcapsules was significantly increased. Very small (<100 μm) microcapsules could be packed to more than 300 microcapsules per microlitre; compared to 20-50 for small microcapsules; and 4-5 for medium microcapsules (Fig 1*c*).

3.4.6.3 Encapsulated Cells

Although cell viability did not differ between small (100-200 μm) and medium (500-700 μm) microcapsules (Fig. 2*a*), the increased packing efficiency of small microcapsules significantly increased the number of cells per volume of packed microcapsules, by up to 9.4-fold at week 2 (Fig. 2*b*). In both microcapsule types, cells grow quickly until reaching a peak capacity (Fig. 2*c*). The capacity of small microcapsule peaked by ten days (770 ± 50 cells per microcapsule), while the larger microcapsules peaked on day 21 with 3-fold more cells per microcapsule (2500 ± 110 cells). Nonetheless, even with a reduced peak number of cells per microcapsule, the small microcapsules sustained a significantly higher number of cells per volume of packed microcapsules.

3.4.6.4 Production of Recombinant Gene Product

β-glucuronidase secretion from the microcapsules was monitored to compare the production of a recombinant gene product from small (100-200 μm) and medium (500-700 μm) microcapsules. Although there was no significant difference in the gene product production rate per cell in both microcapsule types, the higher cell number per volume significantly increased the resulting rate of gene product secretion per volume of microcapsules (Fig. 2 *d*). β-glucuronidase secretion per volume of microcapsules was up to 8.7-fold higher in small microcapsules at week 2 compared to medium microcapsules. Although this level of enzyme production from small microcapsules declined over time, it remained higher than medium microcapsules.

3.4.6.5 Microcapsule Strength

The microcapsule strength of very small (<100 μm diameter), small (100-200 μm), and medium microcapsules (500-700 μm) were compared using the osmotic pressure test (Van Raamsdonk and Chang 2001). Although the strength of very small microcapsules was at, or above, the range of the assay, it was clear that very small microcapsules were significantly stronger than both small and medium microcapsules (Fig. 3*a*). Small

microcapsules were significantly stronger than medium microcapsules ($P<0.001$) with an average 6-fold increase in strength (Fig 3*b-c*). There is an inverse relationship between microcapsule size and microcapsule strength (Fig. 4).

3.4.7 Discussion

The results of this experiment show that small microcapsules confer several advantages over larger microcapsules, and are optimally suited for implantation into the rodent CNS. Overall, the encapsulated cell viability and recombinant gene product secretion per cell were similar between small (100-200 μm) and medium (500-700 μm) microcapsules. However, the 4-fold reduction in diameter allowed roughly 10 times more small microcapsules to pack together. Even though the small microcapsules held only 30% as many cells in each individual microcapsule, the small microcapsules packed up to 9.4-fold more cells per millilitre of microcapsules, resulted in up to 8.7-fold more gene product secretion per milliliter of microcapsules.

In this study, the rate of recombinant gene product secretion from small microcapsules noticeably declined after 10 days. This trend began after the small microcapsules reached a peak number of cells per microcapsule, after which the cell viability and cell number gradually began to decline an average 12% and 17% a week, respectively. Since the small microcapsules were sustained in the same volume of media as medium size microcapsules, the up to 10-fold higher cell number in the small microcapsules may have outgrown the nutrient supply more rapidly that the medium size microcapsules. Even though the media was regularly changed, the medium microcapsules, with fewer cells per volume, could have had an advantage over the small microcapsules. *In vivo*, the improved nutrient supply would temporarily offset this reduction in secretion rate. However, *in vivo* the microcapsules would similarly reach a maximum sustainable rate of nutrient and waste exchange. At such a point, however, the increased surface area to volume ratio of small microcapsules would likely exhibit an improved rate of nutrient and waste exchange compared to larger microcapsules.

The increased strength of smaller microcapsules is likely due to the increased wall thickness relative to the microcapsule size. Although very small microcapsules (<100 μm) appear to be the optimal microcapsule formulation based on strength, their difficult production prevents large-scale production. Alternate methods of alginate microcapsule generation have been used to make smaller microcapsules. A high-voltage electrostatic

system has been used to generate microcapsules that were uniformly sized and smaller than those produced by airflow systems (Hsu *et al.* 1994; King *et al.* 1999). Indeed, electrostatically-generated alginate microcapsules as small as 300 and 350 μm have been characterized (Halle *et al.* 1994; Robitaille *et al.* 1999), and electrostatically-generated alginate microcapsules as small as 50 μm have been produced (Hsu *et al.* 1994).

The production of small and very small microcapsules currently relies on a centrifugation step following each wash. A potential improvement upon this protocol would be to use a mesh or wire filter that allows a wash solution to pass, while holding back the microcapsules. Such a filter could potentially simplify the generation of small microcapsules by eliminating the need for centrifugation after each wash, and could potentially speed up the generation and washing of regular microcapsules as well.

Smaller alginate microcapsules could reduce the size of the microcapsule injection device, thereby reducing the impact of surgery on the brain. As a result, smaller microcapsules could increase the chance of observing intended behavioral changes resulting from therapeutic gene product delivery, versus observing unintended deterioration as a consequence of surgery. Smaller APA alginate microcapsules also demonstrate an improved biocompatibility, as demonstrated by a reduced pericapsular fibrosis reaction when smaller (350 μm diameter) microcapsules were compared to larger (1200 μm) microcapsules for the encapsulation of islets (Robitaille *et al.* 1999). Additional advantages of smaller alginate microcapsules include a potentially improved nutrient and waste exchange, increased number of cells per implant volume, smaller total implant volume, and improved accessibility to the CNS and other implantation sites. With the development of smaller alginate microcapsules for implantation into the intraventricular space of mice, the feasibility of immuno-isolation gene therapy in the CNS may now be examined *in vivo*, towards the development of a treatment for neurodegenerative diseases.

Figure 3.4.1 Production of different microcapsule sizes.

Varying the airflow over the tip of the alginate extrusion needle generated various sizes of alginate microcapsules. A high airflow (>7 L/min) produces very small microcapsules; however, the microcapsules were not uniformly sized, ranging from 5 to 1000 μm diameter (*a*). A slightly lower airflow (6 L/min) produced homogeneously sized small microcapsules (100-200 μm) (*b*). Standard alginate microcapsules (500-1200 μm) are generated with an airflow of 3 L/min (*c*). Scale bar 500 μm.

Figure 1

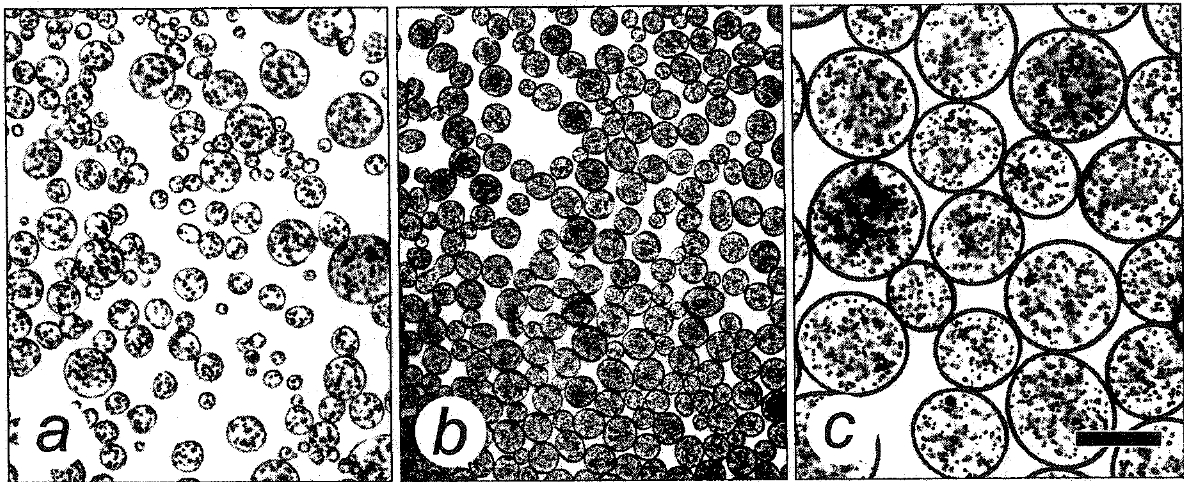


Figure 3.4.2 *In vitro* properties of small microcapsules.

The viability of cells encapsulated within small (100-200 μm) and medium (500-700 μm) alginate microcapsules were similar (*a*). Even though the small microcapsules held only 30% as many cells in each microcapsule (*c*), nearly ten times as many small microcapsules could be packed into the same volume as medium microcapsules. As a result, the small microcapsules had a significantly higher cell density (up to 9.4-fold higher) (*b*), and a significantly higher rate of recombinant gene product secretion per milliliter of microcapsules (up to 8.7-fold higher) (*d*).

Figure 2

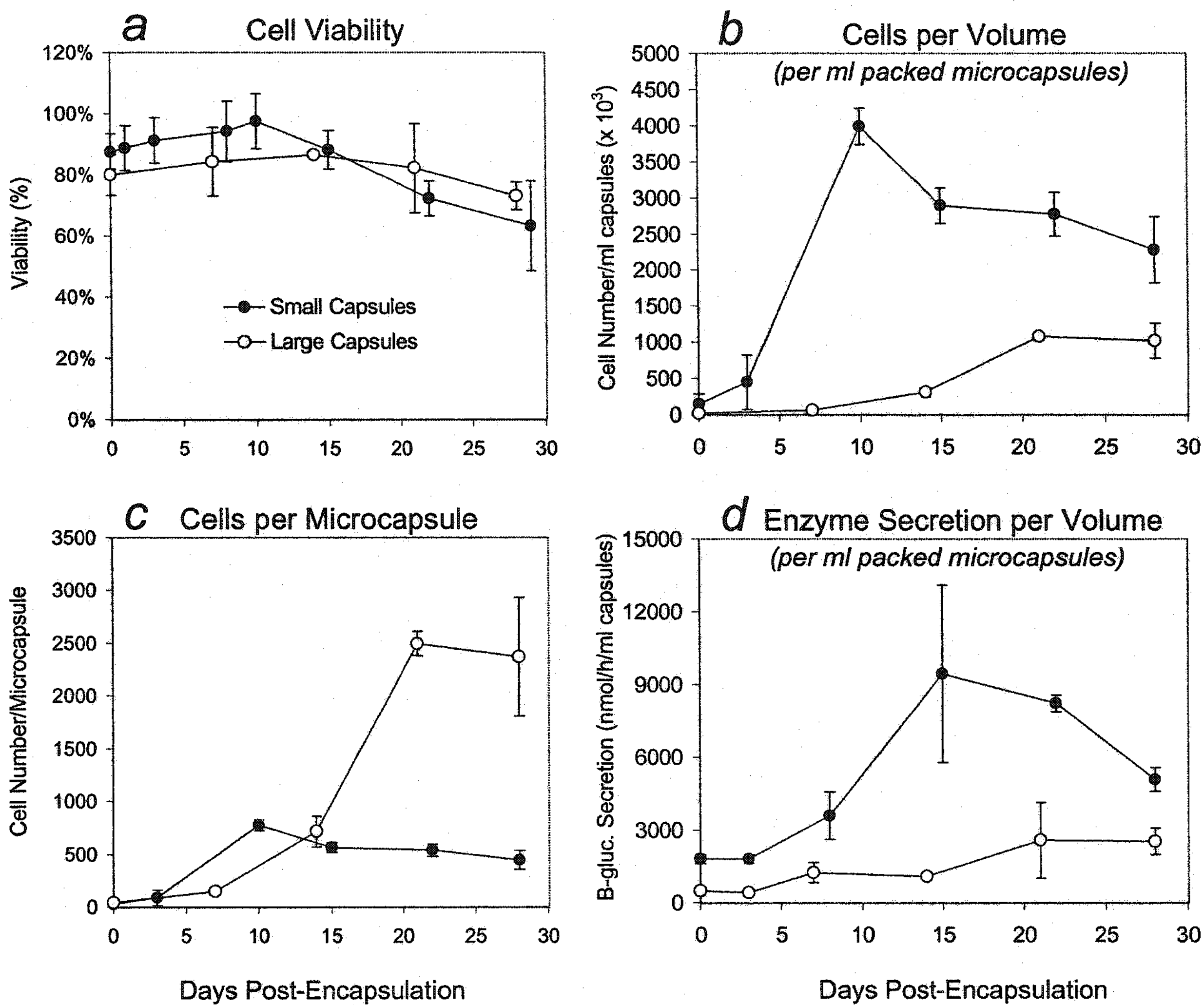


Figure 3.4.3 Strength of varying microcapsule sizes.

The strength of very small (<100 μm), small (100-200 μm), and medium (500-700 μm diameter) alginate microcapsules was compared using the osmotic pressure test. The very small microcapsules (*a*) were clearly the strongest with almost no breakage, and were at least 16% stronger than small microcapsules (*b*) ($P<0.0001$). The small microcapsules were an average 6-fold stronger than the medium microcapsules (*c*) ($P<0.01$). The difference between very small and small microcapsules may have been underestimated because the strength of the very small microcapsules was at, and likely above, the maximum range of the assay.

Figure 3

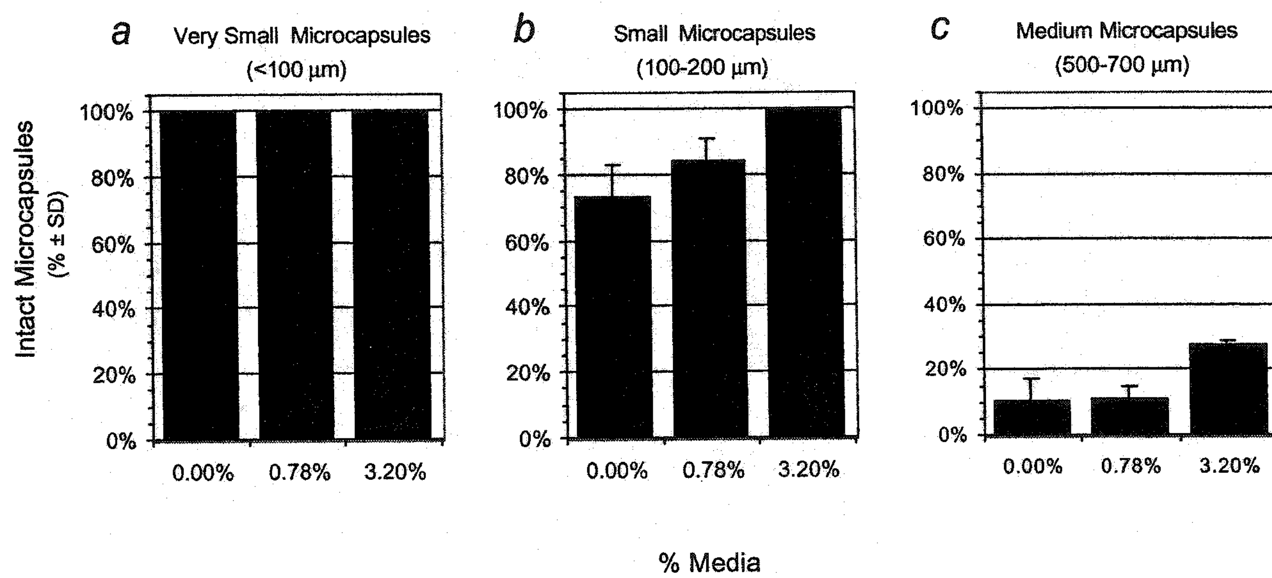
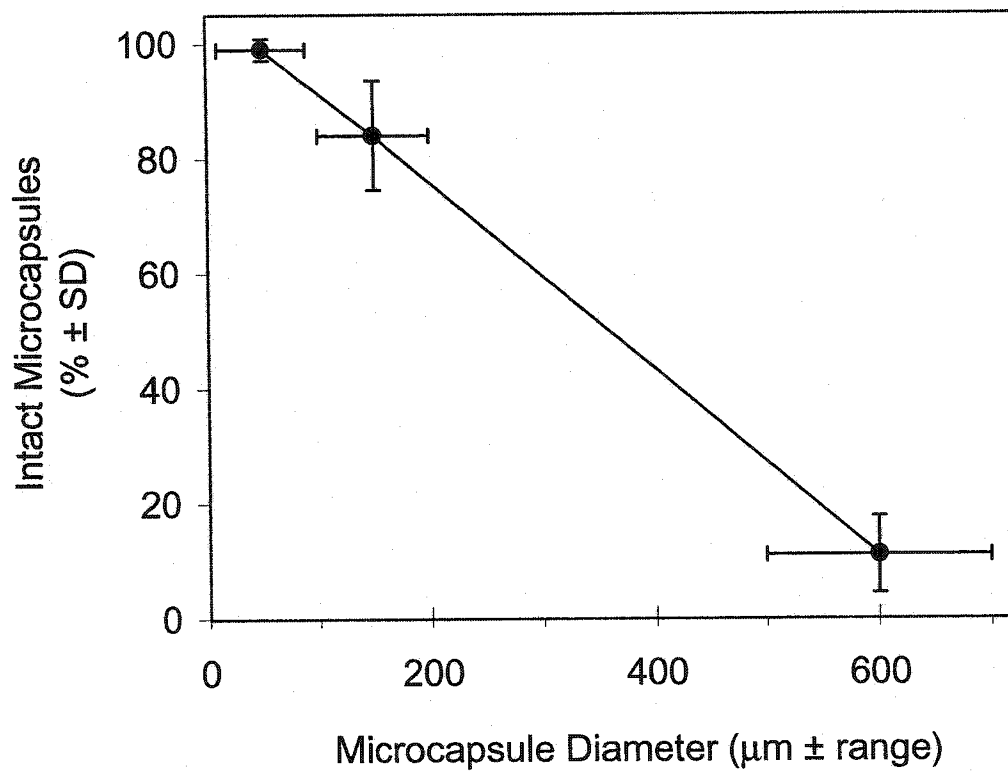


Figure 3.4.4 Microcapsule strength compared to microcapsule size.

The relative strength of the three different alginate microcapsules was inversely proportional to microcapsule size ($r^2 = 0.9998$). The relative microcapsule strength increased roughly 16% per 100 μm decrease in diameter.

Figure 4



3.5 Delivery of recombinant gene products to the CNS with non-autologous cells in alginate microcapsules

Published in *Human Gene Therapy*, **10**:49-59 (1999).

3.5.1 Rationale

Previously, we found that β-glucuronidase could be delivered to the peripheral tissues of MPS VII β-glucuronidase-deficient mice from an intraperitoneal injection of microencapsulated β-glucuronidase-expressing cells (Chapter 3.2). This newly supplied β-glucuronidase was able to improve many clinical features of the affected mice. However, the β-glucuronidase was unable to bypass the blood-brain barrier, and clinical improvements were not observed in the CNS. Subsequently, we investigated the direct implantation of microencapsulated cells in the CNS. Initially, the microcapsule size presented a problem, but this was overcome through the development of smaller microcapsules that could be implanted into the mouse CNS (Chapter 3.4). In this paper, we describe the first *in vivo* application of alginate-microencapsulated recombinant cells delivered to the CNS. Human growth hormone (hGH) was chosen as the marker gene because a sensitive assay for hGH protein was available.

3.5.2 Contributions

This research was accomplished by Colin Ross with the support of Dr. Patricia L. Chang and the assistance of Dr. Martin Ralph for teaching murine CNS surgical methodologies, and maintaining the mouse colony for this experiment.

HUMAN GENE THERAPY 10:49–59 (January 1, 1999)
Mary Ann Liebert, Inc.

Delivery of Recombinant Gene Products to the Central Nervous System with Nonautologous Cells in Alginate Microcapsules

C.J.D. ROSS,[1] M. RALPH,[2] and P.L. CHANG[1,3]

ABSTRACT

Somatic gene therapy using nonautologous recombinant cells immunologically protected with alginate microcapsules has been successfully used to treat rodent genetic diseases. We now report the delivery of recombinant gene products to the brain in rodents by implanting microencapsulated cells for the purpose of eventually treating neurodegenerative diseases with this technology. Alginate–poly-L-lysine–alginate microcapsules enclosing mouse C2C12 myoblasts expressing the marker gene human growth hormone (hGH) at 95 ± 20 ng/million cells/hr were implanted into the right lateral ventricles of mice under stereotaxic guidance. Control mice were implanted similarly with nontransfected but encapsulated cells. Delivery of hGH to the different regions of the brain at various times postimplantation was examined. At 7, 28, 56, and 112 days postimplantation, hGH was detected at high levels around the implantation site and also at lower levels in the surrounding regions, while control mice showed no signal. Immunohistochemical staining of the implanted brains showed that on days 7, 56, and 112 postimplantation, hGH was localized in the tissues around the implantation site. Mice implanted with encapsulated but nontransfected cells showed no signal. Hence, the feasibility of using encapsulated nonautologous cells to deliver recombinant gene products to the brain for extended periods may allow the application of this technology to the treatment of neurodegenerative genetic disorders.

OVERVIEW SUMMARY

The treatment of neurodegenerative diseases is hindered by the blood–brain barrier, preventing the delivery of many therapeutic products to the brain via systemic administration. We now explore the potential of delivering a recombinant product (human growth hormone) as a marker protein in the CNS from a universal myoblast cell line. To prevent the eventual graft rejection even in an "immunologically privileged" site such as the CNS, these recombinant cells are immunologically protected with alginate microcapsules. The results demonstrated the long-term potential of this nonautologous method of gene therapy for the delivery of recombinant products that may be applied to the treatment of neurological disorders.

INTRODUCTION

DELIVERY OF THERAPEUTIC gene products to the CNS may be beneficial for treatments of neurodegenerative diseases. However, the blood–brain barrier impedes the passage of many substances into the brain, thereby limiting the application of most current gene transfer protocols aimed at delivering genes to peripheral organs. Attempts have been made at introducing foreign gene products directly into the CNS, bypassing the blood–brain barrier. Grafting genetically modified cells into the CNS was proposed in 1987 (Gage *et al.*, 1987). In the CNS of rats, primary syngeneic fibroblasts expressing human growth hormone (hGH) have shown hormone delivery for up to 4 weeks, with decreased levels after 6 and 8 weeks (Doering and Chang, 1991). However, since the CNS is not totally immuno-

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logically isolated, the potential cost of having to construct autologous cell lines for each individual patient could be prohibitive. Thus the use of a universal recombinant cell line suitable for implantation into all patients requiring the same therapeutic products is economically attractive (Stockley and Chang, 1997).

To overcome the host immune response against nonautologous cells, immunoisolation devices have been used to protect xenogeneic tissues and cell transplants (Winn *et al.*, 1991; Sagen *et al.*, 1993). This involves encapsulation of cells within a biocompatible, perm-selective membrane that permits exit of the therapeutic product, while excluding immune mediators responsible for tissue rejection. Applying the concept of a perm-selective artificial capsule (Chang, 1964) fabricated from a seaweed extract, alginate (Lim and Sun, 1980; Liu *et al.*, 1993), we have delivered successfully various recombinant gene products to the systemic circulation of rodents (see review by Chang, 1997) such as coagulation factor IX (Hortelano *et al.*, 1996) and human growth hormone (Chang *et al.*, 1993). Furthermore, we have shown that this approach of nonautologous somatic gene therapy with genetically modified nonautologous cells can reverse the disease phenotypes of two rodent models of human genetic diseases. Both the dwarfism of the Snell dwarf mouse (Al-Hendy *et al.*, 1995) and lysosomal storage in peripheral organs of the GUS/GUS mouse (Bastedo *et al.*, 1998) were effectively treated after implantation of nonautologous cells secreting mouse growth hormone and β-glucuronidase, respectively.

Hence we now explore the delivery of a recombinant gene product, human growth hormone (hGH), to the CNS of mice by recombinant mouse myoblasts encapsulated in alginate. The choice of hGH as the marker protein in a rodent recipient permits its specific detection both biochemically and histologically, as it is distinct from the endogenous moiety. The use of myoblasts for such a purpose carries the additional advantage that it can differentiate terminally into myotubes, thus circumventing the potential problem of overgrowth of the proliferative cell line within the confines of an implantable device (Chang and Bowie, 1998). This approach is now shown to be feasible for delivery of a recombinant gene product to the CNS, bypassing the blood–brain barrier for an extended period.

MATERIALS AND METHODS

Cell lines

Cell lines used were mouse C2C12 untransfected myoblasts and also C2C12 myoblasts transfected with pNMG3 expressing human growth hormone (Chang *et al.*, 1990), maintained under the usual tissue culture conditions in Dulbecco's minimal essential medium supplemented with 10% fetal bovine serum, and penicillin (100 U/ml)–streptomycin (100 μg/ml).

Cell encapsulation

Both transfected and untransfected C2C12 cells were encapsulated within alginate–poly-L-lysine–alginate capsules (Sun, 1988), as modified by Liu *et al.* (1993). Encapsulation of cells was carried out at 4–10°C under sterile conditions. Cells were harvested before reaching confluence from 75-cm^2 tissue culture flasks using 0.125% trypsin, resuspended in 0.5 ml of cold saline, and mixed with the desired volume of 1.5% filtered potassium alginate (Keltone LV Lot 17703A, kindly provided by Kelco, San Diego, CA), and the mixture was drawn up into a 20-cm^3 syringe that was placed in an Orion Sage pump (model M362). This cell suspension was extruded through a 27-gauge blunt-end needle (Cat. 7400; Popper & Sons, New York, NY) at a rate of 99.9 cm^3/hr $\times$ 1/100. A concentric air stream (6 liters/min) delivered to the tip of the needle produced small droplets that fell into a bath of cold 1.1% $CaCl_2$. The $CaCl_2$ cross-linked alginate polysaccharide polymers to form solid spherical beads containing embedded cells. These were transferred to a sterile 50-ml conical polypropylene tube and washed successively with 0.55 and 0.28% $CaCl_2$ in saline, 0.9% saline, 0.1% CHES [2(*N*-cyclohexylamino)ethane-sulfonic acid], and 1.1% $CaCl_2$. Washes were followed by brief centrifugations (30 sec, 20 $\times$ *g*, Sorvall [Norwalk, CT] RT6000B, 300 rpm) to ensure that the small capsules had settled prior to the next wash. The droplets were further cross-linked with 0.05% poly-L-lysine (PLL, MW 22,000) for 6 min, washed with 0.1% CHES–1.1% $CaCl_2$–0.85% saline, and coated with another layer of 0.03% alginate for 4 min. This treatment was followed with another wash in 0.85% saline and the unpolymerized polysaccharide in the core of the capsule was dissolved by washing for 6 min in 0.55 m*M* sodium citrate. After two rinses in serum-free medium, the capsules were transferred to complete medium and kept under normal culture conditions.

In vitro *capsule analysis*

Cell viability within capsules was measured using trypan blue exclusion staining based on more that 3 counts of 200 cells per sample.

Cell number per capsule was measured by gently crushing a known volume of capsules containing a known number of capsules into a known volume of trypsin. The trypsin helped break up clusters of cells. Capsules were visually counted prior to crushing. The cells were then counted using a hemocytometer, resulting in values of cells per volume of capsules as well as cells per capsule based on the known number of capsules.

Capsules were assayed for hGH secretion, using a time course assay of cell culture medium samples. A known number of capsules was washed and kept to culture medium. An aliquot of the medium was removed at 0, 1, 2, and 4 hr and assayed for hGH levels with an enzyme-linked immunosorbent assay (ELISA) (Magiwel hGH quantitative ELISA HP-901; UBI, Mountain View, CA).

Implantation of capsules

Mice were anesthetized with a combination of 1.4 units of isofluorane (1-chloro-2,2,2-trifluorethyl difluoromethyl ether; Anaquest, Liberty Corner, NJ), oxygen (0.4 liters/min), and nitrogen oxide (0.7 liters/min) (Vapomatic; Med-Vet Anesthetic Systems, Libertyville, IL) and prepared for surgery by cleaning, shaving, and swabbing the surgery site with iodine. Mice were secured to a stereotaxic device and a 1- to 2-cm incision was made on the skull midline between the ears. A small burr hole was drilled into the right side of the skull. The drilled area was carefully cleaned and checked for any bone fragments before proceeding. A micropipette (Wiretrol 5 μl; Drummond

Scientific, Broomall, PA) was secured to the stereotaxic device, which was zeroed on the brain dura, and then inserted down to the right lateral ventricle. (+2.0 mm right of sagittal suture, −1 mm posterior to the coronal suture, and −3 mm down from dura). Slowly, over a period of 5 min, 5 μl of capsules was implanted into the right ventricle. The micropipette was slowly removed over a period of 2–4 min to allow capsules to spread throughout the implantation site. The surgery site was cleaned and Gelfoam (Upjohn, Mississauga, Ontario, Canada) was placed in the hole of the skull to allow for skull regrowth. The site was stitched two or three times and covered with Hibitane antibiotic/antifungal cream (Ayerst Laboratories, Montreal, Canada). The mice recovered within 30 min after surgery.

In vivo *analysis of implanted capsules*

The implanted right lateral ventricles of mice were compared with the unimplanted left ventricles at 56 days postimplantation by analyzing 100-μm-thick cryosections overlaid with trypan blue, which stained the microcapsules light blue.

In vivo *analysis of reporter gene product in brain*

A quantitative hGH solid-phase ELISA was used to detect hGH in brain tissue. At 1 (n = 6), 4 (n = 3), 8 (n = 3), and 16 (n = 4) weeks postimplantation, mice were sacrificed with CO_2. Brains were removed, placed in aluminum foil boats, snap frozen in a slurry of isopentane and dry ice, and stored at −70°C. They were sectioned midsagittally, and then coronally at the beginning of the striatum, rostal edge of the hippocampus, and the rostal edge of the cerebellum, to produce eight brain sections. Brain sections were sonicated in 300 μl of homogenization buffer (10 m*M* 2-mercaptoethanol, 20 m*M* Tris-HCl [pH 7.5], 140 m*M* NaCl, 0.25% saponin) and centrifuged in a cold microcentrifuge at 13,000 rpm for 30 min, and the supernatant was removed for assay of hGH by ELISAs performed in triplicate, and of total protein content by the method of Lowry *et al.* (1951).

Immunohistochemical localization of gene product in brain

Immunohistochemistry (IHC) was used to visualize the presence of the reporter gene product and implanted cells in the brains of implanted mice, and to estimate the distance of hGH delivery away from the implantation site.

At weeks 1 (n = 2), 8 (n = 2), and 16 (n = 2) postimplantation, mice were sacrificed with CO_2 and perfused with saline and then 4% paraformaldehyde for 4 min. Brains were removed and placed in 4% paraformaldehyde for 30 min and then in 20% sucrose for 16 hr at 4°C. Brains were embedded in O.C.T. Tissue-Tek (Miles, Elkhart, IL) and rapidly frozen in a slurry of dry ice and acetone or liquid nitrogen and stored at −70°C. Frozen brains were cryostat sectioned to 6–15 μm. Sections were air dried for 2 hr and fixed for 30 min in 4% paraformaldehyde, permeabilized for 3 sec in acetone, and then washed in phosphate-buffered saline (PBS). Sections were incubated overnight with primary antibody (1:100 diluted rabbit anti-hGH [Monosan; Cederlane, Hornby, Ontario, Canada] or 1:100 diluted rabbit anti-desmin [Sigma, St. Louis, MO]), 3% goat serum, and 6% normal brain homogenate blocking solution in phosphate buffer. Sections were washed with PBS and then incubated with the secondary antibody (1:50 diluted goat anti-rabbit IgG[H + L] conjugated to Texas Red or fluorescein isothiocyanate [FITC; Jackson Immunoresearch Laboratories, West Grove, PA]). Sections were washed with PBS and distilled H_2O. Sections were air dried, mounted, and viewed and photographed on a Zeiss (Thornwood, NY) Axioskop microscope.

RESULTS

In vitro *capsule analysis*

Transfected and untransfected C2C12 myoblasts were encapsulated at a density of 6.8 ± 0.1 and 7.1 ± 0.1 million cells per milliliter of alginate, respectively. Resulting capsules were 80–150 μm in diameter, with a density of 130 ± 16 microcapsules per microliter of microcapsules. They were assayed for hGH secretion, cell viability, and cell proliferation (Table 1). Microcapsules containing untransfected cells produced no hGH, while their cell viability and cell density were similar to those of the microcapsules containing transfected cells. Encapsulated cells were maintained for more than 74 days postencapsulation *in vitro*. They showed a steady decrease in viability (from 83 to 45%), while maintaining a relatively stable cell number per

TABLE 1. *In Vitro* CAPSULE ANALYSIS[a]

Day	Viability (% ± SD)	No. of viable cells ± SD		hGH secretion (ng/hr ± SD)	
		Per capsule	Per microliter of capsules	Per microliter of capsules	Per 10^6 viable cells
0	84 ± 8	ND	ND	ND	95 ± 20
1	74 ± 5	9 ± 10	1180 ± 1310	ND	ND
3	73 ± 5	9 ± 7	1140 ± 940	95 ± 22	83 ± 19
11	71 ± 5	11 ± 8	1390 ± 360	70 ± 27	50 ± 19
50	68 ± 5	14 ± 13	1810 ± 210	ND	ND
74	45 ± 1	15 ± 2	1970 ± 610	81 ± 23	41 ± 22

[a]C2C12 myoblasts transfected with the hGH gene were encapsulated in alginate on day 0 and kept under standard tissue culture conditions with regular changes of media. At various days postencapsulation, an aliquot of the capsules was removed to estimate the rate of hGH secretion (see Materials and Methods). The capsules were counted and their volume measured before they were crushed to release the encapsulated cells for viability estimation and cell counting.

Abbreviation: ND, Not determined.

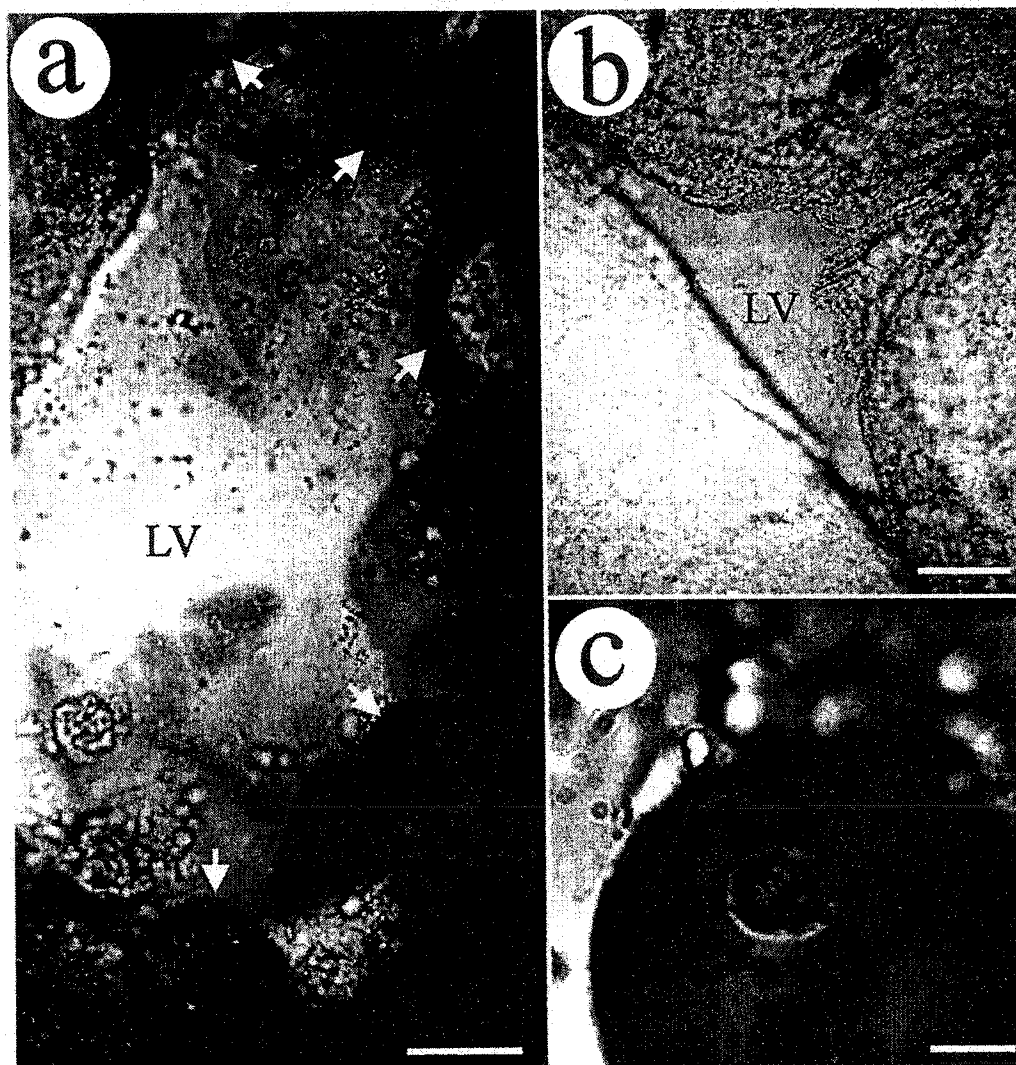


FIG. 1. Histology of the implanted and nonimplanted ventricles of the mouse brain. The implanted right lateral ventricle (LV) of a mouse brain (**a**) was compared with the unimplanted left ventricle (**b**) of the same mouse at 56 days postimplantation. Capsules appeared light blue after staining with trypan blue. Some capsule fragments appeared damaged from sectioning, while some small capsules (arrows in panel **a**) remained intact (**c**). Photomicrographs represent transverse cryosectioned (100 μm) frozen brain of a mouse implanted with microcapsules into the right ventricle. Scale bars: (**a** and **b**) 100 μm; (**c**) 25 μm.

capsule from 9 ± 10 to 15 ± 2 viable cells per capsule. Secretion of hGH per volume of capsules from encapsulated myoblasts was relatively stable for up to 75 days postencapsulation, from 95 ± 22 to 81 ± 23 ng/ml capsules/hr, but secretion of hGH per viable cell appeared to decline from 95 ± 20 to 41 ± 22 ng/10^6 cells/hr.

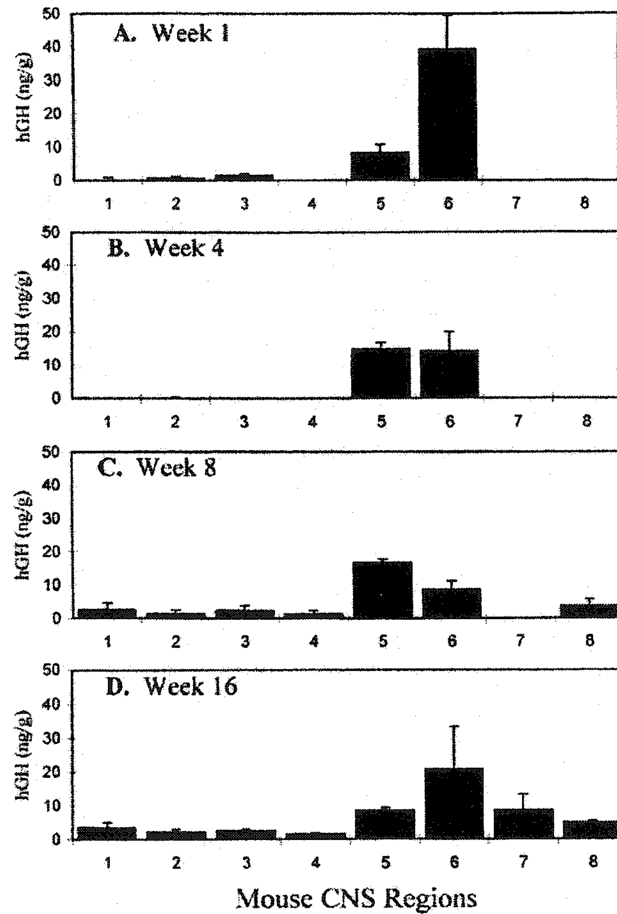
In vivo *analysis of CNS implanted capsules*

Microcapsules containing either hGH-secreting myoblasts or untransfected myoblasts were implanted into the right lateral ventricle of the brains of 27 mice. The histology of the implanted region was compared with that of the nonimplanted left lateral ventricle, showing the appearance of the implanted microcapsules *in situ* (Fig. 1). At least up to 56 days postimplantation, intact capsules were clearly visible at the implantation site.

CNS reporter gene expression from capsules

The reporter gene, encoding hGH, was delivered to the brains of mice at significant levels for up to 16 weeks postimplanta-

tion (Fig. 2). The implanted right lateral ventricle region (section 6 in Fig. 2) showed the highest average levels of hGH, with 40 ± 25 ng/g of hGH per total protein, which decreased to 21 ± 12 ng/g at week 16. Anterior to the implantation site, the right anterior region (section 5 in Fig. 2) showed the second highest levels of hGH, from 8 ± 2 ng/g at week 1 to 9 ± 1 ng/g at week 16, with a peak of 16 ± 1 at week 8. In all other regions of the implanted brains, the hGH levels remained below background



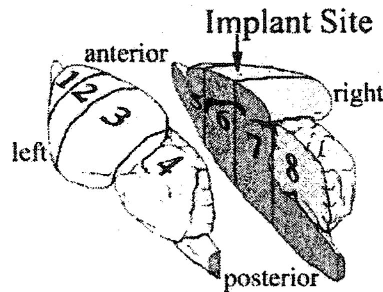


FIG. 2. Delivery of a reporter gene product (hGH) to the CNS. Five microliters of microcapsules containing C2C12 cells was implanted under stereotaxic guidance into the right ventricle. At (**A**) week 1 (n = 6), (**B**) week 4 (n = 3), (**C**) week 8 (n = 3), and (**D**) week 16 (n = 4) postimplantation, brains were removed and sectioned. The eight regions were created by sectioning the brain (diagram) midsagittally, and then coronally at the beginning of the striatum (sections 1 and 5), at the rostal edge of the hippocampus (sections 2 and 6), and at the rostal edge of the cerebellum (sections 3 and 4, and 7 and 8). Each region was homogenized to determine the level of hGH (nanograms) and total protein (grams). Control mice (n = 3) implanted with nontransfected myoblasts were similarly assayed but did not show elevated hGH levels.

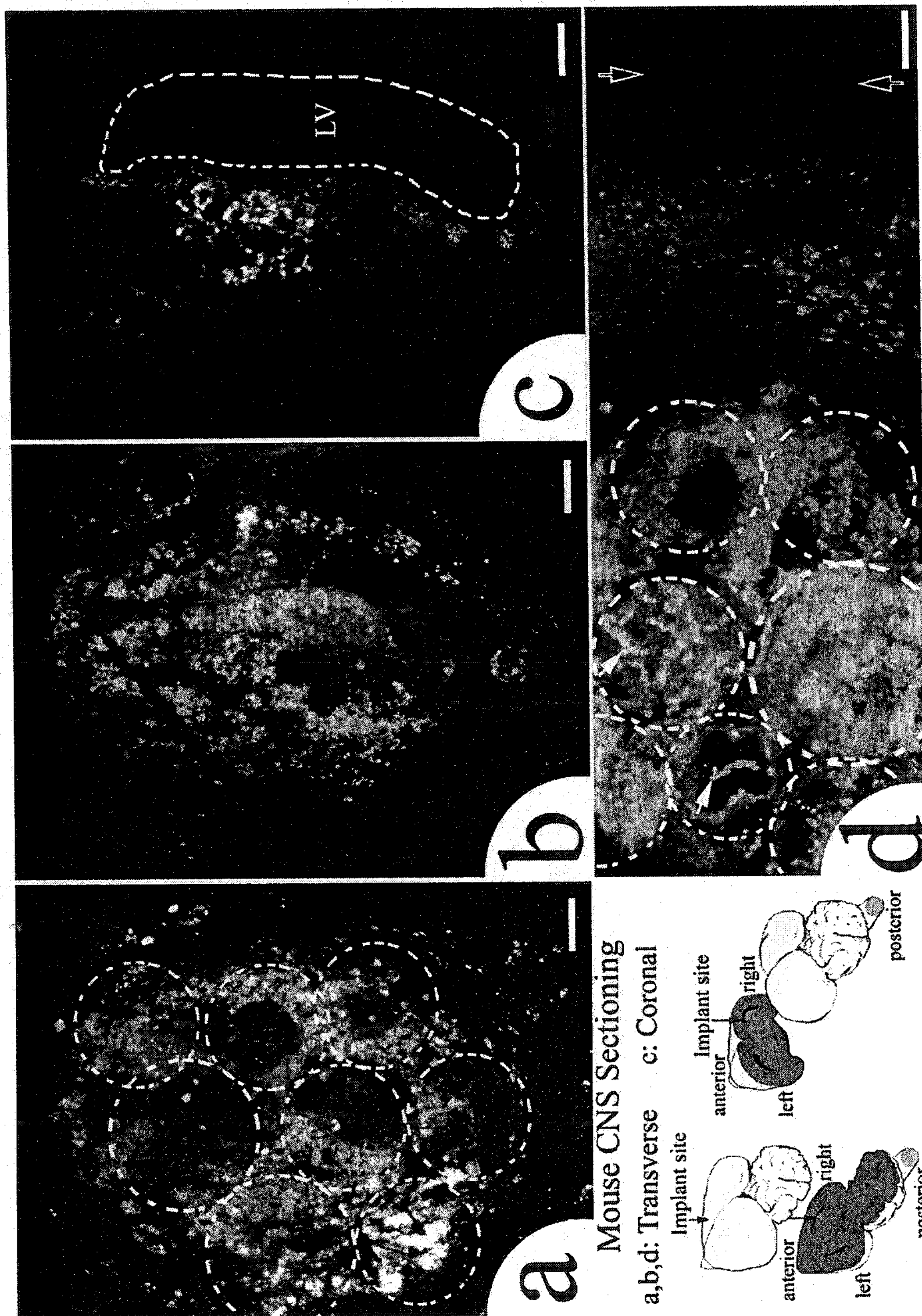


FIG. 3.

levels of the assay, except region 7, which seemed to have acquired the reporter gene product only after a prolonged period of time, at 16 weeks (Fig. 2D). All controls implanted with untransfected, encapsulated cells also did not show any hGH above background levels of the assay.

Immunohistochemical detection of hGH expression from capsules

The presence of the reporter gene product (hGH) and implanted cells as well as the distance of hGH delivery away from the implantation site were assessed with immunohistochemistry. Immunoreactive hGH was detected at all time points up to 16 weeks postimplantation (Fig. 3a–d). The hGH was highly localized to a circular region around the implantation site, extending to a diameter of 700–800 μm. A lower intensity of the hGH signal was detected over a wider region, spreading from the core to form a larger zone with a diameter of 1500–2000 μm (Fig. 3d). Coronal sections at week 16 showed positive hGH signals adjacent to the lateral ventricle (LV) and on the outside of the lateral ventricle lining (Fig. 3c). However, the hGH was not detected within the ventricle, suggesting that any hGH that was delivered to the ventricular space must have diffused and dissipated throughout the cerebral spinal fluid instead of remaining stagnant in the ventricle.

To localize the site of implanted myoblasts, a muscle-specific marker, desmin, was used for immunohistochemical detection of the implanted myoblasts (Fig. 4). Desmin-positive areas of the brain showed a pattern similar to that of the hGH localization in Fig. 3b and d, except there was no desmin immunoreactivity outside the 700-μm core shown in Fig. 3d.

DISCUSSION

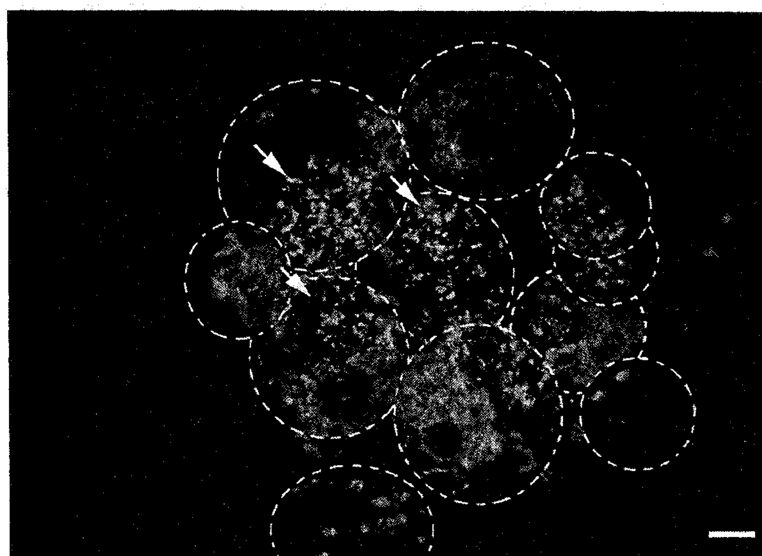
Evidence from *in vitro* culture (Table 1) and *in vivo* implantation (Figs. 2 and 3) showed that the encapsulated myoblasts maintained long-term viability and transgene expression for at least 16 weeks. All 16 experimental mice showed levels of hGH significantly above background, particularly around the implantation site. This localization was further confirmed by immunohistochemistry showing the presence of immunologically reactive hGH near the implantation site at all time points from 1 to 16 weeks postimplantation (Fig. 3). Furthermore, the presence of the transgene product can be attributed directly to the implanted myoblasts as shown by the colocalization of the hGH with a muscle-specific marker, desmin (Fig. 4).

Alginate–poly-L-lysine–alginate (APA) microcapsules provide immunoisolation as well as permit secretion of functional insulin (O'Shea *et al.*, 1984; O'Shea and Sun, 1986; Sun *et al.*, 1996) and recombinant products, such as human factor IX (Liu *et al.*, 1993), human growth hormone (Chang *et al.*, 1993), human adenosine deaminase (Huges *et al.*, 1994), and mouse β-glucuronidase (Bastedo *et al.*, 1998).

In spite of the immunologically privileged status of the CNS, the encapsulation of cells prior to implantation in the CNS is critical for long-term survival of the nonautologous cells when implanted. In adult guinea pig brain striatum, implantation of PAN-PVC-encapsulated, xenogeneic PC12 cells conferred survival for at least 12 weeks, while unencapsulated cells did not survive for even 2 weeks (Aebischer *et al.*, 1991). Moreover, cell encapsulation can inhibit the formation of tumors (Winn *et al.*, 1991; Tresco *et al.*, 1992; Hoffman *et al.*, 1993; Schinstine *et al.*, 1995), which has been seen as a result of implantation of immortalized cells within the CNS (Jaeger, 1985; Cunningham *et al.*, 1991; Hatton *et al.*, 1992; Miyazono *et al.*, 1995). In addition to the potential economic advantage, encapsulated cell transplantation methods are not complicated by tissue availability, or by the ethical concerns associated with the use of human fetal cells for cell transplantation.

Encapsulated cells implanted within the CNS have been shown to be physiologically effective in relieving pathological conditions. For example, analgesic pain control in rats was achieved with xenogeneic PAN-PVC-encapsulated bovine chromaffin cells (Sagen *et al.*, 1993) and with encapsulated cells secreting β-endorphin (Saitoh *et al.*, 1995). Behavioral recovery in 6-hydroxydopamine-lesioned rats after intrastriatal implantation was obtained with implantation of encapsulated dopamine-secreting cells (Winn *et al.*, 1991; Tresco *et al.*, 1992; Emerich *et al.*, 1993). The neuroprotective effects of nerve growth factor (NGF) was demonstrated in lesioned rats with polymer-encapsulated fibroblasts (Hoffman *et al.*, 1993; Emerich *et al.*, 1994a; Winn *et al.*, 1994, 1996), and schwannoma cells (Schinstine *et al.*, 1995), and in monkeys with polymer-encapsulated human cells (Emerich *et al.*, 1994b; Kordower *et al.*, 1994). However, since the putative therapeutic products in such cases cannot be readily distinguished from the

FIG. 3. Immunohistochemistry of reporter gene product (hGH) at the site of capsule implantation. At week 1 (**a**), week 8 (**b** and **d**), and week 16 (**c**) postimplantation, brains were removed for cryosectioning (6–10 μm) and immunostained for hGH. (**a**) Transverse section: FITC conjugate for hGH signal. At week 1 postimplantation, fluorescent signal was detected at high levels in a 700- to 800-μm zone. Within this zone, circular regions (tentatively outlined in white dashed circles) of activity correlated with the size of implanted microcapsules. (**b**) Transverse section, Texas Red conjugate for hGH signal. At week 8 postimplantation, fluorescent signal was still detected in the same region as in (**a**). (**c**) Coronal section; FITC conjugate for hGH signal. At week 16 postimplantation, hGH-positive signal was still detected in the same region, although at lower intensity than in (**a**), adjacent to the lateral ventricle (LV) marked in the white dashed outline. (**d**) Transverse section: stained as for (**b**). At week 8 postimplantation the hGH-positive zone still forms a core region of high delivery within a diameter of 700–800 μm, as in (**a**). Immunopositive cells that appear to be myotubes are indicated with white arrows. The circular clumps that correlated with the size of implanted microcapsules are tentatively outlined in white dashed circles. There is an outer region of lower level hGH delivery spreading to a diameter of 1500 to 2000 μm (outer boundary indicated by white open arrows). Control mice implanted with nontransfected myoblasts ($n = 3$) were similarly examined and showed no hGH-positive signals. Photomicrographs were taken using a Zeiss microscope. Original magnification: ×100. Scale bars: 100 μm.



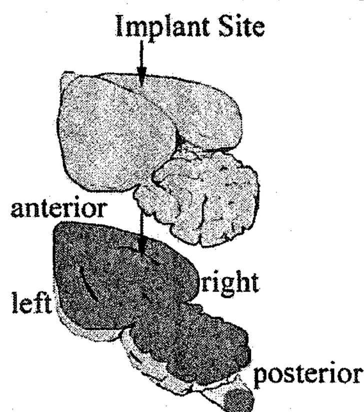


FIG. 4. Immunohistochemistry of a muscle-specific marker at the site of capsule implantation at week 8 postimplantation. Brains were removed for cryosectioning (6- to 10-μm transverse sections) and immunostained for desmin, a muscle-specific marker, to detect the presence of the implanted myoblasts. The desmin signal was localized within the area corresponding to the core region (700- to 800-μm diameter) of high hGH delivery in Fig. 3. Stained cells (arrows) appeared in clumps, which were tentatively labeled and circled as microcapsules because the clumps were circular in appearance and correlated with the size of implanted microcapsules (80–200 μm). Photomicrographs were taken with a Zeiss Axioskop microscope. Scale bar: 100 μm.

endogenous products present in the hosts, the actual delivery of the product could not be accurately assessed. Hence, by using a xenogeneic transgene product such as hGH, which can be quantitatively (ELISA) and qualitatively (immunohistochemistry) distinguished from the host product, further insight can now be provided.

It appeared that the level of hGH in the implanted region decreased slowly over time, from 39.2 ng/g at week 1 to 20.9 ng/g at week 16 (Fig. 2). The half-life ($t_{1/2}$) of hGH in normal mice has been estimated to be 2.2 min in high-turnover areas, 10 min in medium-turnover areas, and 70 min in slow-turnover areas (Turyn and Bartke, 1993). However, the actual $t_{1/2}$ in the mouse CNS is not known. The similarity between the observed values in Fig. 2 and the estimated levels, especially those based on a half-life of 10 min (Table 2), supported the hypothesis that the hGH was able to diffuse from a localized region to remaining parts of the brain after prolonged implantation.

On the basis of immunohistological data, a circular 0.7-mm diameter area with high levels of hGH appears to be located at the implantation site. This region is likely a capsule-rich area resulting from the 0.7-mm-diameter pipette used for microcapsule injections. Spreading of hGH from this site was observed. On transverse sections at weeks 1 and 8 postimplantation, the hGH appears to spread in a gradient of decreasing intensity to

form a 2.7-mm-diameter circle of detectable hGH. These results indicate the hGH is being secreted from the capsules and spreading throughout the brain in a limited manner. This may indicate the limited distance that the hGH can travel away from the capsules within the brain.

The hGH was detected by ELISA in sections other than the implanted section (6 in Fig. 2). The entire implanted region (6 in Fig. 2) was 3 mm in length, anterior to posterior; thus it is possible that some of the hGH within the 2.0-mm-diameter region of immunologically detectable hGH may have overlapped into the anterior section (5 in Fig. 2). Moreover, the hGH detected in the anterior region (section 5 in Fig. 2) may have resulted from hGH or microcapsules spreading from the implantation site within the cerebrospinal fluid (CSF). This is consistent with the anterior direction of CSF flow within the brain away from the choroid plexus. Spreading of hGH through the intraventricular space is suggested by the pattern of immunohistochemical staining of coronal brain sections at week 16, in which hGH was detected on the walls of the lateral ventricle. Thus, implanted microcapsule proximity to lateral ventricles may also have affected observed levels of hGH.

The expected levels of hGH could be estimated with an hGH $t_{1/2}$ range of 2.2–70 min, a constant production rate of hGH from the implanted microcapsules, and a varied volume of hGH delivery throughout the CNS (Table 2). If hGH was delivered to one-quarter of the CNS (to two of the dissected regions), the expected hGH levels would be 3.0–95.2 ng/g. Similarly, a delivery of 6.0–190.4 ng/g would be expected if hGH had been delivered to one-eighth of the CNS (to one of the dissected regions), and 0.7–23.8 ng/g with delivery of hGH to the whole mouse CNS (all eight of the dissected regions). Since the ELISA results in Fig. 2 indicated delivery to two of the dissected regions (5 and 6, Fig. 2) of the mouse CNS (one-quarter of the whole mouse CNS), the observed levels of hGH at the implantation (~10–40 ng/g; Fig. 2) from weeks 1 to 16 were not as high as the maximum expected level, but fell well within the wide range of expected values. These data further strengthened the evidence that suggests the encapsulated cells were still viable and secreting hGH within the brain.

Such long-term delivery of a recombinant gene product in the CNS thus extends greatly the spectrum of neurological disorders potentially amenable to nonautologous somatic gene therapy. It has already been shown that survival was increased by up to 40% in progressive motoneuropathy (PMN)-affected mice after implantation of encapsulated ciliary neurotropic factor (CNTF)-expressing cells into the brain (Sagot *et al.*, 1995, 1996), and human trials with xenogeneic encapsulated CNTF-producing recombinant cells to treat amyotrophic lateral sclerosis have also begun (Aebischer *et al.*, 1996).

Thus, cellular encapsulation may be a safe and useful strategy offering exciting potentials for gene therapy of diseases affecting the CNS. We now showed that intraventricular implantation of small, 80- to 150-μm-diameter microcapsules containing genetically modified, nonautologous myoblasts pro-

TABLE 2. EXPECTED hGH LEVELS IN CNS[a]

Relative volume of brain receiving hGH (V_d)	*Half-life of hGH ($t_{1/2}$ in min)*	*Level of hGH expected (X' in ng/g protein)*
One-eighth	70	190.4
	10	27.2
	2.2	6.0
One-quarter	70	95.2
	10	13.6
	2.2	3.0
Whole brain	70	23.8
	10	3.4
	2.2	0.7

[a]The expected levels of hGH delivered to the CNS were estimated with first-order pharmacokinetics (Ledley and Ledley, 1994) based on the equation (Goldstein *et al.*, 1974)

$$X' = \frac{Q}{(K_e)(V_d)}$$

These estimated values were consistent with the observed values of hGH in the brains of implanted mice. The expected levels of hGH were estimated with an hGH $t_{1/2}$ range of 2.2–70 min, a constant production rate of hGH from the implanted microcapsules, and a varied volume of hGH delivery throughout the CNS. Expected hGH levels were estimated to be 3.0–95.2 ng/g with a delivery of hGH to one-fourth of the CNS (to two of the dissected regions); 6.0–190.4 ng/g with delivery of hGH to one-eighth of the CNS (to one of the dissected regions); and 0.7–23.8 ng/g with delivery of hGH to the whole mouse CNS (all eight of the dissected regions). X', Estimated hGH level; Q, hGH production rate = 0.47 ± 0.11 ng/hr for the 5 μl of implanted microcapsules; K_e, first-order rate of constant elimination of hGH = $0.693/t_{1/2}$, when elapsed time postimplantation is ∞, $t_{1/2}$, half-life of hGH in mice, ranging from 2.2 to 10 to 70 min in high-, medium-, and slow-turnover ares, respectively (Turyn and Bartke, 1993); V_d, volume of tissue receiving hGH delivery, assuming delivery to reach one-eighth, one-quarter, or the whole brain.

vided sustained delivery of a foreign reporter gene for at least 16 weeks. The transplanted encapsulated cells appeared viable and continued to deliver hGH to surrounding brain tissue around the implantation site. This work has shown the potential of using APA-microencapsulated recombinant cells for the delivery of recombinant gene products into the CNS with "universal" cell lines.

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3.6 Somatic gene therapy for a neurodegenerative disease using microencapsulated recombinant cells.

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3.6.1 Rationale

Previously, we found that an intraperitoneal injection of an optimized microcapsule formulation of encapsulated 2A50 fibroblasts overexpressing β-glucuronidase (Chapter 3.1) could deliver therapeutic levels of β-glucuronidase to the peripheral tissues of adult MPS VII mice (Chapter 3.2). This newly supplied β-glucuronidase was able to improve many clinical features of the affected mice, including a reduction in secondary lysosomal enzymes and a loss of lysosomal storage disease pathology. However, the β-glucuronidase was unable to bypass the blood-brain barrier and clinical improvements were not observed in the CNS. To address this problem we developed small alginate microcapsules optimized for implantation into the CNS (Chapter 3.4), and subsequently showed that this strategy could be used for long term delivery of a marker gene product (hGH) in the murine brain (Chapter 3.5).

In this study we investigate the delivery of a therapeutic gene product in the CNS of MPS VII mice to address the neurodegeneration associated with lysosomal storage diseases. A 10 μl volume of small APA microcapsules containing 2A50 fibroblasts overexpressing β-glucuronidase were intraventricularily implanted into 6-8 week old MPS VII mice. Biochemical, histological, and behavioral parameters were monitored.

3.6.2 Contributions

This research accomplished by Colin Ross with the support of Dr. Patricia L. Chang and the assistance of Dr. Martin Ralph (University of Toronto) who provided access to circadian analysis cages, circadian analysis computers, and maintained the MPS VII mouse colony for this experiment.

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Somatic Gene Therapy for a Neurodegenerative Disease Using Microencapsulated Recombinant Cells

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Neurodegenerative diseases caused by lysosomal enzyme deficiencies are catastrophic illnesses with both peripheral organ and central nervous system abnormalities. The mucopolysaccharidosis type VII mouse with β-glucuronidase deficiency was used to develop an alternate approach to gene therapy, in which a "universal" cell line engineered to secrete the missing enzyme is implanted directly into all recipients requiring the same enzyme replacement. The cells, though nonautologous, were rendered immunologically tolerable by protection in immunoisolating microcapsules. Since the blood–brain barrier impedes the passage of large molecules such as β-glucuronidase, encapsulated cells producing β-glucuronidase were introduced directly into the lateral ventricles of the brain. Based on this strategy, β-glucuronidase was delivered throughout most of the central nervous system, reversing the histological pathology and reducing the previously elevated levels of lysosomal enzymes β-hexosaminidase and α-galactosidase. The effectiveness of this approach was further demonstrated with improvements in the mutant circadian rhythm behavioral abnormalities. Compared to wild-type and heterozygous mice, the mutant mice had an unstable periodicity, fragmented activity, and a sixfold reduction in wheel running activity. After treatment, the mutant behavioral abnormalities were significantly improved with a more stable periodicity and a less fragmented pattern of activity. While the overall total activity level did not increase in the treated mutants, it did not show the deterioration observed in the sham-treated as well as in the untreated mutant mice. Hence, this alternative cell-based gene therapy demonstrates biochemical, histological, and behavioral efficacy and provides a potentially cost-effective and nonviral treatment applicable to all lysosomal storage diseases with neurological deficits. © 2000 Academic Press

Key Words: **central nervous system; behavioral abnormality; lysosomal storage disease; mucopolysaccharidosis VII; circadian rhythm; β-glucuronidase; alginate; immunoisolation.**

INTRODUCTION

Many neurodegenerative disorders such as Huntington, Alzheimer, and Parkinson diseases affecting the central nervous system (CNS) may be amenable to gene product replacement strategies (1). Implantation of encapsulated cells into the CNS is a gene product replacement strategy that has been used to deliver dopamine and neurotrophic factors for the treatment of Parkinson disease (2, 3), β-endorphin for pain (4), and ciliary neurotrophic factor for Huntington disease and amyotrophic lateral sclerosis (5, 6). Previously, we have used alginate-poly-L-lysine-alginate (APA) microcapsules implanted in the CNS to provide immunoisolation for nonautologous cells and permit secretion of a recombinant marker gene product in the CNS (7). The cells are encapsulated in a protective immunoisolation barrier to overcome the host immune response against transplanted nonautologous cells. The biocompatible, permselective membrane permits secretion of the recombinant gene product, while excluding immune mediators responsible for tissue rejection. Thus, a single "universal" recombinant cell line could be used to treat many patients that require the same recombinant gene product (8).

Lysosomal storage diseases are a group of diseases caused by a deficiency of any one of the more than 30 different enzymes responsible for the catabolism of macromolecules: lipids, glycolipids, and complex carbohydrates (9). Most of the lysosomal storage diseases also affect the CNS with severe neurodegenerative manifestations (1). Deficiency of β-glucuronidase causes mucopolysaccharidosis VII (MPS VII), a representative neurodegenerative lysosomal storage disease resulting in progressive lysosomal accumulation of undegraded glycosaminoglycans (10). The murine model of MPS VII closely reflects the human disease with measurable cognitive and behavioral anomalies (11–13) and has been well characterized (14–17) to provide an excellent system upon which to develop novel treatments for neurodegenerative disorders. Efforts to counter the neurodegeneration in the CNS have been

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particularly challenging because treatments capable of long-term peripheral enzyme delivery have not succeeded in crossing the blood–brain barrier (BBB) (18–21), unless initiated prior to BBB development in the perinatal period (22–25). Since most patients with lysosomal storage diseases are not diagnosed until the disease is already at an advanced stage, after the BBB has developed, correction of the storage lesions throughout the CNS will require a widespread, long-term supply of enzyme that is produced from within the CNS (20, 26). Thus, attempts have been made at introducing β-glucuronidase directly into the CNS from recombinant cells (27, 28) and viral vectors (26, 29–32).

In this study, we explore the delivery of a therapeutic lysosomal enzyme secreted from nonautologous, encapsulated, recombinant cells implanted into the CNS of MPS VII mice. The recombinant cells secrete high levels of β-glucuronidase for mannose 6-phosphate receptor-mediated uptake (33) and distribution to diseased sites throughout the CNS (20, 32). We also examine if the therapeutic enzyme delivery results in a behavioral change due to the disease reversal in the CNS.

MATERIALS AND METHODS

Mice. MPS VII mutants bred from heterozygous MPS VII mice were genotyped by PCR and restriction digestion. PCR products encompassing the mutation site (16) were amplified with primers GUS-5′-1k (5′-CTA AAT TAA GGA CCA GGA GAT GTA-3′) and GUS-3′-1k (5′-CCA GAG GCT AAG GGA GAT TGT-3′). The resulting 977-bp product was digested with *Eco*0191I to generate 526-, 251-, and 200-bp fragments from the normal allele and 777- and 200-bp fragments from the mutant allele. Both mutant and normal alleles generate a 200-bp fragment for an internal restriction digest control.

Microcapsules. Mouse 2A50 fibroblasts (gift from W. S. Sly, St. Louis, MO) were a Rec$^-$ cell line deficient in the mannose 6-phosphate receptor and transfected with the plasmid pMSXND-MβG to express mouse β-glucuronidase at 522 ± 40 nmol activity/10^6 cells/h from the metallothionein I promoter and upstream of the cDNA encoding dihydrofolate reductase (34). Mouse 3521 β-glucuronidase-deficient cells were used as a control (gift from M. Sands, St. Louis, MO). Both cell types were encapsulated in alginate (35), a polysaccharide polymer composed of alternating blocks of mannuronic and guluronic acid. Microcapsules of ~100 μm in diameter were generated by extruding a suspension of 50 × 10^6 cells in 10 ml of 1.5% alginate (Kelco, San Diego, CA) through a 27-gauge blunt-end needle (Cat. No. 7400; Popper & Sons, New York) using a modified Orion Sage pump (Model M362). Droplets were sheared off with a concentric air flow of 6 liters/min delivered to the tip of the needle. The droplets fell into a bath of cold 1.1% $CaCl_2$ and ionically cross-linked with Ca^{2+} to form solid spherical hydrogel beads containing embedded cells. The permeability of the gelled beads was controlled by laminating with poly-L-lysine (MW 22,000–35,000). The beads were coated with alginate to confer a biocompatible surface, and the core of the capsule was solubilized with a chelating agent, citrate. The small size of microcapsules was controlled by using a high concentric air flow speed and selecting small microcapsules based on their slow settling time in medium compared to large microcapsules. Encapsulation of cells was carried out at 4–10°C under sterile conditions.

Microcapsule implantation. Mice anesthetized with isofluorane were secured to a stereotaxic device and a 1- to 2-cm incision was made on the skull midline between the ears. A small burr hole was drilled into the left and right sides of the skull. A micropipette (Wiretrol 5 μl; Drummond Scientific, U.S.A.) secured to the stereotaxic device was zeroed on the brain dura and then inserted down to the right lateral ventricle (+2.0 mm right of sagittal suture, −1 mm posterior to the coronal suture, and −3 mm down from dura) (36). Slowly over a period of 5 min, 5 μl of microcapsules (159 ± 54 capsules) containing 280 ± 96 cells were implanted into the right ventricle of each animal. The micropipette was slowly removed over a period of 2–4 min to allow the capsules to spread throughout the implantation site. The same procedure was then repeated for the left ventricle. The surgery site was cleaned and Gelfoam (Upjohn, Canada) was placed in the hole of the skull to allow for skull regrowth. The site was stitched two or three times and covered with Hibitane antibiotic/antifungal cream (Ayerst Laboratories, Canada). Intact microcapsules were later retrieved by dissection of brain slices.

Lysosomal enzyme assays. β-Glucuronidase, β-hexosaminidase, and α-galactosidase activities were assayed using the fluorogenic substrates 4-methylumbelliferyl-β-glucuronide, 4-methylumbelliferyl-*N*-acetyl-β-D-glucosamide, and 4-methylumbelliferyl-α-D-galactoside (Sigma, St. Louis, MO), respectively (37). One unit of enzyme activity corresponds to 1 nmol 4-MU substrate hydrolyzed per hour. Microcapsule β-glucuronidase secretion rates were determined from assays of time-course-collected medium samples. Microcapsule protein was quantified with the method according to Lowry (38).

Histology. Dissected tissues were fixed in glutaraldehyde, embedded in Spurr resin, cut into 0.5-μm sections, stained with toluidine blue, and examined with a Zeiss Axioskop. Cryostat sections were stained histochemically with AS-BI-β-D-glucuronide (16) to detect β-glucuronidase activity.

Circadian behavior analysis. Animals were housed individually with unlimited access to food and water in

TABLE 1

Analysis of Microcapsules

	Day	Viability (%)	Viable cells (per 5 μl microcapsules)	Secretion of β-glucuronidase (units/5 μl microcapsules/h)
In vitro	0	73 ± 5	280 ± 96	0.14 ± 0.05
	7	94 ± 3	1,830 ± 447	2.08 ± 0.83
	28	63 ± 5	19,100 ± 3660	5.07 ± 0.84
	56	38 ± 10	16,400 ± 3460	5.34 ± 2.14
In vivo	56	65 ± 9	5,780 ± 1950	—

Note. Microcapsules containing 2A-50 fibroblasts were implanted into the ventricles of mutant mice or maintained *in vitro* and monitored for viability, cell number, and secretion of β-glucuronidase at various days. At 56 days postimplantation, microcapsules retrieved from mice were analyzed also for cell viability and cell number. The secretion rate of β-glucuronidase was determined by time course assays of culture media. The *in vivo* secretion rate could not be determined because of the small amount of microcapsules implanted and retrieved. Data are the means ± SD.

cages with computerized recording of wheel running activity (36) in compliance with the Canadian Council on Animal Care guidelines and International Animal Care Committee approval. After acclimatization for up to 10 days, mice were placed in constant darkness for 7–10 days to characterize the endogenous rhythm. Following implantation with microcapsules containing therapeutic β-glucuronidase-producing cells or control β-glucuronidase-deficient cells, the animals were allowed to recover for up to 3 days before the activity of the mutant mice was recorded for approximately 30 days to monitor changes in wheel running activity and circadian rhythms.

Statistical analysis. Pre- versus posttreatment comparisons of running activity were performed using paired Student's t tests. Comparisons between genotypes and between groups were made using Student's t tests, assuming separate variances.

RESULTS

Encapsulated Cells

Microcapsules of ~100 μm in diameter containing 2A-50 fibroblasts secreting murine β-glucuronidase were analyzed for their functional status *in vitro* (Table 1). Encapsulated cells remained viable for at least 8 weeks in culture (Table 1). The trypan blue measure of cell viability decreased from 73 to 38% *in vitro*, while hemocytometer cell counting revealed a 57-fold increase in viable cell number from 283 to 16,400 cells per implant. The β-glucuronidase secretion from microcapsules maintained in culture increased 37-fold over the 8-week period, from 0.14 to 5.34 units of β-glucuronidase/5-μl implant vol/h.

When these microcapsules were implanted into the ventricles of the mutant mice on day 0 and then retrieved after 8 weeks, they were free of any cellular overgrowth on the capsule surfaces. The cell viability of the retrieved encapsulated cells decreased from 73% preimplantation to 65% after 8 weeks *in vivo*, whereas the total number of viable encapsulated cells increased 20-fold to 5780 cells/implant (Table 1). Due to the small implantation volume, an insufficient quantity of microcapsules was retrievable for assay of β-glucuronidase secretion from these *in vivo* microcapsules.

CNS Implantation

The biochemical consequence of intraventricular implantation of encapsulated β-glucuronidase-secreting cells into the CNS of mutant MPS VII mice was studied over an 8-week period (Fig. 1). Within 3 weeks of implantation, the β-glucuronidase had spread throughout much of the CNS with an average 33-fold higher than normal level, except in the far regions more than 5 mm from the implantation site with lower levels of 4 to 195% of normal. The peak region of β-glucuronidase delivery near the implantation site reached 7320 ± 950% of normal. By weeks 7 and 8 postimplantation, the β-glucuronidase decreased to 1 to 2% of normal levels throughout the CNS, but still maintained elevated levels of 65 to 401% near the implantation site. This biochemical profile was corroborated by histochemical staining for β-glucuronidase activity, demonstrating high levels of β-glucuronidase activity in the corresponding sections, particularly around the implantation site (Figs. 2T–2W).

Concomitant to the delivery of β-glucuronidase, there was also reduction of the secondarily elevated lysosomal enzymes (Fig. 1). In the untreated mutant mice, β-hexosaminidase and α-galactosidase enzyme levels were abnormally elevated 6.6- and 2.6-fold higher than normal, probably as a compensatory mechanism for the increased glycosaminoglycans storage. In the treated mutant mice, both β-hexosaminidase and α-galactosidase were reduced to normal levels in regions of high β-glucuronidase delivery (Fig. 1). Throughout the experiment, β-hexosaminidase was reduced more than 3-fold within 4 mm of the implantation site in most (66%) of the CNS. The high levels of β-glucuronidase at weeks 3 to 5 correlated with a

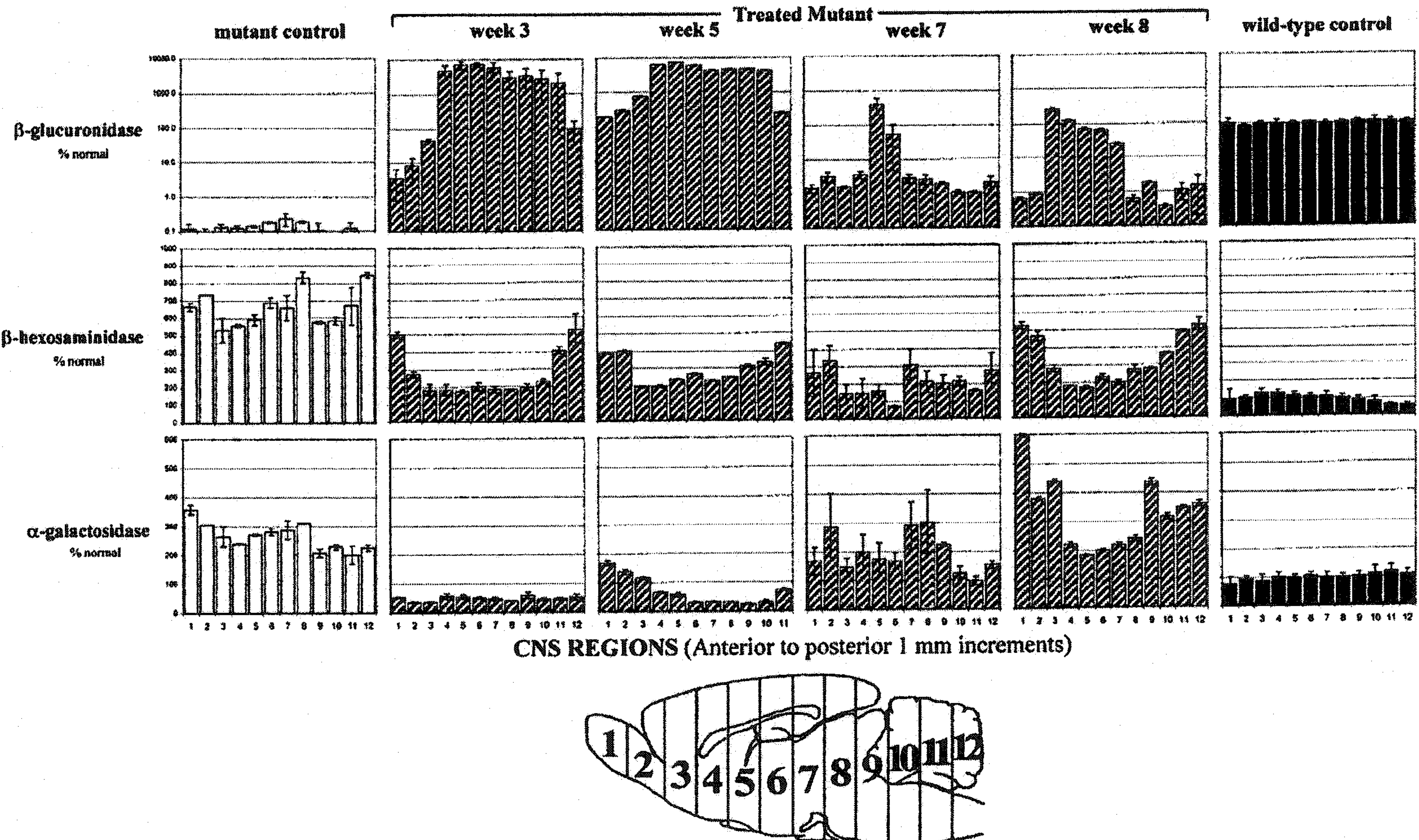

FIG. 1. Correction of enzymatic defects in the brains of CNS-treated MPS VII mice. β-Glucuronidase, β-hexosaminidase, and α-galactosidase enzyme levels in the brains of treated MPS VII mutant mice at week 3 (N = 3), 5 (N = 1), 7 (N = 2), and 8 (N = 1) after intraventricular implantation were compared to those of normal (N = 6) and untreated mutant MPS VII mice (N = 3). All brains were sectioned into 12 regions from anterior to posterior in 1-mm increments for each hemisphere of the brain (region 5–6, corresponding to implantation site) and assayed for lysosomal enzyme activities and protein concentration. All data are means ± SD, or average ± range ($N < 3$), or average ± SD of a single determination assayed in triplicate if N = 1. *Note.* Enzyme activity is represented in logarithmic scale.

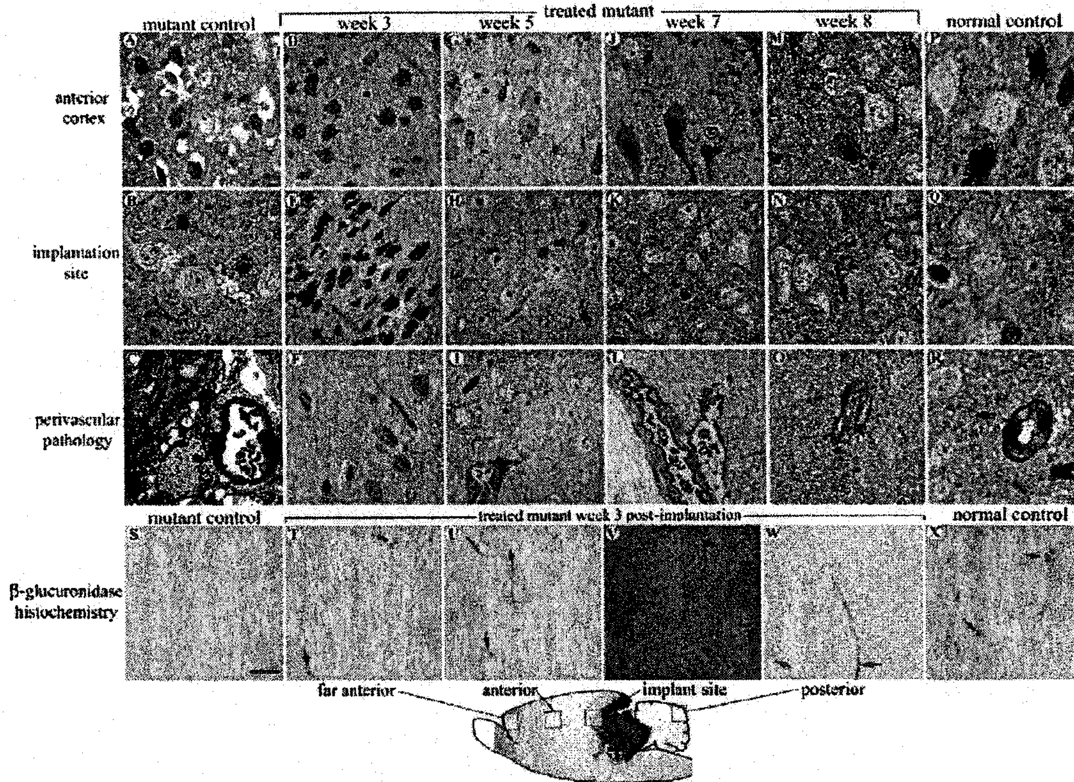

FIG. 2. Histological sections of the CNS showed decreased lysosomal storage in treated mutants for up to 8 weeks postimplantation. Toluidine blue-stained sections were compared from the brains of control mutant (A–C), normal (P–R), and treated mutant MPS VII mice at week 3 (D–F), 5 (G–I), 7 (J–L), and 8 (M–O) postimplantation. Control mutant MPS VII mice showed extensive lysosomal storage disease pathology with distended lysosomes (arrows, in A and B). Storage material was found throughout the brain in many cell types with many distended lysosomes in cortical neurons (A and B, many smaller distended lysosomes), neurons (B, large lysosomes), and perivascular distended lysosomes (C). Treated mutant MPS VII mice show reduced lysosomal storage, especially in sections near the implantation site (E, H, K, N) and reduced perivascular storage (F, I, L, O) and reduced storage at sites far from the implantation site (D, G, J, M). Original magnification 1000×. Histochemical activity of β-glucuronidase was detected in various brain regions of the CNS-treated mutants. Normal controls (X) showed a slight red hue overall, with a speckled dotting of red staining throughout the CNS (arrows), whereas mutant controls (S) showed no staining. At week 3 postimplantation, treated mutants showed intense staining near the implantation site (V), with more diffuse staining throughout the areas anterior (T and U) and posterior (W) to the implantation site (arrows). Scale bar in S, 200 μm.

greater than 4-fold reduction of α-galactosidase throughout the CNS. However, the lower delivery of β-glucuronidase at weeks 7 to 8 correlated with a more localized 30% reduction of α-galactosidase levels within 2.5 mm of the implantation site in 42% of the CNS.

A loss of the lysosomal inclusions characteristic of the disease phenotype was observed in the CNS of all the treated mutant mice (Fig. 2). Three weeks postimplantation, the histology of neurons, glial, and perivascular cells appeared normal within 3 mm anterior and posterior of the implantation site (Figs. 2D–2F). Outside this area, the accumulated lysosomal storage remained, but at significantly reduced levels. By 5 weeks postimplantation, the histology of neurons and glial cells was almost indistinguishable from that of the normal mice throughout the CNS (Figs. 2G–2I). After 7 and 8 weeks postimplantation, there was still a significant reduction in lysosomal storage throughout the CNS, with the highest level of correction apparent in the neurons, glial, and perivascular cells within 3 mm of the implantation area (Figs. 2J–2O). Although some lysosomal storage was observed in the cerebellar Purkinje cells after week 7, this was less severe than that of untreated mutant mice. The histological correction of the lysosomal inclusions in the brain was consistent with the histochemical demonstration of β-glucuronidase enzyme activity in all regions of the brain (Figs. 2T–2W), with exceptionally high levels around the implantation site (Fig. 2V).

An abnormal circadian rhythm behavioral deficit was recently discovered in mutant MPS VII mice.

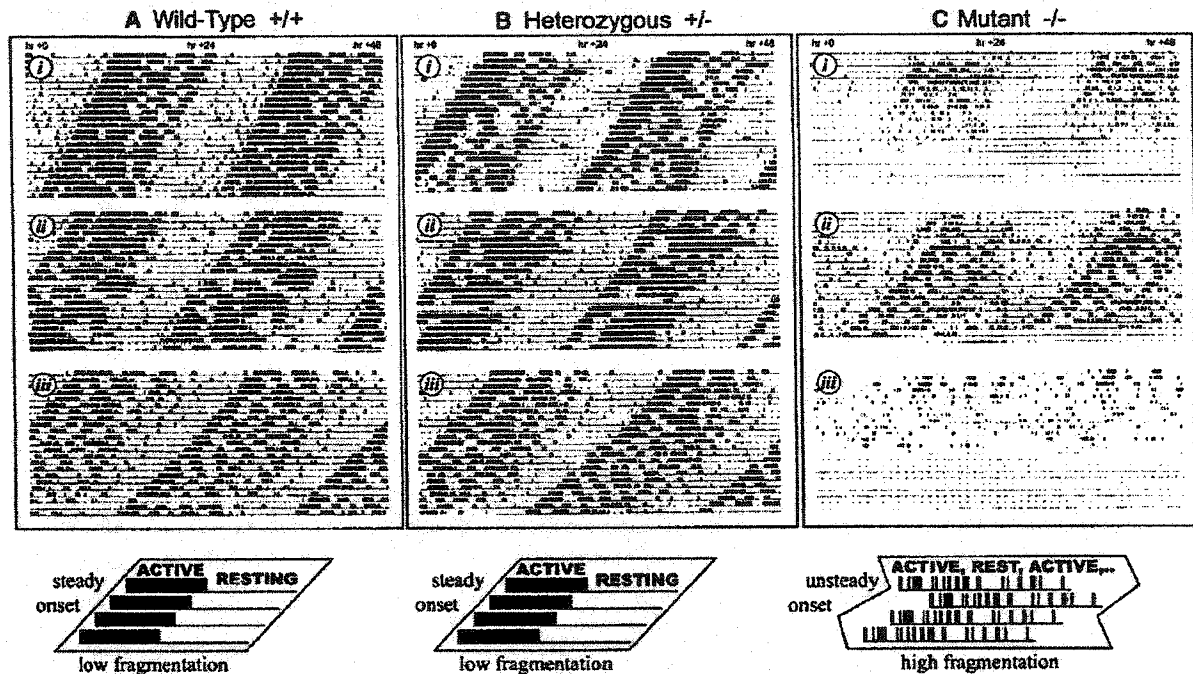

FIG. 3. Circadian rhythms for normal (A), heterozygous (B), and MPS VII mutant (C) mice. Representative graphs of three mice (i, ii, iii) in each group show daily wheel running activity represented as dark bars for a 48-h period from left to right. Each line from top to bottom represents 24 h. The circadian rhythms of normal and heterozygous mice had a steady period with a stable daily onset of a relatively continuous active phase, followed by a generally uninterrupted resting phase. In contrast, the mutant MPS VII mice had an unsteady period and more fragmented periods of activity and inactivity.

Wild-type and heterozygous mice have a periodicity of about 22 h, dividing the active and resting periods almost equally within each period (Figs. 3A and 3B). There were no significant differences in circadian rhythm or running activity between wild-type and heterozygous mice. By 10 weeks of age, the mutant MPS VII mice had a significant sixfold reduction in activity ($P < 0.001$). The activity decreased a further 75% during the equivalent 28-day period of time for which the treated mice were monitored. The mutant mice also exhibited abnormal circadian rhythms with an unstable periodicity that fluctuated between 20 and 24 h with an unstable daily onset of the active and resting phases (Fig. 3C) and a discontinuous active phase with fragmented periods of activity and inactivity.

After intraventricular implantation with 10 μl of microencapsulated cells secreting β-glucuronidase, the behavioral abnormalities were significantly reduced in the MPS VII mutant mice (Fig. 4, Table 2). A more stable period, with a rhythmicity around 22 h, and a less fragmented pattern of activity were restored. While there was still significant fragmentation compared to the wild-type mice, the activity phase in the treated mutants was clearly more frequent and continuous than before. Although the overall running activity of the treated mutant mice declined 28% after treatment, this was not significantly different from pretreatment levels ($P < 0.05$). Overall, the circadian rhythm parameters improved in five (57%) of the treated mutant mice and remained stable in one mutant (m7) that had begun with an exceptionally healthy circadian rhythm. After treatment with non-β-glucuronidase-producing microcapsules, sham-treated mutant mice exhibited a significant 81% reduction in running activity and an increased degree of fragmentation. A similar progressive deterioration of circadian rhythm and activity was also observed in the untreated control mutant mice older than 10 weeks, whose running activity declined by an average 75% during the course of the experiment (Table 2).

DISCUSSION

The MPS VII mouse closely resembles the human disease and provides an excellent model upon which to develop novel treatments for neurodegenerative disorders. The BBB impedes passage of β-glucuronidase into the CNS in peripheral-treated MPS VII mice. For example, enzyme replacement therapy initiated at birth, when the BBB is nonfunctional, is highly effec-

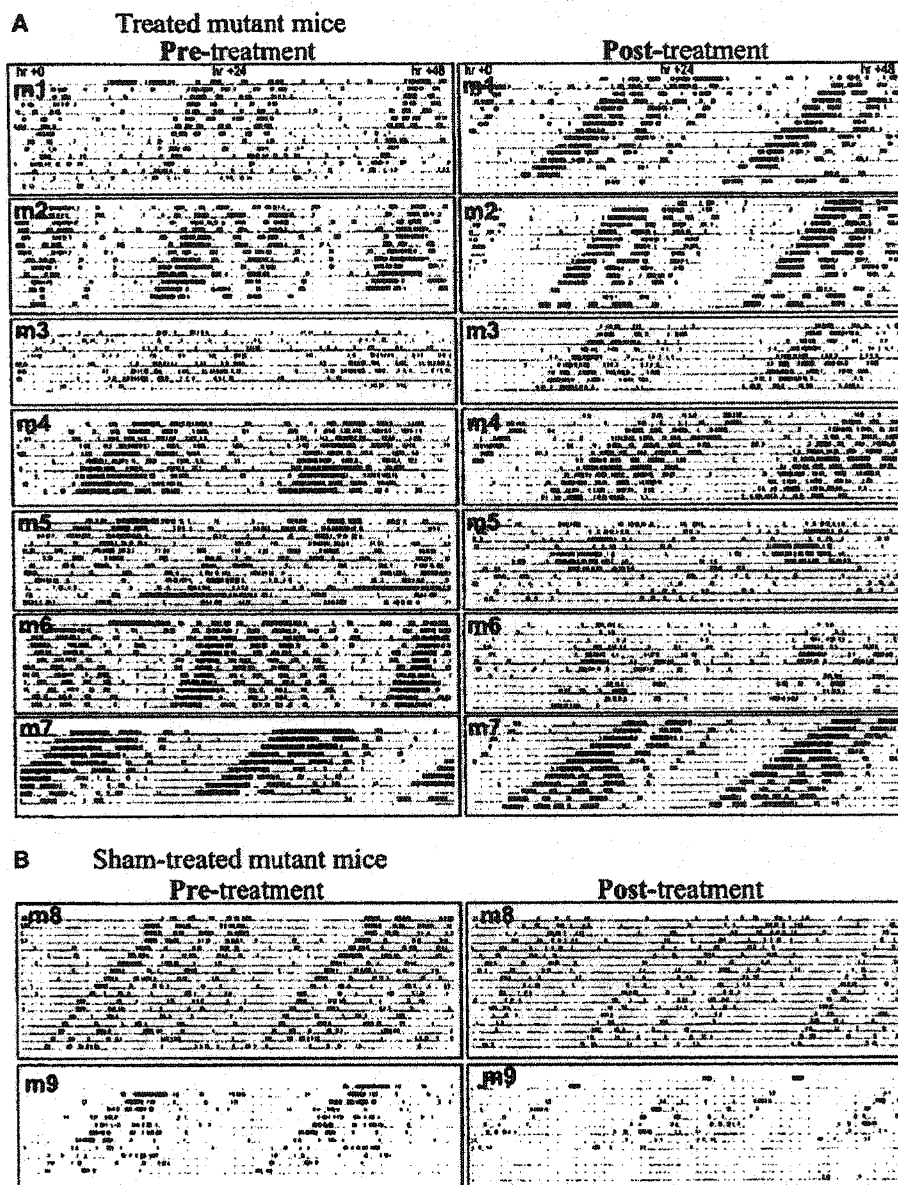

FIG. 4. Circadian rhythms of treated mutant mice (m1–7) and sham-treated mutant mice (m8 and 9) were recorded before and after implantation of microencapsulated cells. Treated mutant mice showed either an improvement (m1–4) or a deterioration (m6) or no change (m5, m7) in the circadian rhythm parameters of a stable period and degree of fragmentation. In contrast, sham-treated mutant mice showed only a deterioration in fragmentation postimplantation (m8 and 9) similar to untreated mutant controls.

tive until the BBB develops. However, by 12 weeks after initiation of therapy, β-glucuronidase becomes undetectable in the CNS and lysosomal distention becomes evident in neurons (24). Recently, this was well illustrated in a series of staggered enzyme injections, beginning 0 to 35 days after birth, in which a positive response to treatment in the CNS was found only when treatment was initiated in mice younger than 14 days (25), corresponding to the development of the BBB in rodents at 10–14 days (39). Therefore, in this study we bypassed the BBB and introduced β-glucuronidase directly into the brain using immunoisolated recombinant cells engineered to produce a long-term supply of β-glucuronidase in the CNS.

TABLE 2

Activity and Circadian Behavior of MPS VII Mice

	Wheel running (cycles/day)			Stable period		Fragmentation	
	Treatment: Pretreat.	Posttreat.	Change	Pretreat.	Posttreat.	Pretreat.	Posttreat.
Wild-type ($N = 6$)	**11,300 ± 1810**			+++		+++	
Heterozygous ($N = 7$)	**12,600 ± 2080**			+++		+++	
Mutant ($N = 6$)	**2062 ± 1980**	**(520 ± 554)***	**(−74.8%)***	−/+	(−/+)*	−/+	(−/+)*
Treated mutants ($N = 7$)							
m1	731 ± 123	3040 ± 319	+316%	−	++	+	++
m2	3342 ± 319	2770 ± 265	−17%	+	+++	+	+++
m3	392 ± 159	900 ± 113	+129%	−	++	−	++
m4	2911 ± 569	1350 ± 296	−54%	+	++	+	++
m5	2900 ± 446	1440 ± 249	−50%	−	−	−	−
m6	3760 ± 253	114 ± 32	−97%	+	−	+	−
m7	4650 ± 444	3820 ± 111	−18%	+++	+++	+++	+++
Total	**2668 ± 590**	**1921 ± 499**	**−28%**	+	++	+/−	++
Sham-treated mutants ($N = 2$)							
m8	1140 ± 197	130 ± 54	−89%	+	+	−	−
m9	1060 ± 162	205 ± 22	−81%	−	−	−	−
Total	**1096 ± 81**	**167 ± 64**	**−81%**	−/+	−/+	−	−

Note. The activity of MPS VII mice was measured with computerized wheel recording and the data were used to derive the circadian characteristics of a stable period and degree of fragmentation. A stable period was scored as a consistent straight line of daily activity onset (+++), a straight line with small fluctuations (++), an irregular onset with large fluctuations (+), or an undiscernable period (−). Fragmentation was scored as a clear separation between active and resting periods with a relatively continuous active period (+++), a well-defined but discontinuous active period (++), a recognizable active period (+), or an unrecognizable active period (−). There were no significant differences between the wild-type and the heterozygous mice. The treated mice were monitored for an average of 27 days postimplantation, and sham-treated mice were monitored for 14 and 28 days (m9 and m8).

* For untreated mutant controls, the activity and behavior postimplantation represent the average change during a period of time equivalent to the time that treated mice were monitored, 28 days after an initial 7-day characterization in mice older than 10 weeks. Data are ±SEM or range ($n < 3$).

Our data confirm previous results in which a relatively small number of cells can produce enzyme that reaches a large proportion of the CNS (32, 40). These results show favorable implications in the development of gene product replacement therapies for lysosomal storage disorders in which a supply of enzyme can be dispersed throughout the CNS. It is likely that the high level of expression combined with the long half-life of the enzyme (2–3 days) (41) helps disperse the enzyme throughout the CNS. The large size of the human brain compared to the mouse will require a widespread dispersal of the therapeutic enzyme. This treatment also reduced the elevated secondary lysosomal enzymes β-hexosaminidase and α-galactosidase (Fig. 1), indicating that these surrogate measures of disease correction have been significantly improved.

In addition to the biochemical and histological correction, we have also demonstrated the effectiveness of using this approach to reverse the behavioral abnormality in the MPS VII mouse (Fig. 4). The correction of neurological deficits is an important consideration in treating chronic neurodegenerative disorders, in which alleviation of the peripheral organ pathology and neurological dysfunction are both critical in improving the quality of life. A behavioral improvement in circadian rhythm was observed in 57% of the treated animals. The mammalian circadian rhythmicity is generated by neurons in the suprachiasmatic nucleus (SCN) of the hypothalamus, an area approximately 3 mm from the implantation site. The mechanism behind the circadian improvement presumably began with localized diffusion of β-glucuronidase through the extracellular matrix near the implantation site and widespread dispersal through the cerebrospinal fluid. This was possibly followed by mannose 6-phosphate receptor-mediated endocytosis and neuronal transport. Upon entry into cells, such as neurons in the SCN, the β-glucuronidase likely metabolized excess glycosaminoglycan storage, as indicated by the histological improvements (Fig. 2), leading to a restoration in cell function and behavioral change. Although 29% of the treated mutants did not show improved circadian rhythms, this was likely due to the deteriorating health of the mutant mice (Table 2, untreated mutants) in combination with the surgical trauma (Table 2, sham-treated mutant mice), which was not well tolerated in mutant mice with advanced disease. Without treatment they all showed a deterioration in circadian rhythms and/or activity levels (Table 2, Fig. 4).

The BBB has also been bypassed with injection of viral vectors directly into the CNS for treatment of neurodegeneration in the MPS VII mouse (29–32, 40,

42–46). The results of these viral-based experiments have recently improved with the introduction of enhanced vectors (32) and new strategies, such as transiently opening the BBB with hyperosmolarity during virus administration (26). However, cell encapsulation may offer several advantages as a viable alternative to viral-mediated gene therapy. The simplicity of this approach permits rapid manufacture of the encapsulated cells, proceeding from cultured cell to a finished injectable dose for implantation in 2 h. Thus, combined with the use of a single well-defined universal cell line, scale-up of this approach is technically feasible for manufacture of large quantities for both human application and thorough quality control monitoring for safety. This translates into a potentially cost-effective treatment for patients suffering from the same disease. The recent success of *in vivo* gene therapy with AAV vectors indicates the possibility of a long-term gene therapy with this approach (32, 42–45). However, scaling up of this very promising form of therapy for human application can be technically challenging and is a potential obstacle to clinical application (47). Finally, in this study the high level of gene product delivery from a 10-μl dose reached potentially excessive levels, successfully demonstrating the delivery potential of this approach, but also highlighting the need for reversible or regulated gene product delivery. Compared to viral vectors, the practically unlimited cloning capacity of the recombinant encapsulated cells may be advantageous for the incorporation of large regulatory elements necessary for precise regulation (48, 49). Moreover, the cell encapsulation approach is reversible, with removal of the encapsulated cells from the host, compared to the uncontrolled dissemination and irreversible infection from viral-mediated *in vivo* gene therapy. Given the similar level of efficacy achieved with this cell-based form of gene therapy compared to viral-mediated approaches, the potential for scale-up and increase in safety are important assets to consider for human application.

The use of unencapsulated genetically modified autologous or syngeneic cells implanted in the CNS of MPS VII mice has also been explored with very promising results (20, 28). The encapsulation of nonautologous cells prior to implantation in the CNS may confer some additional advantages for a safe and economical treatment. A single nonautologous cell line could defray the prohibitive costs associated with the labor-intensive and time-consuming construction of individual recombinant autologous cell lines. Encapsulated cell transplantation methods are not complicated by limited tissue availability nor the controversial ethical concerns associated with the use of human fetal material for fetal cell transplantation. Cell encapsulation can inhibit the formation of tumors from implanted immortalized cells (50–52), shield the encapsulated cells from the inflammatory process resulting from surgical trauma (53), and enhance the survival and long-term expression from encapsulated cells compared to unencapsulated nonautologous cells (2, 54).

Despite the demonstrated clinical efficacy at the somatic and CNS levels, several potential problems of this technology require resolution before human application. These include failure of the microcapsules due to breakdown of the membrane biomaterial, a gradual loss of cell viability (Table 1), loss of gene expression (Fig. 1), and escape of recombinant cells with tumorigenic potential. Conceptually, these problems could be addressed by repeat implantations of fresh microencapsulated cells, improved biomaterial chemistry, appropriate expression vector construction, and a judicious choice of nontransformed cell lines. Immune graft rejection of nonautologous cells even in the CNS is a potential safeguard against the escape of cells from broken microcapsules. Finally, cells optimally suited for encapsulation with the potential for terminal differentiation could be used (55). Cells such as myoblasts could be differentiated prior to implantation, thereby limiting cell overgrowth and avoiding a starved microcapsule environment with potentially diminished expression levels.

In conclusion, using an alternative and potentially cost-effective approach to somatic gene therapy with nonautologous encapsulated recombinant cells, we have shown its efficacy in treating a neurodegenerative form of lysosomal storage disease. The concomitant improvements in biochemical, histological, histochemical, and behavioral outcomes are supportive of further development of this technology toward human clinical application.

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3.7 Systemic delivery of β-glucuronidase to MPS VII mice from alginate microencapsulated cells.

3.7.1 Rationale

Previously, we found that intraperitoneal injection of 2A50 fibroblasts encapsulated in optimized alginate microcapsules (Chapter 3.1), could deliver β-glucuronidase to the peripheral tissues of adult MPS VII mice (Chapter 3.2). This newly supplied β-glucuronidase was able to reduce peripheral lysosomal storage disease pathology. However, the β-glucuronidase was unable to bypass the blood-brain barrier and clinical improvements were not observed in the CNS. To address this problem, microencapsulated cells were implanted directly into the CNS using small microcapsules (Chapter 3.4) optimized for CNS implantation (Chapter 3.3), resulting in significant β-glucuronidase delivery to the CNS and improved behavior in over 50% of the animals (Chapter 3.6).

In this study we investigate the combination of both peripheral (age: 4 weeks, 1 ml subcutaneous injection) and CNS implantations (age: 3-5 weeks, two 0.5 μl intraventricular injections) of immuno-isolated cells expressing high levels of β-glucuronidase as a proof-of-concept for the systemic treatment of a lysosomal storage disease using immuno-isolation gene therapy.

3.7.2 Contributions

This work was accomplished by Colin Ross with the support of Dr. Patricia L. Chang.

3.7.3 Abstract

Lysosomal enzyme deficiencies are potentially catastrophic illnesses with no generally available treatments. We use the β-glucuronidase deficient mouse model of mucopolysaccharidosis type VII (MPS VII) to develop an alternate approach to therapy. A "universal" cell line engineered to secrete the missing enzyme is implanted in recipients requiring the same enzyme replacement. The cells, though non-autologous, are rendered immunologically tolerant by encapsulation in alginate microcapsules that provide protection from immune mediators. Using this strategy, we have successfully delivered β-glucuronidase to the peripheral organs of MPS VII mice from intraperitoneally implanted microcapsules and we have delivered β-glucuronidase into the CNS from intraventricular implanted microcapsules. In this study, we investigate the combination of both peripheral and CNS-directed treatments. MPS VII mice were implanted with alginate encapsulated 2A50 fibroblasts secreting 403 ± 73 units of β-glucuronidase/hr/1 ml subcutaneous implant and 5 ± 3 units of β-glucuronidase/hr/1 µl intraventricular implant. After 1 week, β-glucuronidase activity was detected in the plasma at 9.1% of normal, reaching 14.7% by three weeks post-implantation. At four weeks post-implantation significant levels of β-glucuronidase were detected in the spleen (0.4%), kidney (0.6%), liver (2.4%), and muscle near the implantation site (61%), with concomitant reductions in secondarily elevated lysosomal enzymes and a normalization of the peripheral lysosomal storage pathology. In the CNS, β-glucuronidase spread throughout the brain with an average level of 17% of normal, reaching 89% near the implantation site. Varying reductions in secondarily elevated lysosomal enzymes reflected different levels of β-glucuronidase delivery to different regions of the brain. Lysosomal storage disease pathology was cleared in all regions of the brain, except the cerebellum, where low levels of storage remained. This novel cell-based immuno-isolation gene therapy demonstrates a potentially cost-effective and non-viral treatment alternative applicable to both peripheral and neurodegenerative lysosomal storage diseases.

3.7.4 Introduction

Mucopolysaccharidosis type VII (MPS VII; Sly syndrome) is caused by deficiency of the lysosomal hydrolase β-glucuronidase and is one of over 40 autosomal recessive lysosomal storage disorders resulting from an inherited deficiency of a lysosomal enzyme (Sly *et al.* 1973; Neufeld and Muenzer 1989). In MPS VII patients the incomplete break-down and progressive accumulation of glycosaminoglycans causes systemic cell and organ dysfunction, including hepatomegaly, splenomegaly, skeletal deformities, cataracts, and auditory, cognitive, and neurological deficits in the central nervous system (Birkenmeier *et al.* 1989; Vogler *et al.* 1990a; Chang *et al.* 1993a; Sands *et al.* 1995; Levy *et al.* 1996). The murine model of MPS VII has been well characterized and used to evaluate experimental strategies for lysosomal storage diseases, including bone marrow transplantation, enzyme replacement therapy, and gene therapy (Birkenmeier *et al.* 1989; Wolfe *et al.* 1992a; Wolfe *et al.* 1992b; Marechal *et al.* 1993; Sands and Birkenmeier 1993; Moullier *et al.* 1993a; Bastedo *et al.* 1994; Li and Davidson 1995; Lau *et al.* 1995; Sands *et al.* 1995; Wolfe *et al.* 1996; Naffakh *et al.* 1996; Snyder and Wolfe 1996; Ohashi *et al.* 1997; Taylor and Wolfe 1997a; O'Connor *et al.* 1998; Ghodsi *et al.* 1999; Stein *et al.* 1999; Vogler *et al.* 1999; Skorupa *et al.* 1999; Daly *et al.* 1999a; Daly *et al.* 1999b).

As an alternative to viral-mediated gene transfer in which direct genetic modification of the recipient is required, we have investigated immuno-isolation gene therapy for the delivery of a therapeutic recombinant gene product from an encapsulated universal cell line for the treatment of MPS VII. To prevent the eventual graft rejection, these recombinant cells are immunologically protected with alginate microcapsules. By enclosure within these immuno-isolation devices with controlled permeability, these cells can be protected from graft rejection. Influx of large immune mediators such as complement (>10^6 M.W.) or lymphocytes will be inhibited while smaller molecules including recombinant products with a molecular weight range from 22,000 to 300,000 (Peirone *et al.* 1998b), nutrients and metabolic waste can diffuse through the devices freely. Hence, a single, well-characterized "universal" cell line will serve the need for all patients requiring the same product replacement (Chang 1995).

MPS VII mice receiving immuno-isolation gene therapy through intraperitoneal injections of immuno-isolated non-autologous cells expressing high levels of murine β-glucuronidase attain high levels of β-glucuronidase in the plasma and peripheral organs with concomitant reductions in the disease pathology (Ross *et*

al. 2000a). The secreted β-glucuronidase is taken up into diseased tissues by mannose-6-phosphate receptor-mediated uptake (Taylor and Wolfe 1994). However, the β-glucuronidase was unable to bypass the blood-brain barrier and clinical improvements were not observed in the CNS. To address this problem we developed alginate microcapsules optimized for implantation into the rodent CNS (Ross *et al.* 1999) and with this strategy demonstrated therapeutic delivery of β-glucuronidase into the MPS VII mouse brain to reduce lysosomal storage neuropathology (Ross *et al.* 2000b).

In this study we investigate the combination of both peripheral and CNS implantation of immuno-isolated 2A50 fibroblasts expressing β-glucuronidase. This study is a proof-of-concept for the systemic treatment of a lysosomal storage disease using immuno-isolation gene therapy.

3.7.5 Methods

3.7.5.1 Mice

The genotypes of MPS VII mice were determined as described in Chapter 2, Methods.

3.7.5.2 Microcapsules

Mouse 2A-50 fibroblasts (Gift from W.S.Sly, Saint Louis University School of Medicine, St. Louis, MO) expressing high levels of mouse β-glucuronidase (Grubb *et al.* 1993) were encapsulated in small, 100-300 μm diameter, alginate-poly-L-lysine-alginate (APA) microcapsules for intraventricular implantation (Ross *et al.* 2000a) and medium 300-500 μm solid APA microcapsules for subcutaneous implantation. Control microcapsules were generated with 3521 β-glucuronidase deficient fibroblasts (Gift from M. Sands, Washington University School of Medicine, St Louis, MO) for implantation into control MPS VII mice. Cell encapsulation was carried out at 4-10°C under sterile conditions as described in the Chapter 2, Methods.

3.7.5.3 Microcapsule Implantation

Four-week old MPS VII mice were anesthetized with isofluorane and subcutaneously injected with 1.2×10^6 of β-glucuronidase secreting cells in 1 ml of microcapsules (5500 ± 500 capsules) through a 23-gauge needle into the scruff of the neck as described in the Chapter 2, Methods. Instead of intraperitoneal implantation, the feasibility of subcutaneous microcapsule implantation was investigated because in larger animal models this route

was found to be more effective (Stockley *et al.* 2000). Mice were intraventricularily implanted with 1 μl of microcapsules in two 0.5 μl injections of 26 ± 2 capsules containing 20,400 ± 2,100 cells one week before or after the subcutaneous implantation. Intraventricular implantation was performed as described in Chapter 2, Methods. Control MPS VII mice were similarly implanted with microcapsules containing 3521 β-glucuronidase deficient cells.

3.7.5.4 Weekly Monitoring

Treated mice were monitored with weekly 50-100 μl blood samples collected under anesthesia. Plasma samples were assayed for β-glucuronidase activity. Mice were sacrificed at 3 and 4-weeks post-implantation (2 and 5 weeks post-intraventricular implantation) to obtain blood and organ samples for enzyme assay and histological analyses.

3.7.5.5 Lysosomal Enzyme Assays

β-Glucuronidase, β-hexosaminidase, and α-galactosidase activities and protein levels were assayed as described in Chapter 2, Methods. Biological significance was established at the 95% confidence level. Data are presented as mean ± SEM.

3.7.5.6 Histology

Dissected tissues were fixed in glutaraldehyde, embedded in Spurr resin, cut into 0.5 μm sections, stained with toluidine blue, and examined and photographed with a Zeiss Axioskop microscope.

3.7.6 Results

3.7.6.1 Encapsulated Cells.

MPS VII mice were implanted at 4 weeks of age with APA encapsulated 2A50 fibroblasts secreting 324 ± 25 units of β-glucuronidase/million cells/hr. The subcutaneously implanted microcapsules produced 403 ± 73 units of β-glucuronidase/hr/ml implant volume. The microcapsules implanted into the CNS lateral ventricles produced 5 ± 3 units of β-glucuronidase/hr/μl implant volume.

3.7.6.2 Peripheral Implantation

One week post implantation, β-glucuronidase activity was detected in the plasma at 9.1% of normal, reaching 14.7% by three weeks post-implantation (Fig. 1). Within three weeks of implantation, low levels of β-glucuronidase activity were detected in the spleen (0.04%), kidney (0.1%), liver (0.4%), and muscle near the implantation site (2.4%) (Fig. 2). At four weeks post-implantation higher levels of β-glucuronidase were detected in the spleen (0.4%), kidney (0.6%), liver (2.4%), and muscle near the implantation site (61%). The secondary elevations of β-hexosaminidase and α-galactosidase were concomitantly reduced by as much as -85%, except in the muscle (Fig. 2).

In the treated mutants, a reduction of the lysosomal inclusions characteristic of the disease phenotype was observed in liver, kidney, and muscle by 3 weeks post-implantation, and the spleen was cleared of lysosomal storage pathology (Fig. 3). By 4 weeks post-implantation, the histology of the organs was almost indistinguishable from that of the normal, with disappearance of the lysosomal vacuoles from the Kupffer cells and hepatocytes in the liver, the parenchymal and sinusoidal cells in the spleen, and the parenchymal and glomerular cells of the kidney, and the muscle myofibers.

3.7.6.3 CNS Implantation

Within two weeks of microcapsule implantation into the lateral ventricles of the brain, β-glucuronidase had spread throughout much of the CNS with an average 6.5% of normal activity (range: 0.7 - 40%) (Fig. 4). Five weeks after implantation, the levels of β-glucuronidase had increased to an average 17.3% in the CNS (range: 0.5 - 89 %). The majority of the β-glucuronidase was detected within 2-3 mm of the implantation site, although low levels of enzyme were detected throughout in the CNS. Along with the delivery of β-glucuronidase, there was also a reduction of the secondarily elevated lysosomal enzymes (Fig. 4). In the sham-treated mutant mice, β-hexosaminidase and α-galactosidase enzyme levels were abnormally elevated 6.6- and 2.7-fold higher than normal as a compensatory mechanism for the increased glycosaminoglycans storage. In the treated mutant mice, both β-hexosaminidase and α-galactosidase were reduced in regions of high β-glucuronidase delivery (Fig. 4). By week 5, β-hexosaminidase was significantly reduced by an average -60% in the CNS, and reduced by more than -73% in a large (6 mm) region around the implantation site. Although α-galactosidase throughout the CNS was only slightly

reduced at week 2 (-4.5%), the higher levels of β-glucuronidase at week 5 correlated with a greater -36% α-galactosidase reduction.

A reduction of the characteristic MPS VII lysosomal inclusions was observed in the CNS after 2 weeks (Fig. 5). A complete resolution of lysosomal storage throughout most the CNS was observed after 5 weeks, including regions near the implantation site, the anterior cortex, and the surprachiasmatic nucleus (SCN) of the hypothalamus. Although some lysosomal storage was observed in the cerebellum, this was less severe than that of untreated mutant mice.

3.7.7 Discussion

This study demonstrates a proof-of-principle for the first systemic treatment of adult MPS VII mice by simultaneously delivering therapeutic recombinant gene products to the peripheral organs and the brain to address both the peripheral and neurological consequences of a lysosomal enzyme deficiency. MPS VII mice were implanted with 1 μl of immuno-isolated cells in the lateral ventricles of the CNS and 1 ml of immuno-isolated cells subcutaneously in the scruff of the neck. The cells produced and secreted high levels of β-glucuronidase into the β-glucuronidase deficient mice, resulting in significant levels of enzyme in the plasma, peripheral organs, and the CNS. Moreover, a partial restoration of secondary enzymes was observed in all of the organs, except the muscle, along with a near normalization of lysosomal storage pathology throughout the animal, including the CNS. The muscle appeared refractory to reductions in secondary enzymes, even though the muscle had high levels of β-glucuronidase activity and became clear of lysosomal storage pathology. In muscle, the individual levels of β-hexosaminidase and α-galactosidase are an average 15-fold lower than in other tissues and small variations between animals make comparisons less informative.

The results of this study confirm previous reports where a relatively small number of cells can produce enzyme that reaches a large proportion of the CNS or peripheral organs and that low levels of β-glucuronidase are sufficient for restoration of a normal histological phenotype (Ghodsi *et al.* 1998; Skorupa *et al.* 1999; Ross *et al.* 2000b). The long half-life of β-glucuronidase (2-3 days) (Smith and Ganschow 1978; Vogler *et al.* 1993) combined with the ability of cells to secrete, take-up, and re-use enzyme (Taylor and Wolfe 1994) has favorable implications

for gene product replacement therapies of lysosomal storage disorders where a supply of enzyme can be dispersed throughout the animal. Nonetheless, the large size of the human brain compared to the mouse will require a significantly larger dispersal than mice. In larger animal models a widespread distribution of enzyme might be achieved with additional sites of microcapsule administration throughout the CNS.

This study demonstrates the first successful treatment of both the peripheral and neurological effects of non-neonatal treated MPS VII mice. Previously, the systemic disease of murine MPS VII was dramatically improved with neonatal administration of an adeno-associated viral (AAV) vector carrying the β-glucuronidase gene (Daly *et al.* 1999b). However, neonatal administration of this vector does not reflect the real-world barriers to treating a human lysosomal storage disease. This neonatal treatment strategy was based upon the non-functional blood-brain barrier (BBB) and the immature immune system of neonatal mice (Stewart and Hayakawa 1987; Vogler *et al.* 1999). In contrast, humans are born with a functional BBB and immune system (Saunders *et al.* 1999; Saunders *et al.* 2000; Dziegielewska *et al.* 2000). Consequently, AAV-mediated correction of neonatal MPS VII mice does not address the primary obstacles of treating MPS VII in humans: the BBB and the potential immune response against β-glucuronidase. In humans, *in utero* administration of AAV would be required to mimic the neonatal AAV-mediated treatment strategy. Currently, however, many lysosomal storage disease patients are not diagnosed until the disease is at an advanced stage and beyond the therapeutic window of *in utero* administration (Snyder and Wolfe 1996; Ghodsi *et al.* 1999). In this study, the treatment of young 3-4 week old MPS VII mice more accurately models the human disease and the obstacles that must be overcome for a successful treatment.

Immuno-isolation gene therapy offers several advantages as a viable alternative to viral-mediated gene therapy. The simplicity of this approach permits rapid manufacture of the encapsulated cells, and combined with the use of a single well defined universal cell line, potentially cost-effective scale-up of this approach is technically feasible. In contrast, scaling up viral vector production, such as AAV, can be technically challenging and is a potential obstacle to clinical application (Linden and Woo 1999). The recent discovery that neonatal administration of recombinant AAV to MPS VII mice resulted in liver tumours in 60% of mice after 1.5 years highlights the serious drawbacks of the uncontrolled dissemination and irreversible infection from integrating viral vectors (Sands *et al.* 2001). Compared to viral vectors, the practically unlimited cloning capacity of the recombinant

encapsulated cells may be advantageous for the incorporation of large regulatory elements necessary for precise regulation (Hagihara *et al.* 1999; Rivera *et al.* 2000).

In spite of the demonstrated clinical efficacy, several potential problems of this technology require resolution before human application. These include failure of the microcapsules due to breakdown of the membrane biomaterial (Peirone *et al.* 1998a) and escape of recombinant cells with tumorigenic potential. These problems are currently being investigated with improved biomaterial chemistries (Van Raamsonk 1999; Van Raamsonk *et al.* 2001), regulatable vector constructs, and a judicious choice of non-transformed cell lines with the potential for terminal differentiation that are suited for encapsulation (Chang 1995; Deglon *et al.* 1996; Chang and Bowie 1998; Hortelano *et al.* 2001). Cells such as myoblasts could be differentiated prior to implantation, thereby limiting cell overgrowth, and avoiding a starved microcapsule environment with potentially diminished expression levels.

In summary, the findings of the present study demonstrate a proof-of-principle for the systemic treatment of a lysosomal storage disease using immuno-isolation gene therapy. The combination of both peripheral and CNS implantation of immuno-isolated cells expressing β-glucuronidase resulted in widespread β-glucuronidase delivery and systemic clinical improvements.

Figure 3.7.1 Therapeutic levels of β-glucuronidase in the plasma of treated MPS VII mice.

One week after subcutaneous implantation of immuno-isolated cells expressing β-glucuronidase, MPS VII mice showed significant levels of plasma β-glucuronidase activity (9.1 ± 7.0% of normal), reaching 14.7 ± 3.4% by three weeks post-implantation. The background level for β-glucuronidase detection in plasma is 1.3% of normal.

Figure 1.

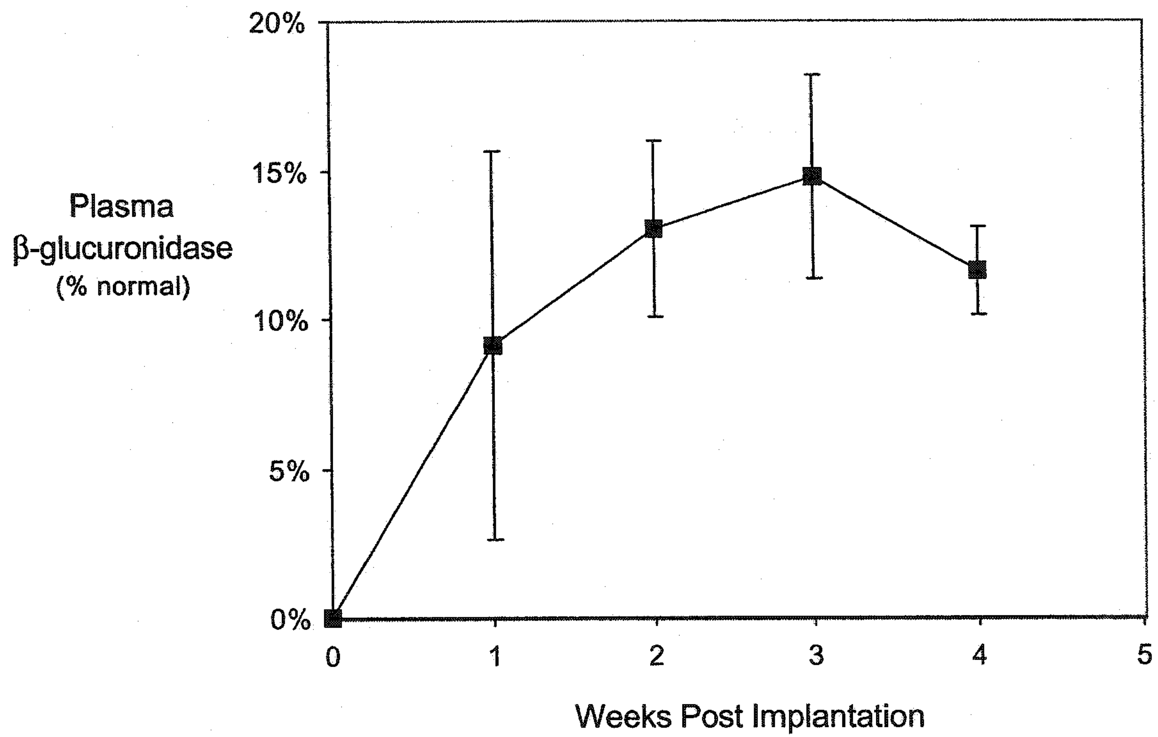


Figure 3.7.2 Partial correction of enzymatic defects in the peripheral organs of treated MPS VII mice.

At various times post-implantation, the lysosomal enzyme activities of organs were compared between treated MPS VII mice (N = 1 per time point), untreated normal mice (N = 3) and sham-treated MPS VII mice (N = 1). In untreated MPS VII mutant mice, β-glucuronidase activity is virtually undetectable in the spleen (0.002 ± 0.000% normal), kidney (0.001 ± 0.020%), liver (0.010 ± 0.003%), and muscle (0.1 ± 0.1%). In the treated MPS VII mice at three weeks post-implantation, low but significant levels of β-glucuronidase were detected in the spleen (0.040 ± 0.004% normal), kidney (0.10 ± 0.02%), liver (0.35 ± 0.3%), and muscle near the implantation site (2.4 ± 0.2%) ($P < 0.05$). At four weeks post-implantation higher levels of β-glucuronidase were detected in the spleen (0.41 ± 0.01% normal), kidney (0.58 ± 0.02%), liver (2.35 ± 0.08%), and muscle near the implantation site (61 ± 2%) ($P < 0.01$).

Figure 2.

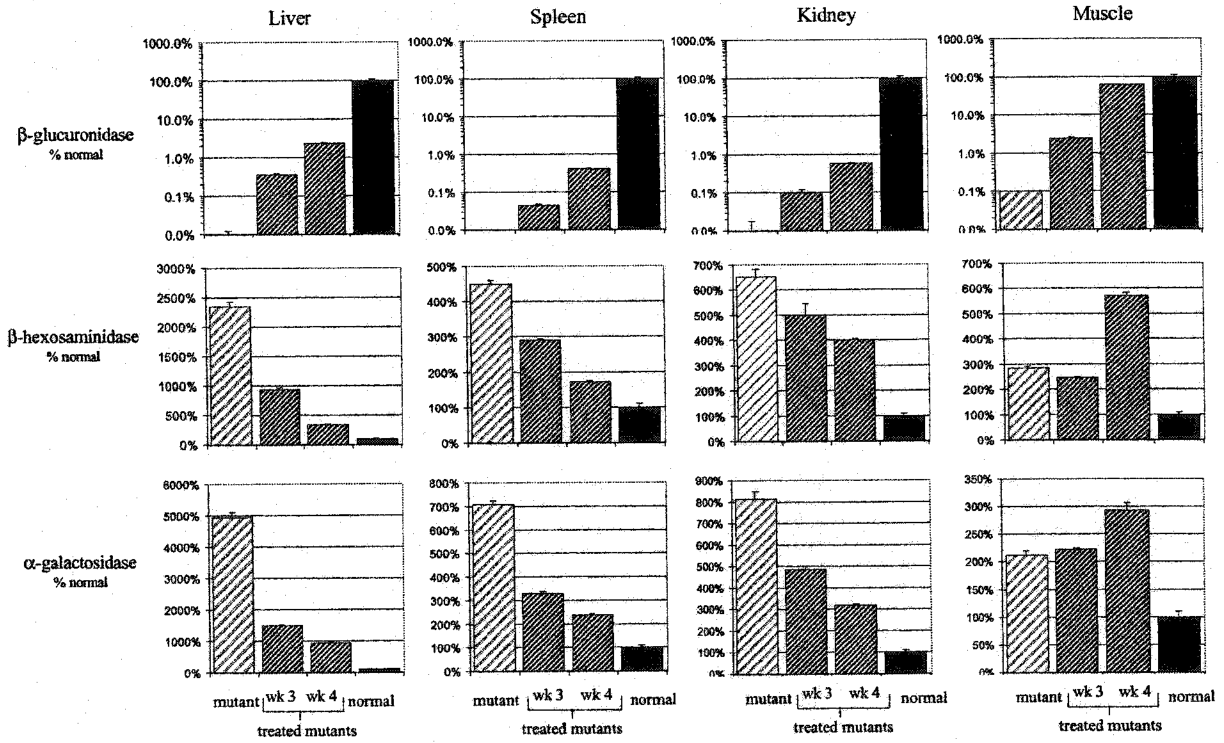


Figure 3.7.3 Correction of peripheral organ lysosomal storage in treated MPS VII mice.

Toluidine blue thin sections were prepared from tissue samples to show the lysosomal storage accumulation. Lysosomal storage pathology was evident in sections of untreated MPS VII mouse liver (A), spleen (E), kidney (I), and muscle (M) compared to normal controls (D, H, L, P). Three weeks after treatment, a reduction in lysosomal inclusions was observed in liver (B), kidney (J), and muscle (N), and a complete resolution of lysosomal storage was observed in the spleen (F). By 4 weeks, the histology of the treated mice was almost indistinguishable from that of the normal, with disappearance of the lysosomal vacuoles from the Kupffer cells and hepatocytes in the liver (C), the parenchymal and sinusoidal cells in the spleen (G), and the parenchymal and glomerular cells of the kidney (K), and throughout the muscle myofibers (O). Original magnifications 200X (A-L) and 1000X (M-P).

Figure 3.

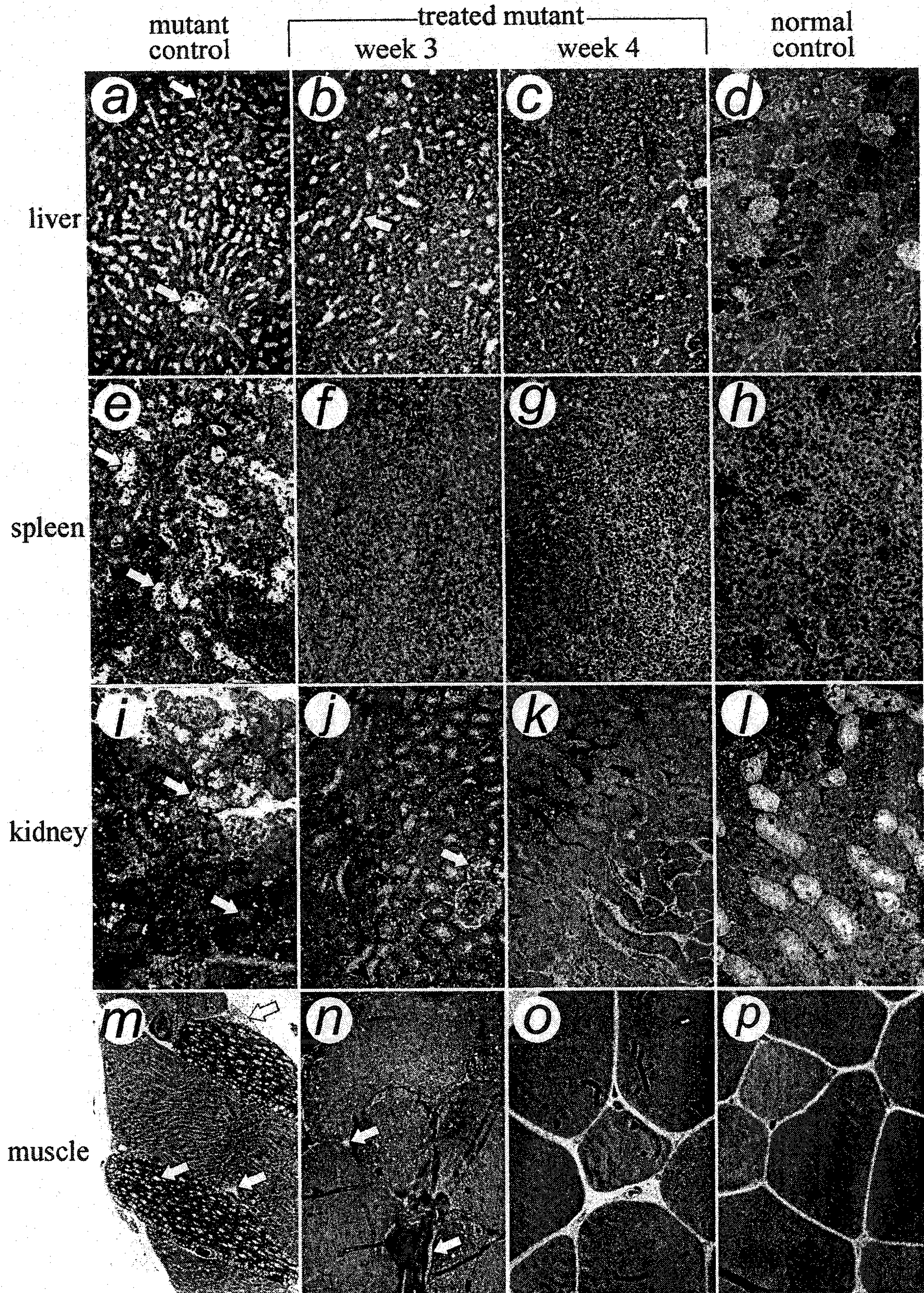
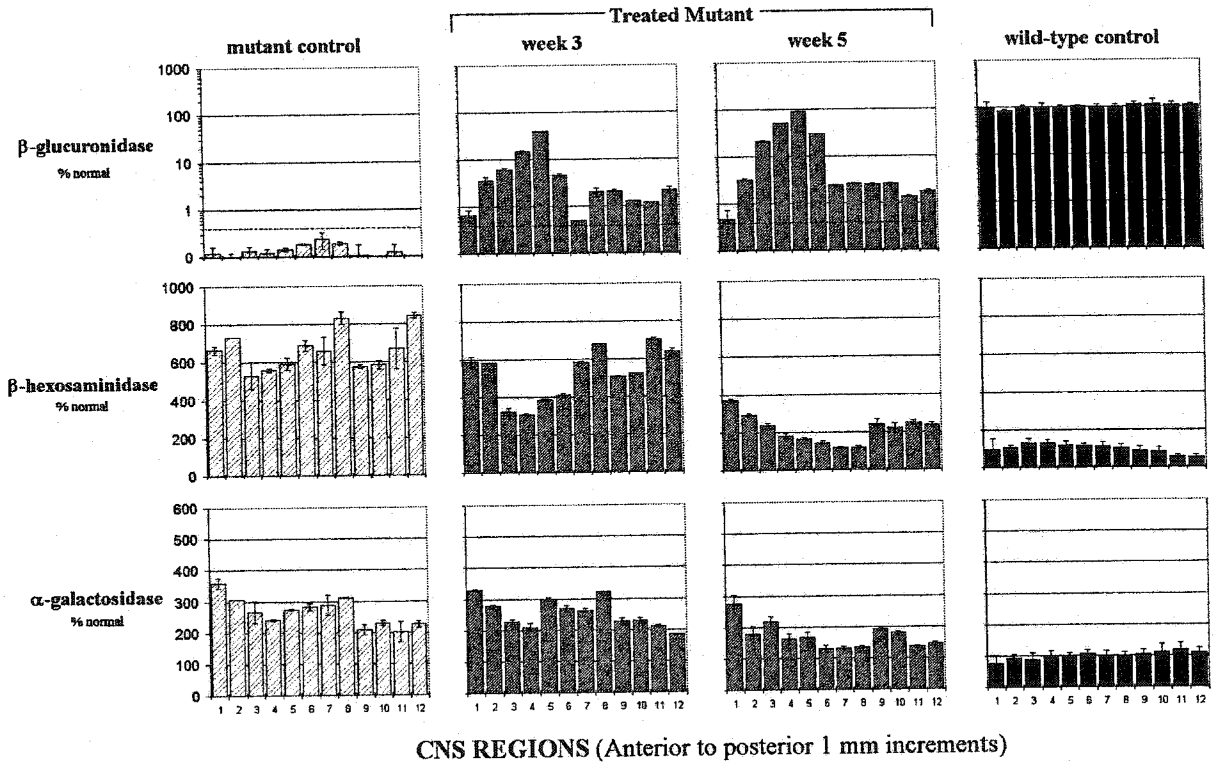


Figure 3.7.4 Partial correction of enzymatic defects in the brains of treated MPS VII mice.

β-glucuronidase, β-hexosaminidase, and α-galactosidase enzyme levels in the brains of treated MPS VII mutant mice at week 2 (N=1) and 5 (N=1) after intraventricular implantation of immuno-isolated cells were compared to normal (N=6) and sham-treated mutant (N=1). All brains were sectioned into 12 regions from anterior to posterior in 1 mm increments for each hemisphere of the brain (region 5-6 corresponding to the implantation site) and assayed for lysosomal enzyme activities and protein concentration. β-glucuronidase was detected throughout much of the CNS in treated MPS VII mice with the majority of the enzyme localized to within 3 mm of the implantation site. Secondary elevated lysosomal enzymes β-hexosaminidase and α-galactosidase were reduced, especially in regions with high levels of β-glucuronidase delivery. All data are means ± S.D.

Figure 4.



CNS REGIONS (Anterior to posterior 1 mm increments)

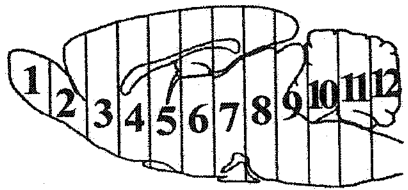
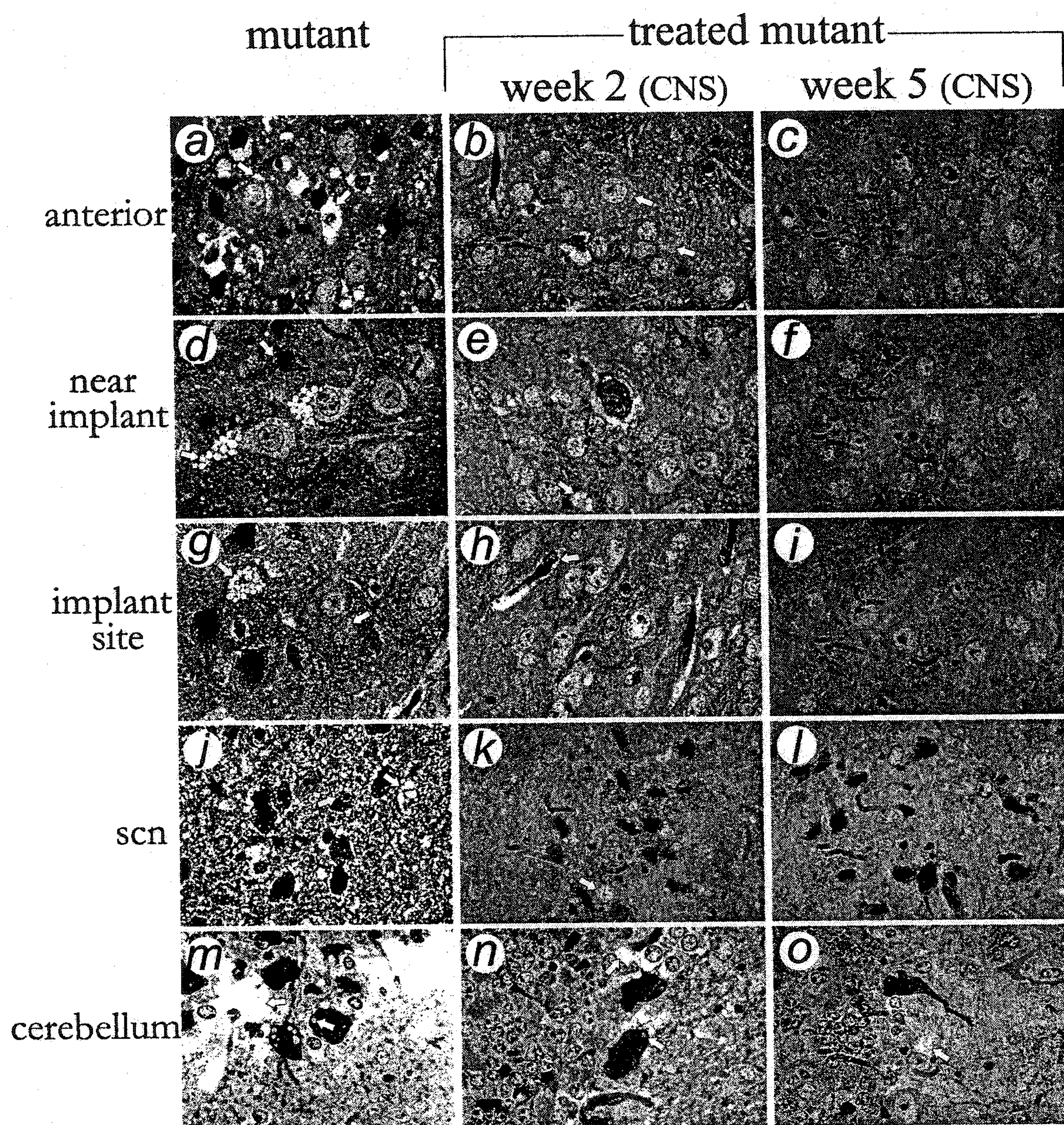


Figure 3.7.5 Decreased lysosomal storage in the CNS of treated MPS VII mutants.

Toluidine blue stained sections from the CNS of a sham-treated control mutant MPS VII mouse showed extensive lysosomal storage disease pathology with distended lysosomes (white arrows) in the anterior cortex (A), near the implantation site (D), within the implantation site (G), SCN (J), and the cerebellum (M). Storage material was found throughout the brain in many cell types, including neurons, glial cells, and perivascular storage. Treated mutant MPS VII mice show reduced lysosomal storage two weeks after CNS-implantation. After 5 weeks, treated MPS VII mice showed a dramatic loss of lysosomal storage throughout the entire CNS, except in the cerebellum, where low levels of lysosomal storage remained, albeit at significantly reduced levels compared to the sham-treated MPS VII control. Original magnification 1000x.

Figure 5.



4.0 DISCUSSION

The aim of this research was to develop a gene therapy for the treatment of a lysosomal storage disease. The over 50 recessive lysosomal storage diseases carry a significant burden of disease, affecting over 1 in 7700 people, and there are no clinically accepted treatments for all but the mildest form of one lysosomal storage disease (Gaucher's disease, type I) (Meikle *et al.* 1999). The lysosomal storage diseases are particularly amenable to gene therapy because low levels of resupplied enzyme result in significant therapeutic improvements (Chabas *et al.* 1991), lysosomal enzymes are generally very stable (Smith and Ganschow 1978), and most enzymes can be secreted and taken up by different cells via the mannose-6-phosphate receptor (Brooks *et al.* 1991; Taylor and Wolfe 1994; Thomas 1994). Many animal models of lysosomal storage diseases exist, including the well-characterized MPS VII mouse. The MPS VII mouse has been widely used as a model to develop novel therapies for lysosomal storage diseases, including bone marrow transplantation, enzyme replacement therapy, and gene therapy.

We hypothesized that immuno-isolation gene therapy could continuously re-supply β-glucuronidase from microencapsulated recombinant cells in order to overcome the genetic deficiency of MPS VII mice. To prevent the eventual graft rejection, these recombinant cells were immunologically protected with alginate microcapsules. These microcapsules inhibit the entry of large immune mediators such as complement or lymphocytes, while allowing nutrients and metabolic waste to freely diffuse through the devices. Hence, a single, well-characterized "universal" cell line could serve the need for all patients requiring the same gene product replacement (Chang 1995).

4.1 Peripheral Treatment of MPS VII

An optimized microcapsule formulation was determined for the encapsulation of a recombinant mannose-6-phosphate receptor deficient 2A50 fibroblast cell line that expressed and secreted extremely high levels of β-glucuronidase (Chapter 3.1). It was important to determine which microcapsule formulation should be used in

future experiments for the 2A-50 cell line. Hollow calcium APA, solid barium APA, and solid barium alginate microcapsules were compared. The results of this study showed that human growth hormone (MW=22,000), human factor IX (MW=57,000), and murine β-glucuronidase (MW=300,000) were able to traverse the three microcapsule formulations at similar rates. However, the hollow APA microcapsule formulation appeared to be the most suitable formulation for the *in vitro* proliferation of 2A50 cells. Although the hollow APA microcapsules were weaker than solid microcapsules, this was not a critical factor for intraperitoneal implantation in mice.

Based on these results, mutant MPS VII mice were treated intraperitoneally with 3 ml of hollow APA microencapsulated cells secreting high levels of β-glucuronidase (Chapter 3.2). Significant β-glucuronidase activity was detected in plasma and peripheral organs, including liver and spleen, along with significant reductions in secondary lysosomal enzymes and lysosomal storage histopathology for the duration of the 8-week experiment. However, the results of this experiment revealed three significant obstacles to this treatment. First, all of the mice developed a strong humoral immune response with anti-β-glucuronidase antibodies directed against the re-supplied mouse β-glucuronidase limiting the effectiveness of the treatment. Second, this treatment did not address the early onset effects of the disease. The MPS VII mice were already severely affected at the time of treatment at 6-8 weeks of age. Third, the β-glucuronidase was unable to cross the blood-brain barrier (BBB) into the CNS, thereby limiting the effectiveness of the treatment because MPS VII is a progressive neurodegenerative disease.

4.2 Transgene Immune Response

The formation of antibodies against the murine β-glucuronidase demonstrated the potential response in patients with a null mutation who similarly react against the replaced enzyme as a foreign antigen. In humans, purified infusions of therapeutic enzyme result in the development of antibodies in 3% of severe hemophilia B patients, 25-50% of severe hemophilia A patients, 40% of MPS I patients, and 13-40% of Gaucher patients (Grabowski *et al.* 1995; Whittington and Goa 1995; Rosenberg *et al.* 1999; Lusher 2000; Kakkis *et al.* 2001a). Antibodies can lead to significant problems such as hypersensitivity and anaphylactic reactions, enzyme inactivation, and enzyme degradation.

To address the problem of anti-β-glucuronidase antibodies, MPS VII mice were administered a single immunosuppressive treatment of purified anti-CD4 monoclonal antibody at the time of microcapsule implantation (Chapter 3.2). This resulted in increased levels and duration of β-glucuronidase delivery. As a control, treated heterozygous mice maintained elevated levels of β-glucuronidase throughout the experiment and did not develop antibodies. Although antibodies did eventually develop in immunosuppressed MPS VII mice, this was not unexpected because the mice had received only a single round of transient immunosuppression.

After the development of antibodies in the treated MPS VII mice, the decrease in β-glucuronidase levels and localization of the β-glucuronidase in the liver to the Kuppfer cells and not the hepatocytes suggested a decrease in receptor-mediated endocytosis, substituted instead with an immune-mediated clearance of the enzyme from circulation. In spite of the development of antibodies, a dramatic reduction of storage lesions was observed for at least 8 weeks. This may be due to small amounts of undetected β-glucuronidase that remain in the hepatocytes. With the long half-life of β-glucuronidase in the liver (2-3 days) (Smith and Ganschow 1978; Vogler *et al.* 1993) the expected level of enzyme would decrease slowly but could remain high enough to maintain a normal phenotype without any additional supply of enzyme. It is also possible that intracellular GAG exchange (Yanagishita 1992) between the hepatocytes and the corrected Kupffer cells resulted in some clearance of storage material in the hepatocytes. Finally, the re-accumulation of lysosomal storage is a gradual process that would not begin until after the supply of β-glucuronidase was fully exhausted. It was previously demonstrated in enzyme replacement studies that lysosomal storage did not re-accumulate in the liver and spleen for 4 to 12 weeks after the last enzyme injection (Vogler *et al.* 1996).

Recently it was found that affected MPS VII mice exhibit an altered immune response (Daly *et al.* 2000). The abnormal lysosomal function in affected MPS VII mice produces a defect in immune system antigen processing. This has become apparent with the surprisingly limited generation of antibodies against human β-glucuronidase in enzyme replacement and early gene therapy studies (Daly *et al.* 2000). Similarly, Wolfe *et al.* reported an increase in the toxicity of both wild-type and recombinant herpes vectors after administration to MPS VII mice (Wolfe *et al.* 1996). In addition, generation of B-cell hybridomas from untreated MPS VII mice is very difficult; while, pretreatment with human β-glucuronidase before immunization greatly enhances the number of hybridomas

generated (W. S. Sly, St. Louis University School of Medicine, 1998, personal communication in Daly et al., 2000). Thus, MPS VII mice may be unable to properly mount an immune response in a diseased state. Consequently, a correction of the MPS VII disease phenotype with the delivery of β-glucuronidase would likely correct the immunological deficits as well. Thus, a successful treatment of MPS VII may be self-limiting if the function of the immune system is restored and eventually develops anti-β-glucuronidase antibodies. The lack of an immune response in the early MPS VII mouse treatments may actually be a characteristic of the inability of these treatments to restore normal immune function.

4.2.1 Strategies to Inhibit the Transgene Immune Response

The immune response directed against a foreign therapeutic transgene poses a significant problem to the long-term efficacy for all gene therapies using a foreign transgene. Several immunosuppressive agents have been used to overcome the transgene immune response in response to gene therapy, such as FK506, cyclophosphamide, anti-CD40, and anti-CD4 antibodies (Potter *et al.* 1998; Stein *et al.* 1999). Hence, the use of anti-CD4 antibody or similar immunosuppressive strategies to prevent early development of the antibodies should be an effective adjunct strategy to resolve this immunological issue. However, an alternative to long-term immunosuppression would be valuable. Possible alternatives include intra-thymic implantation (Garrovillo *et al.* 1999; Sprent and Kishimoto 2001), oral tolerance with improved high dose or IL-10 protocols (Benson *et al.* 2000; Slavin *et al.* 2001), transient immunosuppression (Peduto *et al.* 2000; Rinsch *et al.* 2001), novel tolerance regimes using transient immunosuppression with gradual dose reduction (Nemes and Pitlik 2000), or low level immunosuppression produced directly from the implanted or encapsulated cells (Carmen *et al.* 1991; Zhan *et al.* 2000; Sutherland *et al.* 2000). Interestingly, recent studies in adult immunocompetent mice receiving human factor VIII and in humans receiving enzyme replacement therapy for Gaucher's disease suggest that long-term high levels of the foreign gene product may eventually induce tolerance, even after the development of high antibody titres (Rosenberg *et al.* 1999; Chao and Walsh 2001).

4.3 Early Onset of MPS VII

To address the early onset of MPS VII, mutant mice were implanted within 0 to 5 days of birth with 25 μl of hollow APA microcapsules (Chapter 3.3). This study demonstrated the first application of immuno-isolation gene

therapy in neonatal mice. Low but significant levels of β-glucuronidase were detected in the plasma and organs, resulting in a significant delay in the onset of lysosomal storage pathology compared to untreated controls. However, by week 4, anti-β-glucuronidase antibodies were detected in some mice, eventually inhibiting the effectiveness of the therapy in 100% of the animals. Nonetheless, these results demonstrate that in conjunction with an effective means of controlling the immune response, treatment can be initiated at the earliest time possible after diagnosis in order to address the early onset of lysosomal storage diseases.

The neonatal administration of microcapsules did not result in β-glucuronidase tolerance, as generally observed following AAV-mediated neonatal gene transfer, even though both treatments resulted in significant β-glucuronidase delivery and reversal of the disease pathology. AAV-mediated delivery of β-glucuronidase produced antibodies in 0% and 14% of mice following intramuscular and intravenous injections, respectively. Several possibilities could explain this discrepancy.

Adjuvant contaminants in the implanted microcapsules could have promoted the development of antibodies. Alginate probably did not lead to the formation of antibodies because it has been reported that it does not act as an adjuvant (Kulseng *et al.* 1999). In a recent phase I/II clinical trial using encapsulated cells in humans, antibodies directed against components of the fetal bovine serum (FBS) used to culture the encapsulated cells were found in the cerebrospinal fluid of treated patients (Zurn *et al.* 2000). Thus, FBS could act as an adjuvant. However, in the research described in this thesis, the encapsulated cells were incubated in FBS-free medium prior to implantation (except in Chapter 3.2). Nonetheless, this demonstrates the potential for carryover of substances adsorbed to the surface of the cells or the microcapsule membranes.

The levels of β-glucuronidase delivery to the mice did not appear to influence the development of antibodies. The AAV-mediated approach produced a wide range of β-glucuronidase levels in the blood after one week, from 0.5% of normal via intramuscular injection to 2000% of normal from intravenous injection (Daly *et al.* 1999a; Daly *et al.* 1999b), while microcapsule-treated neonatal MPS VII mice showed an intermediate level of 3.6%. In contrast, the number of sites within the animals producing enzyme was significantly different between AAV-treated and microcapsule-treated mice. Microcapsule-treated mice produced β-glucuronidase from a single subcutaneous injection site, producing antibodies in all of the treated mice. In contrast, AAV-intramuscular

injected mice produced β-glucuronidase from 4 injection sites and the vector was also disseminated to the liver, and likely other organs as well, resulting in antibodies in just one of seven (14%) mice. In addition, AAV-intravenously injected mice which did not produce any antibodies produced β-glucuronidase throughout the animal, including liver, spleen, heart, lung, kidney, and brain. Thus, it is possible that the widespread dissemination of the AAV vector, possibly to the thymus, for example, could have been responsible for the tolerance to β-glucuronidase in these mice. Alternatively, the slow onset of AAV-derived β-glucuronidase expression, generally increasing slowly over 2-8 weeks, may have promoted β-glucuronidase tolerance as well (Danos 2001).

4.4 Treatment of MPS VII Neurodegeneration

To address the third problem of gene product delivery to the CNS, the direct intraventricular implantation of microcapsules into the CNS was investigated. Alginate microcapsules had not previously been implanted into the CNS, and the current microcapsules were too large for implantation into the mouse CNS; therefore, a smaller alginate microcapsule formulation was developed and tested *in vitro* (Chapter 3.4). Unexpectedly, the smaller microcapsules were significantly stronger and able to sustain a higher number of cells per volume of microcapsules compared to large microcapsules. In addition, the small microcapsules secreted up to 10-fold higher levels of β-glucuronidase per volume of microcapsules compared to larger microcapsules. A common marker gene, hGH, was used to develop a protocol for the intraventricular implantation of microcapsules into the murine CNS under stereotaxic guidance, resulting in detectable hGH expression for at least 16 weeks (Chapter 3.5).

After 10 μl of small alginate microcapsules producing β-glucuronidase were implanted into the CNS of MPS VII mice, significant levels of β-glucuronidase were detected throughout the brain. Within three weeks of implantation, β-glucuronidase had spread throughout much of the CNS with an average 33-fold increase over wild-type levels (range 4 -7320% of normal). By weeks 7 and 8 post-implantation, β-glucuronidase decreased to an average 40% of normal levels with higher levels localized within 5 mm of the implantation sites (24-410%), and lower levels (1-2%) throughout the rest of the CNS. The reconstituted enzyme produced significant reductions of secondary lysosomal enzymes and lysosomal storage pathology, and behavioral abnormalities in the treated mutant mice were significantly improved compared to sham-treated and untreated animals (Chapter 3.6). These results

demonstrated the first delivery of alginate microcapsules in the CNS for the treatment of a disease, and the first application of immuno-isolation gene therapy to treat the neurodegenerative manifestations of a lysosomal storage disease.

These results established the potent capability of immuno-isolation gene therapy to deliver extremely high levels of recombinant products to the CNS. The results of this research confirmed similar reports where a relatively small number of cells could produce β-glucuronidase that reached a large proportion of the CNS and that low levels of β-glucuronidase were sufficient for restoration of a normal histological phenotype (Ghodsi *et al.* 1998; Skorupa *et al.* 1999). The long half-life of β-glucuronidase in tissues (2-3 days) (Smith and Ganschow 1978; Vogler *et al.* 1993) combined with the ability of cells to secrete, take-up, and re-use enzyme (Taylor and Wolfe 1994) has favorable implications for gene product replacement therapies of lysosomal storage disorders where a supply of enzyme can be dispersed throughout the CNS. Nonetheless, the large size of the human brain compared to the mouse will require a significantly larger dispersal of the therapeutic enzyme.

4.5 Systemic Treatment of MPS VII

For a clinically relevant systemic treatment for a lysosomal storage disease in adult mice, the combination of both peripheral and CNS implantations of immuno-isolated cells expressing β-glucuronidase was investigated (Chapter 3.7). MPS VII mice were treated subcutaneously with 1 ml of solid APA microcapsules and intraventricularly with 1 μl of small hollow APA microcapsules. The immune response to β-glucuronidase was not addressed in this short-term study. The implanted cells produced and secreted high levels of β-glucuronidase in the treated MPS VII mice, resulting in significant levels of enzyme in the plasma, peripheral organs, and the CNS. The treated animals showed a reduction in both the peripheral and CNS manifestations of the disease, an achievement that has not previously been accomplished with any other treatment of adult MPS VII mice.

A range of therapeutic strategies have been used to separately address either the peripheral or CNS pathology of MPS VII (Table 4.6.1). However, there have been no reports of a treatment for both the peripheral and neurological effects of the adult MPS VII mouse. Although BMT in adult mice improved the lysosomal storage pathology in the peripheral organs, improvements in the CNS were limited to the meninges and perivascular cells. The observed low levels of enzyme in the brain (1.6 to 6% of normal) may have resulted from

Table 4.5.1 Experimental treatments for adult MPS VII mice.

		Enzyme Activity (%)				Improved Pathology		
Therapy	*Site/Notes*	*Liver*	*Spleen*	*CNS*	*Duration*	*Periph**	*CNS**	*References*
Peripheral								
BMT *(0.5-10 Gy)*	IV	14	70	6.0	55 wk	+++	-	Birkenmeier, 1991
Rv-BMT *(2 Gy)*	IV	1	2	0	16 wk	+++	-	Wolfe *et al.*, 1992
Rv-Fibroblast	IP	2	0.3	0.1	8 wk	+++	-	Moulier, 1993
BMT *(8.5 Gy)*	IV	10	99	6.0	28 wk	+++	-	Porthuis, 1994
Rv	Intestine	nd	nd	nd	0.6 wk	nd	nd	Lau *et al.*, 1995
Rv-Myoblast	IM	1	0.3	nd	4 wk	+++	-	Naffakh, 1996
AAV	IV or IM	low	low	0	16 wk	+	-	Watson *et al.*, 1998
Adeno	IV	1	1	0	4 wk	+++	-	Ohasi *et al.*, 1997
Adeno	IV	85	nd	nd	16 wk	+++	-	Stein *et al.*, 1999
Rv-Macrophages	IV	3	0	0	4 wk	+++	nd	Freeman, 1999
ER	IV	25	5	0	6 wk	+++	-	Vogler *et al.* 1999
Rv	IV	1	0.4	nd	14 wk	+	nd	Gao *et al.*, 2000
Lenti	IV	38	5.7	nd	3 wk	+	nd	Stein et al., 2001
Microcapsules	**IP**	**12**	**19**	**0**	**2 wk**	**+**	**-**	**Ross *et al.*, 2000a**
Microcapsules	**IP**	**43**	**60**	**0**	**4 wk**	**+++**	**-**	**Ross *et al.*, 2000a**
Microcapsules	**IP**	**0.7**	**0.5**	**0**	**8 wk**	**+++**	**-**	**Ross *et al.*, 2000a**
CNS-Directed								
Herpes	CNS	nd	nd	v. low	16 wk	nd	-	Wolfe *et al.*, 1992
CNS-Progenitors	CNS	nd	nd	9	24 wk	nd	+	Snyder *et al.*, 1995
Rv-Fibroblast	CNS	nd	nd	1	8 wk	nd	+	Taylor, 1997
Adeno	CNS	nd	nd	v. low	2 wk	nd	-	Ohashi *et al.*, 1997
Adeno	CNS	nd	nd	high	12 wk	nd	+	Ghodsi *et al.*, 1998
Adeno	CNS	nd	nd	high	3 wk	nd	+++	Ghodsi *et al.*, 1999
Adeno	CNS	nd	nd	high	16 wk	-	+++	Stein *et al.*, 1999
AAV	CNS	nd	nd	low	12 wk	nd	+	Elliger *et al.*, 1999
AAV	CNS	nd	nd	12	20 wk	nd	+++	Skorupa, 1999
Microcapsules	**CNS**	**nd**	**nd**	**3050**	**3 wk**	**nd**	**+++**	**Ross *et al*, 2000b**
Microcapsules	**CNS**	**nd**	**nd**	**3720**	**5 wk**	**nd**	**+++**	**Ross *et al*, 2000b**
Microcapsules	**CNS**	**nd**	**nd**	**40**	**7 wk**	**nd**	**+++**	**Ross *et al*, 2000b**
Microcapsules	**CNS**	**nd**	**nd**	**45**	**8 wk**	**nd**	**+++**	**Ross *et al*, 2000b**
Combined Peripheral + CNS								
Microcapsules	**SC/CNS**	**2.4**	**0.4**	**17**	**4-5 wk**	**+++**	**+++**	**Ross, 2001**

Legend: Abbreviations: BMT: Bone Marrow Transplant; Rv: Retroviral vector; Adeno: ΔE1/E3 adenoviral vector; AAV: adeno-associated viral vector; ER: enzyme replacement; IV: Intravenous; IM: Intramuscular; IP: Intraperitoneal; SC: subcutaneous; "Periph": peripheral organs including kidney, liver, spleen, and muscle. Enzyme activity as a % of normal or v. low (very low), low, and high as described by authors. Histopathology was unimproved (-), partially improved (+), or improved (+++).

contaminating enzyme in circulation, a disrupted BBB, or radiation-induced toxicity of the myeloablation prior to BMT. The BBB may be weakened in MPS VII mice due to the severe perivascular lysosomal storage and disruption of vessel architecture in the CNS. However, this would be self-limiting for brain recovery because once the lysosomal storage was reduced in the BBB and normal function regained, the brain would again be starved of the therapeutic enzyme. In addition, BMT radiation-induced toxicity severely impaired behavior, and the behavioral deficits of the MPS VII mice were not corrected (Sands *et al.* 1993; Bastedo *et al.* 1994; Poorthuis *et al.* 1994). Thus, immuno-isolation gene therapy demonstrates the first clinically promising treatment for both the peripheral and neurological deficits of adult MPS VII mice.

The systemic disease of MPS VII has been improved following BMT, enzyme replacement, or AAV vector-administration in neonatal mice (Sands *et al.* 1993; Daly *et al.* 1999b). Although these results were interesting, they were not of clinical significance because in humans the BBB and the immune system are functional prior to birth (Saunders *et al.* 1999; Saunders *et al.* 2000; Dziegielewska *et al.* 2000). These treatments relied upon the non-functional BBB and immature immune system of neonatal mice (Stewart and Hayakawa 1987; Vogler *et al.* 1999). In human fetuses, tight junction barriers to proteins at the BBB are present very early in development (Saunders *et al.* 1999; Dziegielewska *et al.* 2000). In humans, early *in utero* administration of AAV would be required to mimic the neonatal AAV-mediated treatment strategy. Currently, however, many lysosomal storage disease patients are not diagnosed until the disease is at an advanced stage, beyond the *in utero* therapeutic window (Snyder and Wolfe 1996; Ghodsi *et al.* 1999). Nonetheless, several groups have begun to investigate *in utero* gene therapy (Lutzko *et al.* 1999b; Lipshutz *et al.* 2001). Recently, retroviral-modified syngeneic or allogeneic hematopoietic stem cells were transplanted *in utero* into MPS VII mice; however, a low engraftment (0.1%) limited the therapeutic effects of the treatment (Casal and Wolfe 2001).

4.6 Advantages of Immuno-Isolation Gene Therapy

The use of *ex vivo* genetically modified autologous or syngeneic progenitor cells implanted in the CNS of MPS VII mice have shown promising results (Wolfe *et al.* 1992b; Taylor and Wolfe 1997a; Serguera *et al.* 2001). *In vivo* gene therapy for MPS VII has also reduced lysosomal storage pathology in MPS VII mice (Sakiyama 1995; Zammarchi *et al.* 1996; Evers *et al.* 1996; Wolfe *et al.* 1996; Ohashi *et al.* 1997; Ghodsi *et al.* 1998; Stein *et al.* 1999;

Skorupa *et al.* 1999; Daly *et al.* 1999a; Gao *et al.* 2000a). The results of these experiments have recently improved with the development of enhanced vectors, such as AAV, and new strategies, such as transiently opening the BBB with hyperosmolarity during virus administration (Ghodsi *et al.* 1999; Skorupa *et al.* 1999). However, immuno-isolation gene therapy using alginate microcapsules offers several advantages as a viable alternative to both *in vivo* and *ex vivo* gene therapy strategies.

The simplicity of alginate immuno-isolation gene therapy permits rapid manufacture of the encapsulated cells. In contrast, the cost of enzyme production and purification for enzyme replacement strategies is a serious deterrent to commercial development to treat relatively rare single-gene disorders, no matter how effective the therapy may be. Immuno-isolation gene therapy is not complicated by the ethical and political concerns of using fetal material for fetal and stem cell transplantation. Compared to autologous gene therapy approaches, a single universal cell line could defray the prohibitive costs associated with the labor intensive and time consuming construction and safety characterization of thousands of individual recombinant autologous cell lines. In addition, viral vector production scale-up can be technically challenging and is a potential obstacle to clinical application with viral vectors such as AAV (Linden and Woo 1999). On the other hand, immuno-isolation gene therapy provides an efficient and potentially cost-effective treatment for patients suffering from the same disease (Chang *et al.* 1993b).

Compared to viral vectors, the practically unlimited cloning capacity of recombinant encapsulated cells may be advantageous for the incorporation of large genes or genomic sequences. This large capacity allows the incorporation of safety elements and large regulatory sequences for regulated gene product delivery (Deglon *et al.* 1996; Hagihara *et al.* 1999; Rivera *et al.* 2000; Urlinger *et al.* 2000). On the other hand, AAV, for example, is restricted to a 4.6 kb insert DNA capacity.

The uncontrolled dissemination and infection of viral vectors and the integration of *ex vivo* modified autologous or non-autologous stem cells are irreversible. In contrast, immuno-isolation gene therapy is repeatable and potentially reversible with the addition or removal of the encapsulated cells from the host. Emerging technologies like gene therapy may lead to unforeseen problems and side effects. A gene therapy clinical trial has already caused the death of one healthy patient, Jesse Gelsinger (Somia and Verma 2000; Teichler 2000), an

enzyme replacement clinical trial generated unexpected severe side-effects after intraventricular NGF infusion in the CNS (Eriksdotter *et al.* 1998), and, as mentioned, AAV resulted in liver tumours in 60% of MPS VII mice after 1.5 years (Sands *et al.* 2001). Consequently, an irreversible treatment could pose a significant risk to patients.

Immuno-isolation gene therapy may be administered repetitively without clearance by the immune system, while viral approaches are limited to one administration, due to neutralizing anti-capsid immune responses. In addition, up to 85% of the population carry pre-existing antibodies against AAV and adenovirus, precluding treatment with these vectors for most patients (Manno *et al.* 2000).

Immuno-isolation gene therapy also has several important safety advantages over other forms of treatment. Immuno-isolation gene therapy does not carry the risk of an errant insertional mutagenesis because the cells of the patient remain unchanged. Viral vectors, on the other hand, carry a putative risk of insertional mutagenesis capable of permanent transmission to the germ-line, and tumour-suppressor inactivation or oncogene activation to cause tumours. The ability of wild-type AAV to site-specifically integrate (>70%) into chromosome 19q13.3qter initially made it an attractive candidate for long-term human gene therapy (Samulski *et al.* 1991). However, AAV loses the ability to site-specifically integrate when genetically modified as a vector for gene therapy (Young *et al.* 2000). Instead, recombinant AAV vectors randomly integrate. Moreover, this random-integration of recombinant AAV occurs at a very high frequency, in up to 10% of AAV-transduced cells, carrying a significant risk of insertional mutagenesis (Yang *et al.* 1997; Kay 2001). The recent finding that neonatal administration of recombinant AAV to MPS VII mice resulted in liver tumour formation in 60% of the mice after 1.5 years highlights the potentially serious drawbacks of an integrating viral vector (Sands *et al.* 2001).

Should immuno-isolated cells escape from microcapsules, they will be quickly eliminated from the body by the immune system because of their non-autologous nature. Lentiviral vectors have been used to partially reduced the peripheral lysosomal storage in MPS VII mice (Serguera *et al.* 2001). However, the biosafety of viral vectors is of utmost concern. Reports that children participating in a lentiviral gene-therapy experiment were almost exposed to replication competent HIV has added to the growing alarm about lentiviral gene therapy (McCarthy 2000). Third generation of lentiviral vectors are deleted of all lentiviral genes essential for pathogenesis that are not required for gene transfer, leaving less than 22% of the original HIV genome (Zufferey *et al.* 1997; Kafri *et al.*

1997). However, the unlikely chance of recombination and generation of replication-competent virus should not be underestimated. In an early non-human primate study using a Mo-MLV retroviral vectors, the unexpected production of replication-competent virus resulted in rapidly progressive lethal T-cell lymphomas (Donahue *et al.* 1992). At that time, wild-type retrovirus was not known to be a primate pathogen. Wild-type HIV, on the other hand, is clearly a human pathogen.

Given the similar level of efficacy achieved with immuno-isolation gene therapy compared to viral-mediated approaches, the potential for scale-up, potential saving in cost, and safety advantages are important assets to consider for human application.

4.7 Future Directions

In spite of the demonstrated clinical efficacy, several potential problems of this technology require resolution before human application. These include the need for regulated gene expression, escape of recombinant cells with tumorigenic potential (Hortelano *et al.* 2001), and failure of the microcapsules due to breakdown of the membrane biomaterial (Peirone *et al.* 1998a). The long-term storage of microcapsules may also be beneficial for clinical application. These problems are currently being addressed and should be the focus of future research.

4.7.1 Vector Construction

The vector formulation for immuno-isolation gene therapy is critical for the future development of immuno-isolation gene therapy. The vector used to deliver the therapeutic gene into the universal cell line should be capable of long-term *in vivo* expression. We have shown that loss of selection pressure can result in significantly reduced levels of therapeutic gene expression *in vitro* (Chapter 3.2), and similar *in vivo* studies have reported up to a 1500-fold reduction in expression in the absence of selection pressure (Palmer *et al.* 1991; Caplen *et al.* 1995). The use of regulatable vectors could further increase the safety of immuno-isolation gene therapy. In the current research, the levels of therapeutic β-glucuronidase reached potentially excessive levels, up to 70-fold higher than normal levels (Chapter 3.5). Fortunately, the MPS VII mouse is resilient to high levels of β-glucuronidase and transgenic mice with over 10-fold higher than normal β-glucuronidase levels are phenotypically normal (Kyle *et al.* 1990). Although these results successfully demonstrated the delivery potential of this approach, these results also highlighted the need for reversible or regulated gene product delivery. The Tet regulatory system allows stringent

regulation of target genes over a range of 4 to 5 orders of magnitude and has been used to control gene expression of implanted encapsulated cells in a dose-dependent manner in peripheral tissues and in the CNS (Saitoh *et al.* 1998; Hagihara *et al.* 1999; Urlinger *et al.* 2000). For optimal safety, the cells should be equipped with a safety mechanism capable of killing the encapsulated cells. The presence of the herpes simplex thymidine kinase gene (HSV-tk) in an expression vector has been used to eliminate dividing encapsulated myoblasts upon exposure to ganciclovir, substantially increasing the safety of the encapsulation technology (Deglon *et al.* 1996). Compared to viral vectors, the practically unlimited cloning capacity of the recombinant encapsulated cells may be advantageous for the incorporation of these large regulatory elements.

4.7.2 Cell Lines

4.7.2.1 Non-transformed

For small animal studies, encapsulation of transformed cell lines is advantageous for repeat controlled experiments. However, the choice of cell lines for immuno-isolation gene therapy should eventually include the use of non-transformed cell lines.

4.7.2.2 Terminal Differentiation

Long-term immuno-isolation gene therapy may also benefit from the use of conditionally mitotic cells to avoid the overgrowth observed with continuously dividing cell lines. Myoblast lines have all the advantages of a dividing cell line, including unlimited availability, potential for *in vitro* screening for the presence of pathogens, suitability for stable gene transfer, and clonal selection. Furthermore they can be terminally differentiated into a non-mitotic stage upon exposure to low serum-containing medium to avoid microcapsule overgrowth. (Al-Hendy *et al.* 1995; Deglon *et al.* 1996; Chang and Bowie 1998; Hortelano *et al.* 2001).

4.7.2.3 Mannose-6-Phosphate Receptor Deficient

In order to increase the efficiency of immuno-isolation gene therapy for lysosomal storage diseases, the lysosomal enzyme internalization pathway should be disrupted to increase lysosomal enzyme secretion. This was achieved in the current research using a naturally occurring murine mannose-6-phosphate-receptor (MPR) deficient fibroblast cell line. This could also be achieved using other currently available murine MPR deficient cell lines (Ludwig *et al.* 1994) or through the isolation of new cell lines from MPR46 and MPR300 knockout mice (Koster *et al.* 1993; Wang *et al.* 1994). Currently, there are no human MPR deficient cell lines, except for two tumor

cell lines (De Souza *et al.* 1995). Mucolipidosis II patient cells have a phenotype similar to the murine MPR knockout cells with increased secretion of lysosomal enzymes (Lau *et al.* 1994). However, recombinant mucolipidosis II cells would not boost treatment efficiency because the increased lysosomal enzyme secretion results from decreased application of the M6P-signal on lysosomal enzymes, and would not be taken up by other cells. A human MPR knockout cell line that could potentially boost the secretion of lysosomal enzymes could be generated with targeted disruption of the MPR gene as it has been done before in a mouse cell line (Ludwig *et al.* 1993).

4.7.3 Microcapsule Formulations

4.7.3.1 Microcapsule Strength

Microcapsule strength and stability *in vivo* are important issues and further improvements in stability will be required for human application (Peirone *et al.* 1998a; Stockley *et al.* 2000). The optimal choice of microcapsule should be the strongest, least immunogenic formulation available. A variety of methods to increase alginate microcapsule stability without compromising the immuno-protective properties have been investigated, such as higher alginate concentrations, poly-L-lysine free, additional second layers of alginate, using agarose, solid microcapsules without core solubilization, and small microcapsules (Chapter 3.4)(Zekorn *et al.* 1992; Soon-Shiong *et al.* 1992b; Klock *et al.* 1994; Tashiro *et al.* 1998; Peirone *et al.* 1998a). As well, a variety of newly developed alginate microcapsules formulations have shown beneficial properties for *in vivo* application, such as alginate-polyphosphazene and alginate-poly-L-arginine microcapsules (Van Raamsonk 1999; Van Raamsonk *et al.* 2001).

4.7.3.2 Microcapsule Size

The use of smaller microcapsules (100-200 μm diameter) has shown the potential for further applications. These small microcapsules can pack more tightly together and sustain a higher density of cells per volume of microcapsules, presumably because of the increased surface area to volume ratio that allows greater diffusion of nutrients and waste. As a result, these microcapsules exhibited increased levels of gene product production per volume of microcapsules. For instance, the small microcapsules destined for CNS-implantation in chapter 3.7 produced more than 10-fold higher levels of β-glucuronidase per volume than the larger solid microcapsules destined for subcutaneous delivery. Small microcapsules also carry benefits of increased strength, a smaller

implantation volume, a smaller implantation needle, and reduced immunogenicity (Chapter 3.4) (Robitaille *et al.* 1999). Furthermore, small microcapsules are effective for *in vivo* gene product delivery (Chapters3.3, 3.5 , 3.6, 3.7)

4.7.3.3 Microcapsule Cryopreservation

Although not essential, a long-term storage method for microencapsulated cells would be highly beneficial for clinical application of immuno-isolation gene therapy. An optimized cryopreservation strategy using 300 μm alginate microcapsules in 15% DMSO (3 min.), 20% fetal calf serum, and DMEM resulted in a 39 ± 12% viability for human kidney 293 cells after freezing in liquid nitrogen (Read *et al.* 1999). Moreover, the cells retained a proliferative capacity and could repopulate the capsules. Although the reagents required for this cryopreservation would not be suitable for clinical application, these results show the potential for this approach. In a different study, transplantation of fragile encapsulated primary islets that were cryopreserved in a Bio Cool III freezing machine showed that cryopreservation was successful in 6 out of 10 recipients (Zhou *et al.* 1997). Thus, cryopreservation could potentially be applied to large quantities of encapsulated cells that are stored and ready for injection. This approach could be used to standardize immuno-isolation therapeutic procedures.

4.7.4 Animal Models

The MPS VII mouse provides an exceptional animal model in which to study and compare novel treatments for lysosomal storage diseases. The development of two new MPS VII animal models makes this model even more valuable, and will be important in future research. A new mouse model of MPS VII was generated with a missense mutation in the active site of the β-glucuronidase gene, resulting in a MPS VII mouse that does not produce antibodies against re-supplied human β-glucuronidase (Sly *et al.* 2001). This animal model may facilitate the development of therapies for patients that do not develop antibodies against β-glucuronidase, but it cannot address the potentially serious immune responses in patients with null alleles that are likely to develop antibodies. In addition, a second new mouse model of MPS VII has been developed with a less severe phenotype. These mice may provide a better opportunities to study MPS VII because the affected mice are able to bear live young, raise litters, and live significantly longer than the original MPS VII mutant mice, yet the affected mice still exhibit symptoms of increased lysosomal storage in many tissues (Gwynn *et al.* 1998). Future pre-clinical studies will also have to examine the efficacy of immuno-isolation gene therapy in larger animal models. The canine models of

MPS I and MPS VII provide excellent models of lysosomal storage diseases with phenotypes similar to the human diseases (Shull *et al.* 1982; Haskins *et al.* 1984). Increased financial support will likely be required for the future development and pre-clinical studies of immuno-isolation gene therapy in larger animal models. Commercial financial support should be investigated as a means to augment funding of these studies.

4.8 Proposed Treatment Protocol

The results of this research show that immuno-isolation gene therapy can provide an effective treatment for the systemic effects of a lysosomal storage disease in small animals. The next steps will be to show that this treatment is efficacious and safe in a larger animal model prior to clinical application. The feasibility of this approach has recently been demonstrated with the transient delivery of a marker gene in the canine CNS (Barsoum 2000). However, several important factors must be incorporated into large animal model preclinical studies for the treatment of lysosomal storage diseases.

The formulation of vector, cell line, and microcapsule will be critical. The vector used to deliver the therapeutic gene into the universal cell line should be capable of long-term regulated *in vivo* expression (Chapter 3.2), and for optimal safety the cells should be equipped with a safety mechanism capable of killing the encapsulated cells. The judicious choice of cell line must include a non-transformed cell that is capable of terminal differentiation to avoid microcapsule overgrowth (Al-Hendy *et al.* 1995; Chang and Bowie 1998; Hortelano *et al.* 2001). For the delivery of lysosomal enzymes, a mannose-6-phosphate receptor deficient cell line would be optimal for efficient secretion of the therapeutic gene product from the cells. The optimal choice of microcapsule should be the strongest, least immunogenic formulation available. Current and ongoing improvements in microcapsule formulations should be incorporated (Van Raamsonk 1999; Van Raamsonk *et al.* 2001). For peripheral implantations in large animals, either subcutaneously or intraperitoneally, the strength of the microcapsules will be crucial (Peirone *et al.* 1998a; Stockley *et al.* 2000). For CNS implantation, a small microcapsule size will be important to minimize surgical trauma with the smallest diameter injection needle. The use of smaller microcapsules should also be considered because of their increased levels of gene product secretion, increased strength, smaller implantation volume, smaller implantation needle, reduced immunogenicity, and a potentially increased dispersal at the implantation site (Chapter 3.4).

The treatment protocol should be initiated at the earliest time possible following diagnosis in order to address the early onset of lysosomal storage diseases (Chapter 3.3). For peripheral implantation in large animal models subcutaneous injection is currently the optimal site of administration (Stockley *et al.* 2000). Multiple injections should be used to maximize enzyme dispersal. Dogs would need roughly 300 ml of microcapsules secreting an equivalent quantity of enzyme as was subcutaneously injected in mice. However, using small microcapsules this volume could potentially be reduced to 30 ml, and thirty 1 ml subcutaneous injections would be feasible. In the CNS, the large size of the dog and human brain compared to the mouse brain will require significantly more enzyme dispersal than in mice. In larger animal models a widespread distribution of enzyme would have to be achieved with additional sites of microcapsule administration throughout the CNS. Compared to the mice treated in these studies, the implantation volumes would be feasible for dogs (70-700 μl) and possibly for humans (1 - 13 ml), and the use of multiple implantation sites in the CNS would have to be investigated. Finally, the treatment protocol would have to address the potential immune response against the transgene product. The subjects should be pre-screened to determine if they carry pre-existing antibodies against the transgene. Immunosuppression or alternative methods to achieve tolerance, such as long-term delivery, should be considered. If the immune response against the therapeutic gene product is not controlled, the treatment will likely be ineffective in null allele patients who develop antibodies against the transgene.

During the course of therapy, the subjects should be carefully monitored for antibody formation and levels of therapeutic gene expression. The optimum level of gene expression could be controlled through additional microcapsule implantations, microcapsule removal, or vector regulation. Routine biopsies analyzed for lysosomal enzyme levels and disease pathology would be required to assess the ongoing effectiveness of the treatment. In the CNS, analysis of CSF could potentially be used to assess the level of gene expression in the brain. The CSF data could be correlated with the results of necropsied animals to estimate the levels of therapeutic enzyme in the brain. Overall, the results of this research have shown that immuno-isolation gene therapy can provide an effective treatment for the systemic effects of a lysosomal storage disease in small animals, and these recommendations should help form the protocol for scale-up studies in larger animal models.

4.9 Conclusion

Immuno-isolation gene therapy demonstrated clinical efficacy in treating the systemic manifestations of a lysosomal storage disease in a murine model of MPS VII. This approach was capable of reducing both the peripheral and CNS manifestations of the disease in adult animals, an achievement that has not previously been accomplished for the treatment of MPS VII. Although the treatment was abrogated by an anti-transgene immune response that limited the duration of the treatment to 8-12 weeks, immunosuppression was capable of circumventing this response. Nonetheless, the results from this study highlight the fact that immunologic reactions to the therapeutic transgene product may be a significant impediment to gene therapy in some patients and effective alternatives to systemic immunosuppression are needed. In summary, the findings of the present study demonstrate a proof-of-principle for the systemic treatment of a lysosomal storage disease using immuno-isolation gene therapy. The combination of both peripheral and CNS implantation of immuno-isolated cells expressing β-glucuronidase resulted in widespread β-glucuronidase delivery and systemic clinical improvements. The concomitant improvements in biochemical, histological, and behavioral manifestations of this disease are supportive of further development of this technology towards human clinical application.

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