THE DELIVERY OF HUMAN GROWTH HORMONE TO DOGS USING MICROENCAPSULATED NON-AUTOLOGOUS CELLS

By

MICHAEL PEIRONE, B.Sc.

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TITLE: The Delivery of Human Growth Hormone to Dogs Using Microencapsulated Non-Autologous Cells

AUTHOR: Michael Peirone, B.Sc. (McMaster University)

SUPERVISOR: Dr. P.L. Chang

EXAMINING COMMITTEE: Dr.A.Fletch
Dr.B. Golding

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ABSTRACT

Many of the presently approved somatic gene therapy protocols involve re-implantation of genetically engineered autologous cells into a patient. A potentially more cost-effective approach to the delivery of therapeutic gene products is the use of a universal recombinant cell line that can be implanted into a number of patients with the same product requirements. Enclosure of these non-autologous cells inside a permselective microcapsule membrane would permit the diffusion of the recombinant product but prevent entry of the host's immune mediators. The clinical efficacy of this approach has been demonstrated by the implantation of recombinant fibroblasts and myoblasts to correct mutant phenotypes in murine models of diseases such as dwarfism (Al-Hendy et al., 1995) and lysosomal storage disease (Bastedo, 1994). In the first part of this thesis, a new microcapsule type was created that incorporated a combination of traits from both alginate-poly-L-lysine-alginate and barium-alginate microcapsules. The new, barium-poly-L-lysine-alginate microcapsule was cross-linked with $BaCl_2$ and received a poly-L-lysine, and a second alginate coat. The three different types of microcapsules were compared with respect to encapsulated cell viability, proliferation and secretion *in vitro*. Results of these analyses demonstrated that cells inside alginate-poly-L-lysine-alginate microcapsules had higher viability and a greater proliferation rate than did cells inside either barium-alginate or barium-poly-L-lysine alginate microcapsules. However, secretion from the alginate-poly-L-lysine alginate microcapsules was lower than from either of the barium-alginate types, and the two types of barium-alginate microcapsules, formulated with a higher alginate concentration, were more resistant to well-defined fluid shearing forces, than was the calcium alginate microcapsule. No significant difference in any of the parameters measured was observed between the barium-alginate and barium-poly-L-lysine alginate microcapsule types.

In the second part of this thesis the three different types of microcapsules, each containing canine MDCK cells secreting ~20 ng/10^6 cells/hr of human growth hormone (hGH) were implanted into the peritoneal cavities of a large animal model. The

microencapsulated cells were able to deliver recombinant human growth hormone to the circulation of dogs at levels nearly 100 % higher than human physiological levels. In contrast, implantation of unencapsulated recombinant cells resulted only in short-term delivery of hGH to the dogs. The level of titre of anti-hGH antibodies was monitored in the experimental and control animals, and its increase was determined to be associated with the disappearance of the human growth hormone from the circulation of the dogs. The $BaCl_2$ cross-linked capsules with the higher alginate concentration lasted longer *in vivo*, confirming their superior mechanical integrity relative to the alginate-poly-L-lysine alginate type. The presence of the microcapsules in the peritoneum of the dogs was associated with localized inflammation of the omentum, and mild lymphadenitis. This pathology, combined with varying degrees of fibrotic overgrowth of the microcapsules with increasing time *in vivo*, suggests that modifications must be made in order to improve the biocompatibility of alginate microcapsules.

In conclusion, modifications of alginate microcapsules, such as the cross-linking with barium cations, the use of higher alginate concentrations and lamination with poly-L-lysine alginate have contributed to the mechanical stability of the capsules and permitted the long-term delivery of recombinant gene products using non-autologous cells. This study has highlighted some of the issues to be addressed during pre-clinical studies in large animal models, in order to determine the efficacy of this new technology for humans.

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Abbreviations

APA	alginate-poly-L-lysine alginate microcapsules
BaAlginate	barium-alginate microcapsules
$BaCl_2$	barium chloride
BMT	bone marrow transplantation
BPA	barium-poly-L-lysine alginate microcapsules
$CaCl_2$	calcium chloride
CFTR	cystic fibrosis transmembrane conductance regulator
CHES	2-(N-Cyclohexylamino)ethanesulfonic acid
EDTA	ethylenediaminetetraacetic acid disodium salt
ELISA	enzyme-linked immunosorbent assay
H_2O	water
HEPES	4-(2-Hydroxyathyl)-1-piperazin-athansulfonsaure
hGH	human growth hormone
IL	interleukin
K Alg	potassium alginate
LB	Luria Broth
MPS	mucopolysaccharidosis
NaCl	sodium chloride
O.D.	optical density
O.R.	operating room
PBS	Phosphate buffered saline
PBS/T	Phosphate buffered saline with Tween 20 (Sigma)
PLL	poly-L-lysine
rpm	rotations per minute
T_H	Helper T lymphocyte
TNF	tumor necrosis factor
Tris	Tris(hydroxymethyl)aminomethane

1.0 INTRODUCTION

Gene therapy is the treatment of disease through the introduction of genes using various strategies to correct somatic cell defects in the patient. Advances in the characterization of gene defects at the DNA level, and the development of delivery vectors for corrective genetic material have increased the pace of approvals for human clinical trials of gene therapy protocols. Many governing bodies are involved in the development and approval of the clinical trials, such as the National Institute of Health (NIH) Recombinant DNA Advisory Committee (RAC), and the Food and Drug Administration (FDA) in the United States. The gene therapy trials eventually aspire to meet a number of goals, including identification of possible side effects and risks to the patients and efficacy of treatment. The vectors or delivery systems that are used in the presently approved human clinical trials include retrovirus, adenovirus, liposomes and the direct injection of DNA. The current state of these different vector systems will be reviewed in this section.

Gene delivery into patients can occur either *ex vivo* or *in vivo*. *Ex vivo* gene therapy involves the removal, modification *in vitro*, and subsequent reintroduction of cells into the patient. With *in vivo* gene therapy, the genetic modification takes place within the patients themselves with the direct introduction of functionally active genes into the somatic tissues or organs of the patients.

1.1 *Ex Vivo* Gene Therapy

1.1.1 Retroviral vectors

Retroviral vectors are retroviruses from which viral genes have been removed making them replication-incompetent, so that no viral proteins can be produced when this vector infects a host cell. The use of packaging cell lines, which provide viral proteins but do not produce infectious virus particles, allows for the replication of the non-infective virus particles. Retrovirus has a transduction frequency of up to 30%, resulting in a high frequency of gene transfer and stable integration of genetic material into replicating cells. The introduced genetic material stably integrated into hematopoeitic stem cells and epithelial cells.

Disadvantages of retrovirus as a vector for gene therapy are its inability to integrate into non-dividing cells, eliminating quiescent cell types such as muscle and nerve tissues as potential targets, and the inability to produce the vector synthetically. Since retroviral vectors must be produced in cultured cells, it is difficult to obtain homogeneous purified preparations of the vector, making extensive testing for replication-competent viruses necessary. Additionally, retroviruses are capable of only low infectivity, necessitating the use of high concentrations of viral particles to deliver clinically significant amounts of the gene product of interest.

Retroviral mediated gene transfer was used as an *ex vivo* approach in the first gene therapy human clinical trial undertaken by Blaese et al. that commenced in 1990. This trial involved the transfer of the adenosine deaminase (ADA) gene into lymphocytes of patients with severe combined immunodeficiency. Prior to this treatment attempt, some patients had been treated with allogeneic bone marrow transplantation while others received treatments with ADA-containing erythrocyte infusions or ADA conjugated to polyethylene glycol (PEG-ADA). Patients were accepted to take part in this trial only if they did not have a sibling with antigen-matched lymphocytes, who could provide donor bone marrow. T-cells were obtained from the blood of the patients and were expanded in culture. The cells were transduced with a retroviral vector carrying the adenosine

deaminase cDNA, and reinfused into the patients. Two years after the discontinuation of this treatment, T and B lymphocytes, bone marrow cells and granulocytes continued to express ADA, resulting in the development of a functional immune response in these patients (Bordingnon et al., 1995, Blaese et al., 1995). These patients, however, have continued to receive enzyme replacement therapy in the form of PEG-ADA, albeit at lower levels than before the gene transfer was performed.

Another recent clinical trial involving retrovirus was used to treat familial hypercholesterolemia (FH) (Grossman et al., 1994). Retroviruses containing the cDNA for the low-density lipoprotein (LDL) receptor were used to transfect autologous cultured hepatocytes. These transfected hepatocytes were reinfused into the partially hepatectomized patient where they caused an increase in the proportion of LDL receptors on the liver.

1.1.2 Adenoviral Vectors

As in retroviral vectors, the elements necessary for the adenovirus to replicate and produce a systemic infection have been removed. The development of E1-deleted replication-deficient adenoviral vectors has allowed for the propagation of this vector inside of cells engineered to express E1 genes (Jones and Shenk, 1979). The major advantages to gene therapy using adenovirus is its ability to carry large inserts of DNA and to infect non-dividing cells.

One of the disadvantages to using adenovirus as a gene transfer vector is the presence of some adenoviral genes whose products may stimulate an immune response in the host. As a result, gene delivery to the recipients by adenoviral vectors is often short-lived. Additionally, the adenovirus remains episomal in the hosts cells as it produces its recombinant gene products, resulting in its eventual loss during cell division. Integration of the adenoviral DNA sequence into the host cells may occur in high multiplicities of infection, but such integration is inefficient and not a vital part of the adenoviral life cycle. For this reason, adenovirus is a more appropriate vector for use in terminally differentiated cells.

A complex composed of adenovirus conjugated to DNA and poly-L-lysine has been used to transfect various cell types *in vitro* (Lozier et al., 1994). These complexes resulted in high levels of transgene expression from cultured fibroblasts and bone marrow stromal cells, but only for as long as 2 weeks after transfection. This protocol, using an adenoviral vector in a complex, may be useful only for short-term gene therapy.

1.1.3 Engraftment of Recombinant Cells

A number of other approaches have been proposed for the delivery of recombinant genes. Many involve the implantation of naked cells into the recipients, and have been used to treat a number of diseases with varying success. Genetically modified fibroblasts from a rabbit have been clonally selected and implanted into the subcapsular space in the kidney of mice to deliver human growth hormone to the sera for over 500 days (Heartlein et al., 1994). Since these cells represented a primary cell line and were not immortalized, there was no threat of tumor formation. Additionally, the ability of fibroblasts to induce vascularization resulted in the prolonged survival of these cells. In another study, cultured mouse Ltk^- fibroblasts transfected with a plasmid secreting human growth hormone (hGH) were implanted intraperitoneally into mice (Selden et al., 1987). In this case, transkaryotic implantation was successful at delivering hGH to the circulation for less than 2 weeks, regardless of the site of implantation. Transplant rejection by the recipients' immune mediators occurred quickly, and the use of immunosuppression in these animals resulted in death due to the development of plaques on the peritoneal organs and to increased peritoneal cell mass (ascites).

As an alternative to plasma protein replacement therapy for the treatment of haemophilia B, transduced C2C12 myoblasts secreting human factor IX have been injected into skeletal muscles of mice (Yao and Kurachi, 1992). When the transfectant cells were injected into skeletal muscles of mice, they fused with the existing muscle fibres. When injected concurrently with immunosuppressant therapy, the fused muscle fibres were able to deliver human factor IX to the circulation of the mouse for 4 weeks,

before disappearing from the circulation. The injection of naked DNA into the muscle of the mice, however, failed to result in any of the human factor IX entering the circulation.

The above studies of cell implantation relied on the isolation of syngeneic or autologous cells from the recipients receiving the implants, and the administration of immunosuppressive agents for the prolonged survival of the implanted tissues. In the case of a haematological disorder such as haemophilia B, isolation of tissues by biopsy may not be well tolerated by the patients. Additionally, the immunosuppressants required for the survival of non-autologous tissues can be associated with side effects such as renal damage (Rang et al., 1995).

1.1.4 Bone Marrow Transplantation

Bone marrow transplantation (BMT) is another method of *ex vivo* gene therapy that has been used as an approach to the treatment of many disorders. Lysosomal storage diseases such as the mucopolysaccharidoses have been treated using BMT resulting in significant correction of the disease phenotype. Birkenheimer et al. in 1991 transplanted bone marrow from homozygous normal mice to genetically identical mutant gus^{mps}/gus^{mps} mice affected with Mucopolysaccharidosis Type VII (MPS VII). The dosage of radiation administered to the bone marrow recipients corresponded to the resulting amount of β-glucuronidase delivered and hence the degree of biochemical and histological correction. Mice receiving high doses of radiation not only had improved correction of lysosomal storage, but also increased life span compared to their untreated mutant counterparts. Similarly, in dogs affected with MPS I who suffer from a deficiency of α-L-iduronidase, whole body irradiation followed by bone marrow transplantation from littermate donors has resulted in significant correction of the disease phenotype (Breider et al, 1989, Shull et al., 1988). In these cases, the enzyme was able to cross the blood-brain barrier and result in the amelioration of some degree of lysosomal storage in neurons. However, while BMT in several animal models has been encouraging, musculoskeletal lesions in MPS I children continue to persist after BMT treatment. Additionally, there is a marked variation among patients in the degree of CNS

correction that can be attained through BMT of normal α-L-iduronidase-producing bone marrow cells. Treatment is most effective if administered very early in the life of the patient before significant damage occurs as a result of the storage lesions (Shull et al., 1988, Taylor et al., 1992).

Some disadvantages associated with BMT are the scarcity of genetically compatible donors, and the use of high doses of radiation to ablate the bone marrow of the host. Evidence of radiation-induced toxicity in the central nervous system of neonate mice has been observed where this strategy has been used as part of a BMT procedure (Sands et al., 1993, Bastedo et al., 1994).

1.1.5 Recombinant Neo-Organ Implantation

Implants consisting of one to a number of small collagen lattices filled with transduced cells have been used to deliver missing gene products to small (Moullier et al., 1993) and large animals (Moullier et al., 1995). In earlier studies, neo-organs containing β-glucuronidase-secreting recombinant fibroblasts were implanted into MPS VII mice (Moullier et al., 1993). The implanted cells survived for nearly three months *in vivo*, and continuously secreted the human β-glucuronidase to the circulation resulting in the complete elimination of the lysosomal storage lesions in the liver and spleen. In the dog, skin biopsies were taken from healthy dogs and isolated fibroblasts were transduced with a retroviral vector coding for the human β-glucuronidase gene (Moullier et al., 1995). Lattices were prepared *in vitro* by mixing the fibroblasts with collagen and polytetraflouroethylene (PTFE) fibres treated with heparin and fibroblast growth factor. These lattices, each 5 cm in diameter and containing an average of 10^9 transduced fibroblasts, were surgically implanted into the omentum of normal dogs and their condition *in vivo* were evaluated by laparotomy procedures performed throughout the experiment. The neo-organs became well vascularized but remained free of local inflammation. The enzyme was delivered to the circulation at quantities equivalent to 0.8-3.1% of endogenous levels and was taken up by the liver and spleen of the dog. This

treatment method represents a less-invasive alternative to bone marrow transplantation to treat lysosomal storage diseases, but still involves invasive surgical procedures.

Hollow fibre tubular membranes are another example of an implantable neo-organ used for the isolation of recombinant tissues. These hollow fibre tubes were used to deliver ciliary neurotropic factor (CNTF) to 6 human patients with amyotrophic lateral sclerosis (ALS) using baby hamster kidney cells (Aebischer et al., 1996). Genetically modified xenogeneic cells were enclosed in a 5 cm long, hollow fibre fabricated from poly-ether-sulfone. The device was implanted into the lumbar intrathecal space, and an attached tether was sutured beneath the skin permitting easy removal. The implanted cells continuously delivered CNTF to the patients for over 17 weeks, and was free of cell adhesions upon removal. Although the device prevented the destruction of the implanted xenogeneic tissues in the absence of immunosuppressant administration, the disease continued to progress. This approach shows promise for the delivery of recombinant gene products to the CNS that can overcome the limitations imposed by the blood-brain barrier.

1.2 Implantation of Non-Recombinant Biomaterials

1.2.1 Hollow Fibre Tubular Diffusion Membranes

Tubular diffusion chambers have also been used as immunoisolation devices for the implantation of non-recombinant tissues such as allogeneic or xenogeneic pancreatic islet cells (Lanza et al., 1991 and 1992) and xenogeneic chromaffin cells secreting neuroactive substances (Sagen et al., 1993). In an attempt to circumvent the vigorous immune response to xenogeneic tissue transplants, islet xenografts from discordant species were enclosed in semipermeable tubular membrane diffusion chambers fabricated from an acrylic copolymer, XM-50 (Lanza et al., 1992). In the earlier studies by this group, either canine, bovine or porcine islets were coated with sodium alginate before being seeded into the tubular chambers, where they were gelled in a $CaCl_2$ solution. Once the ends of the chamber were heat sealed, they were randomly implanted

into the peritoneum of streptozotocin-induced diabetic rats where they reversed the diabetic state of all of the recipients for one month. Microcapsules containing xenogeneic islets enclosed in the tubular membranes induced an inflammatory response consisting of a layer of fibroblasts and collagen, and were permeable to immunomodulatory products of lymphocytes and monocytes that damaged the enclosed islets (Weber et al., 1990). Smooth-surfaced neo-organs, however, showed no evidence of inflammatory response (Lanza et al., 1991). In cases where the membrane walls of the diffusion chamber were broken, the interior of the membrane showed evidence of fibroproliferation and immune cell elements. In later studies, Lanza et al.in 1992, produced similar artificial pancreas neo-organs without the use of the alginate coating. Again in this case, the hollow fibre membranes remained free of signs of an inflammatory response and were able to respond to glycemic stress and reduce or supplant exogenous insulin therapy in dogs for more than two months without the use of immunosuppression (Lanza et al., 1992).

Hollow fibre membranes have also been used to immunoisolate xenogeneic adrenal medullary chromaffin cells producing neuroactive substances (Sagen et al., 1993). In this and similar studies, bovine-source chromaffin cells were enclosed in hollow fibres consisting of polyacrylonitrile vinyl chloride (PAN-PVC) and implanted into the CNS of rats. Though the CNS is considered immunologically benign to xenografts, rejection of implanted tissues is still known to occur in this area. This device prevented the rejection of the xenogeneic tissue while maintaining cell viability and allowing the release of the pharmacological agents.

The incomplete biocompatibility of these devices, combined with the surgical intervention associated with their implantation, warrants further development of this technology as an approach to gene therapy.

1.2.2 Intravascular Pancreatic Devices

Intravascular profusion devices have been developed to be implanted as an arteriovascular shunt in the vascular system. With this system, biological materials such as pancreatic islets are enclosed in these devices and have direct access to the

vasculature. This provides an advantage over intraperitoneal or sub-cutaneous implantation, where oxygen and nutrients must be exchanged over large diffusion distances. For use in the treatment of diabetes, islet cells were distributed in a disk-shaped smooth acrylic housing containing a selectively permeable coiled tubular membrane made from poly acrylonitrile-polyvinyl chloride (PAN-PVC). Devices such as these reversed hyperglycemia in spontaneously diabetic rats for more than one year in the absence of immunosuppression (Lanza et al., 1995). When implanted into diabetic dogs, 25% of the enclosed islets remained viable after 1 year *in vivo* (Lanza and Chick., 1996). Insulin independence was achieved for >10 weeks, and devices examined following their removal were generally free of fibrotic overgrowth.

While the biocompatibility and glycemic correction achieved with these devices are promising, the extensive surgical manipulation required for their implantation and retrieval is highly invasive.

1.3 *In Vivo* Gene Therapy

1.3.1 Viral Infusion

Retroviral vectors encoding a human serum protein marker have been directly infused into organs of recipients to transduce cells *in vivo*. In a study by Kay et al. in 1992, a partial hepatectomy was performed on mice to stimulate hepatocytes to proliferate to allow retroviral integration into the host genome. Supernatant from human α-1-antitrypsin-producing recombinant retrovirus was infused into the portal vein of the hepatectomized animals. When infusion of the viral supernatant occurred within 48 hours after hepatectomy, the transduction frequency of the hepatocytes was 1-2%, resulting in the delivery of high levels of the α-1-antitrypsin to the circulation of the mice. Constitutive, low-level expression was achieved for up to 6 months in some of the experimental animals.

A similar procedure using selective perfusion of a regenerating liver following partial hepatectomy was also performed on dogs (Cardoso et al., 1993). To prevent the

spread of the vector into other tissues, the infra- and suprahepatic vena cava, as well as the portal vein were clamped, essentially isolating the regenerating liver lobes from the systemic circulation. When a helper-free retroviral vector encoding β-galactosidase was perfused into the livers of 5 dogs, X-gal staining showed β-gal-positive cells in the livers of 3 of the 5 dogs for up to 6 weeks.

While no ectopic gene transfer was found in these animals, the surgical procedure required to stimulate hepatic regeneration was extremely invasive. In the dogs, 44 to 72% of the liver mass was removed, making this approach to gene therapy prohibitive for the treatment of some diseases, particularly haematological disorders.

Adenoviral vectors have also been used in animal studies, to treat haemophilia B in factor IX-deficient dogs (Kay et al., 1994). A subcutaneous port with a catheter tip was placed in the splenic vein of the dog, and an adenoviral vector encoding canine factor IX was infused into the portal vasculature. The factor IX concentration in one of the dogs reached a level of 300% of normal plasma on day 1 post-infusion. The plasma levels of factor IX decreased within three weeks to 1% of normal levels, before disappearing entirely by 100 days post-infusion. This approach to gene therapy using adenoviral vectors was successful at delivering much more of the gene product of interest than was required for phenotypic correction. However the adenovirus was found to spread to other tissues remote from the target site, and tissue degeneration and necrosis resulting from the viral infusion occurred. Additionally, a second viral infusion following the disappearance of factor IX from the circulation of the dog could not be detected in the circulation. This suggests an immunological block to re-infection, possibly directed at the adenoviral proteins.

Recombinant adenoviral vectors have also been used in a human clinical trial to treat cystic fibrosis (CF). Adenoviral vectors encoding for the human *CFTR* cDNA (Ad*CFTR*) were delivered to the respiratory tract of four patients with cystic fibrosis (Crystal et al., 1994). Because of the branching structure of the airway, the epithelium from these areas cannot be removed and re-implanted, making *ex vivo* gene therapy impossible. In this study involving four patients receiving the Ad*CFTR*, there were no adverse effects observed in three of them following administration of the vector to the

bronchial epithelium. In one patient however, a syndrome developed that was characterized by headache, fever, tachycardia, hypotension and other symptoms. An elevated level of IL-6, believed to be associated with adenovirus administration, was observed in this patient and one other. While this study demonstrated the ability to deliver recombinant adenovirus to the epithelium of the respiratory tract of human patients without viral shedding or the production of replication competent viral particles, it did not address the effect of the therapy on the pathological manifestations of CF in the respiratory tract. Additionally, no CFTR expression could be detected past day 10 post-administration. There is a number of dangers inherent to the use of adenovirus as a vector for gene therapy, such as the "spillover" of vector particles observed on the radiographs of the treated patients in the Ad*CFTR* study. Any other viral infection of the respiratory tract could introduce the missing E1a-like information to the adenoviral vector, possibly leading to the production of replication-competent viral particles.

With both retroviral and adenoviral vectors, the unwitting delivery of replication-competent viral particles to the patient could lead to problems such as down-regulation of host protein synthesis, toxicity to certain cells, insertional and the development of malignancies in the host (Alberts et al., 1989). Additionally, problems such as loss of episomal DNA from the transduced cells and the development of antibodies to components of the viral particles, limit their effectiveness *in vivo* (Kay et al., 1994, Crystal et al., 1994). Research in this area continues, with more patients enrolled in clinical trials involving the use of retroviral vectors.

1.3.2 Protein or DNA Complexed to Carrier Molecules

Direct injection of DNA represents an *in vivo* approach to gene therapy. Cao et al. in 1995 implanted a plasmid expressing the tyrosine hydroxylase (TH) transgene that was complexed to a lipofectin reagent into a rat model of Parkinson's disease (PD). When injected into multiple sites in the brain, the lipofectin allowed for the transfer of the TH gene into striatum cells of the brain. A behavioral test to assess the effect of this gene therapy demonstrated a reduction in rotational behavior, a trait demonstrated by PD-

model rodents, for 15 days post-injection before reverting to pre-treatment state. Drawbacks of this method included non-specific transfection of cell types in the brain and an immunoreaction to the plasmid occurring at the injection site (Cao et al., 1995).

A plasmid containing the normal canine α-L-iduronidase cDNA was prepared for direct injection into muscle tissue of two dogs with mucopolysaccharidosis I (MPS I) (Shull et al., 1996). The plasmid entered muscle either as implanted dried plasmid pellets, or as a suspension for intramuscular injection. In both dogs, blood leukocytes assayed for up to 3 months post-injection were negative for the enzyme. However, later implantations in the same dogs with α-L-iduronidase-secreting recombinant myoblasts failed to deliver the enzyme to the circulation, suggested that the injection with plasmid DNA had been immunogenic. In this case, the direct injection of DNA had been unsuccessful at delivering the enzyme to the MPS I dogs, and had resulted in a severe immune response against later injections of the α-L-iduronidase.

1.3.3 Encapsulation of Biomaterials

The concept of encapsulation of biomaterials was first proposed by T.M.S. Chang in 1964. It was during the 1980's that researchers began investigating the applicability of this approach to the encapsulation of biomaterials such as pancreatic islets to treat diabetes using a capsule made from alginate-poly-lysine-alginate. Since then this approach has been applied to the study of the behavior of a number of encapsulated tissues both *in vitro* and *in vivo*. Microencapsulation has been investigated as a treatment modality not only for endocrine disorders, but also for neurological disorders and the alleviation of chronic pain.

Biological material can be enclosed in either macrocapsules or microcapsules. Droplet techniques are often employed for macroencapsulation wherein biomaterials are mixed with the encapsulation material and extruded into a cross-linking reagent. Hollow fibres containing cells (as described earlier) are another form of macrocapsule. These devices may range in length up to 5 cm and have a diameter of >1.5 mm (Lanza et al., 1991, Aebischer et al., 1996). Most microcapsules are also formed with a droplet

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method, but this method can be modified by the use of a concentric air stream along the extrusion needle, a droplet generator or electrostatic pulses so that the polymer/biomaterial beads are sheared from the end of the extrusion needle more quickly. This modification results in the formation of smaller capsules (250-750 μm in diameter). The smaller microcapsules have the advantage over macrocapsules of having a larger surface area to volume ratio which increases biomaterial loading capacity (Stormo and Crawford, 1992), and the ability to simply inject into recipients and remove by simple lavage and needle aspiration.

Microencapsulation of biomaterials has been used to immunoisolate many different types of tissues for gene therapy. Researchers such as Sefton et al.in 1992, have investigated the use of encapsulation materials such as biocompatible thermoplastic copolymers, which were permselective and allowed for the controlled release of substances secreted by the encapsulated cells (Sefton et al., 1992). The capsules were less than 1 mm in diameter and had a molecular weight cutoff range of ~70-150 kD, which excluded antibodies (IgG = 150 MW) from coming into contact with the enclosed tissue. While the resulting capsules were strong enough to require manipulation with forceps to break them, there were some limitations with respect to parameters other than structural integrity. Either pancreatic islet cells or dopamine-producing PC12 cells were encapsulated using these devices and implanted into the peritoneum of mice and rats. When encapsulated islets were retrieved from the peritoneum after 4 weeks, the capsules were covered in a thin (1-4 layer) fibrous capsule, indicating incomplete biocompatibility. Additionally, during the encapsulation of islets, only 30% of the islets used in the production process became enclosed in the capsules. The islets that were encapsulated secreted insulin at only one third the rate of unencapsulated islets. With the dopamine-producing PC12 cells, the cells were only viable in a ring right inside the capsule wall, suggesting possible nutrient transport limitations.

Alginate has been used as a biocompatible immunoisolating membrane predominantly for the encapsulation of pancreatic islets (Goosen et al., 1985, Mazaheri et al., 1991, Halle et al., 1992, Levesque et al., 1992, Soon-Shiong et al., 1993 and 1994, Grohn et al., 1994, Lanza et al., 1995). With the use of alginate poly-L-lysine-alginate

microcapsules, allogeneic islets were transplanted into diabetic rats with or without cyclosporin (Mazaheri et al., 1991). The encapsulated islets, implanted without supplementary immunosuppressants or anti-inflammatory agents, survived for less than 15 days *in vivo*. When recovered from the peritoneal cavity, these capsules were covered with a thick pericapsular infiltrate (PCI) composed predominantly of fibroblasts and macrophages. When capsules were implanted in rats receiving imunosuppressant therapy, the encapsulated islets survived for 44 days, and were free of any PCI upon removal from the peritoneum. It was found in this study that graft failure seemed to correspond with the formation of the PCI, perhaps due to starvation of the encapsulated tissue of nutrients. Other studies have shown that encapsulated islets showed a reduced capacity for insulin secretion when compared to free islets, though their response to physiological regulation by glucose was identical (Levesque et al., 1992). Later studies using canine islet allografts encapsulated in modified alginate microcapsules demonstrated the ability to reverse diabetes in a diabetic dog for 172 days (Soon-Shiong et al., 1993). The microcapsules implanted in this study were more biocompatible due to a modification in the alginate material used to form the capsules, as evidenced by their freedom from PCI after 6 months *in vivo*. The modification to the microcapsules was the use of an alginate higher in α-L-guluronic acid residues which reduced the mitogenicity of the capsule, as will be discussed later. Subtherapeutic doses of cyclosporin A (CsA) were used during the initial trials in this study to prevent cytokine stimulation in the early transplant period.

Encapsulated human islets were transplanted into the peritoneum of a human patient in 1994, albeit with the administration of low dose immunosuppressants (Soon-Shiong et al., 1994). Within 24 hours of islet transplantation, the patient's exogenous insulin requirements decreased. In the ninth month post-transplant, exogenous insulin therapy was discontinued and the patient reported improvements in his quality of life and was able to resume working after a decade of unemployment.

Recombinant cultured cells have also been encapsulated, and have proliferated and maintained high cell viability while delivering gene products of interest both *in vitro* and *in vivo*.

Chang et al., demonstrated the ability to encapsulate an Ltk$^-$ fibroblast cell line secreting human growth hormone (Chang et al., 1993). When these capsules were implanted into the peritoneal cavity of mice, up to 1.5 ng of human growth hormone (hGH)/ml of serum was detected within the first 2 weeks post-implantation. Starting from day 21, antibodies against hGH appeared in the circulation of the mice and continued to rise for up to 3 months, suggesting its continued production by the encapsulated cells. Capsules recovered after 78 days *in vivo* continued to secrete human growth hormone at pre-implantation levels when recultured, and were free of adhesive cells. Because of the species-specificity of growth hormone, no biological effect was observed in the treated animals in this study. This approach was later applied to the delivery of mouse growth hormone to Snell dwarf mice, a model of a hereditary disorder (Al-Hendy et al., 1995). Allogeneic myoblasts engineered to secrete mouse growth hormone (mGH) were microencapsulated and implanted into the peritoneum of dwarf mice. The microencapsulated cells delivered the mouse growth hormone to the circulation resulting in increases in linear growth, body weight, peripheral organ weights and tibial growth plate thickness in the treated animals. Compared to control mice receiving either encapsulated untransfected myoblasts, or unencapsulated mGH-secreting myoblasts, the mice in the treated group showed significantly higher increases in the parameters measured. Microcapsules retrieved after 6 months *in vivo* remained free of indentations or inflammatory cell adhesions and continued to secrete mouse growth hormone when recultured.

A genetically engineered factor IX-secreting Ltk$^-$ fibroblast cell line has also been successfully encapsulated and delivered the biologically active clotting factor from alginate microcapsules *in vitro* (Lui et al., 1993). To address the possibility of decreased viability inside the microcapsules due to overcrowding of proliferative cells, a similar approach was taken to address the treatment of factor IX deficiency by encapsulating human factor IX secreting myoblasts and implanting them into the peritoneal cavity of mice (Hortelano et al., 1996). Myoblasts, being muscle stem cells, will differentiate into myotubes inside the capsules, preventing overcrowding, and retain a higher viability after long periods in culture than do fibroblasts (Al-Hendy et al., 1995). The encapsulated

myoblasts delivered a peak of 3.8 ng/ml of human factor IX to the circulation of the mouse on day 2, before disappearing from the circulation on day 14 post-implantation. In contrast, unencapsulated recombinant myoblasts failed to deliver any detectable factor IX *in vivo*. In the mice implanted with the encapsulated cells, anti-human factor IX antibodies were detected in the circulation beginning at 2-4 weeks post-implantation and the titre was sustained until the cessation of the experiment at day 213. This sustained antibody titre suggested ongoing human factor IX delivery from the microcapsules. This study demonstrated the potential for microencapsulated cells to deliver clinically relevant levels of gene products of interest, such as human factor IX, while avoiding some of the risks associated with more conventional treatments for haemophilia such as viral infection by blood product administration. Additionally, intra-peritoneal implantation of microencapsulated cells can be achieved with a simple injection using an intravenous catheter (Al-Hendy et al., 1995, Hortelano et al., 1996).

1.3.4 Alginate Microcapsules

Alginate is a polysaccharide composed of homopolymeric regions of 1,4-linked β-D-mannuronic acid (M blocks) and α-L-guluronic acid (G-blocks). The M and G blocks are arranged randomly in the alginate among mixed sequences (M and G) of the two. The structure and molecular weight of the alginate are a function of the different kinds, ages, and parts of the seaweed used (stipe or blade) as well as the different extraction procedure used (Tanaka et al., 1983). Alginate forms a stable gel when interchain chelation occurs between the G-block components of the alginate and divalent cations such as Ca^{2+} and Ba^{2+} (Otterlei et al., 1991, Martinsen et al., 1989, Zekorn et al., 1992). The gel forming properties of alginate are strongly correlated with the proportion and length of contiguous L-guluronic acid residues in the polymeric chain, with alginate strength increasing with increasing numbers of G-blocks. Gel strength also increases in proportion to the square of the alginate concentration (Martinsen et al., 1989). Junctions are formed in the capsule membrane *via* interchain chelation of the divalent cations with the homopolymeric G-blocks.

To control the permselectivity of the capsule, the alginate bead can be coated with copolymers such as poly-L-lysine (PLL) or polyethyleneimine (PEI). In the case of PLL, the positively charged PLL molecules penetrate into the interchain regions of the alginate and react with its negatively charged carboxyl ($-COO^-$) groups (Ma et al., 1994). The molecular weight of the copolymers can affect the strength of the resulting capsule. Longer poly-L-lysine chains (higher MW) have more difficulty penetrating the alginate gel matrix resulting in fewer alginate/PLL interactions (Goosen et al., 1985). The result is a microcapsule with a larger pore size and hence increased permeability, and a greater tendency for swelling if the alginate core of the capsule is liquefied (Goosen et al., 1985, King et al., 1987, Halle et al., 1992).

In the formation of alginate microcapsules, it is the interchain chelation of cations such as Ca^{2+} or Ba^{2+} between polymeric α-L-guluronic acid (G-blocks) that causes the aqueous alginate to form a gel bead (Martinsen et al., 1989). The strength of the alginate bead that forms is directly proportional to the G-block content of the alginate. It has been calculated that alginates with an α-L-guluronic acid content greater than 70% and an average G-block length of 15, form the strongest alginate beads (Smidsrod and Skjak-Braek, 1990). Due to the strong complex formed between alginate and polycation polymers, cross-linking reagents such as poly-L-lysine (PLL) are often used to stabilize alginate gels. In the case of poly-L-lysine, the negatively charged carboxyl groups of the alginate form ionic bonds with the positively charged PLL molecules (Ma et al., 1994). This copolymer coating prevents the dissolution of the alginate microcapsules in the presence of chelating agents such as phosphates and citrate, which have high affinities for Ca^{2+} ions (Smidsrod and Skjak-Braek, 1990, Klock et al., 1994). The molecular weight of the copolymers such as PLL affects the diffusion limits of the microcapsules, with high molecular weight PLL resulting in larger pore sizes (Goosen et al., 1985). This swelling results from the inability of long PLL chains to penetrate the alginate gel matrix, which results in fewer PLL/alginate interactions. Conversely, PLL's with too small a molecular weight are too short to form bridges with the molecular chains of the alginates, resulting in a weak membrane (Ma et al., 1994). Thus one way of adjusting the strength and permeability of microcapsules with a poly-L-lysine coating is to change the molecular

weight of the copolymer being used. Studies of microcapsules prepared using PLL with molecular weights of either 14,000, 24,000 or 57,000, demonstrated that PLL with 24,000 MW formed the strongest membrane with alginate and resulted in a molecular weight cutoff of ~100,000. Such a molecular weight cutoff would be sufficient to exclude antibodies, but not necessarily complement or cytokines which could be stimulated by necrotic cellular debris leaving the microcapsules (Abbas et al., 1994).

A majority of the work on encapsulation of biomaterials has been carried out by using calcium as the divalent cation to gel an aqueous solution of alginate (Halle et al., 1992, Levesque et al., 1992, Mazaheri et al., 1991, Goosen et al., 1985, King et al., 1987, Soon-Shiong et al., 1994). These capsules, formed with a 1.2-1.8% (w/v) alginate solution are gelled in a calcium chloride ($CaCl_2$) bath. The resulting calcium alginate bead is cross-linked with a copolymer such as PLL and covered with another layer of alginate forming **A**lginate-**P**oly-L-Lysine-**A**lginate (APA) capsules. The alginate core of the microcapsule is then liquefied using a solution of sodium citrate, leaving the enclosed cells floating freely in the centre of the capsules. The resulting capsules have a molecular weight cutoff of 60-70 kD (King et al., 1987). Other divalent cations, such as barium and strontium have also been used to produce alginate microcapsules, and offer some advantages over calcium as a cross-linking reagent (Zekorn et al., 1992, Grohn et al., 1994, Klock et al., 1994., Horcher et al., 1994, Park and Khang, 1995). The barium alginate microcapsules are formed using a 2% (w/v) solution of alginate gelled in a barium chloride ($BaCl_2$) bath.

One of the benefits of the use of barium versus calcium is the higher affinity of alginate molecules for barium, resulting in a more stable hydrogel (Tanaka et al., 1988). Additionally, examination of the mitogenicity of the various components of calcium-alginate and **ba**rium-**alg**inate (**BaAlg**) microcapsules demonstrated that the cross-linking of alginate to barium reduces mitogenicity compared to cross-linking with calcium. In contrast to the formation of APA capsules, where a number of washing steps are required (Sun, 1988), the complexion of alginate by barium is a one step process, allowing for the encapsulation of large amounts of tissue in a shorter amount of time. The barium alginate microcapsules retain a solid alginate core, whereas the alginate core of the APA

capsules is liquefied. Thus, the BaAlg microcapsules are essentially solid alginate "beads", with the enclosed cells embedded in the alginate core. The higher concentration of alginate used to form this capsule type also imparts greater strength to the barium alginate microcapsules (Martinsen et al., 1989). Diffusion studies performed on BaAlg microcapsules demonstrated that these capsules were permeable to substances with a molecular weight >100 kD (Horcher et al., 1994, unpublished observation). Barium alginate microcapsules have also been used to deliver missing gene products *in vivo* (Zekorn et al., 1992, Horcher et al., 1994). As in studies using APA microcapsules, the cells enclosed in the barium-alginate capsules demonstrated prolonged secretion of their gene product to the circulation of treated animals while protected from destruction by the host's immune system.

In this study, we introduced a third type of microcapsule which represented a "hybrid" of the APA and BaAlg capsules. The **B**arium-**P**oly-L-lysine-**A**lginate (**BPA**) microcapsule was formed using a 2% (w/v) solution of alginate gelled in a $BaCl_2$ bath, like the BaAlg capsules. However, like the APA capsules, the BPA capsules were further cross-linked with a poly-L-lysine layer and covered with a second layer of alginate. The centre of the BPA capsule remained solid, leaving the enclosed cells embedded in the alginate core. By combining certain properties of the APA and BaAlg capsules, the resulting BPA capsules had the advantage of added stability due to their higher alginate concentration and solid alginate core. Additionally, the membrane permeability could be more tightly regulated using varying concentrations and reaction times with PLL (Goosen et al., 1985, Halle et al., 1992).

1.4 Immunologic Reaction to Alloantigens

The immune responses to many of the implantable devices used for gene therapy, particularly the formation of fibrosis, have been characteristic of a delayed type hypersensitivity reaction (DTH) (Abbas et al., 1994). The DTH reaction elicited by devices such as the microcapsules was predominantly mediated by $CD4^+$ T_H1 T-cells, as the antigens were probably being presented to T-cells in association with class II MHC

molecules. Initially, DTH reactions are initiated by the recognition of a MHC-associated antigen by T-cells. In the case of a class II MHC molecule, the T-cell that recognizes the antigen would be a $CD4^+$ T cell. The activated $CD4^+$ T cell secretes cytokines such as IL-2, which cause proliferation of the activated T cells and self-stimulate the $CD4^+$ T cells to also produce IFN-γ, and TNF. The IFN-γ stimulates an increase in class II MHC molecule expression on antigen presenting cells, while the TNF increases the binding and activation of leukocytes by vascular endothelial cells. The leukocytes leave the vasculature and enter the site of infection. This leukocyte activation and recruitment leads to inflammation. The IFN-γ is also a potent activator of monocytes such as macrophages, which are recruited to the site of inflammation, and is thus a vital mediator of DTH. The activated macrophages stimulate an inflammatory response *via* the secretion of inflammatory mediators such as platelet activating factor, prostaglandins and leukotrienes. In the case of chronic antigenic stimulation, such as microcapsules trapped in the omentum of an implanted animal, the cytokines and growth factors secreted by the activated macrophages modify the local tissue environment around the site of inflammation, destroying the native tissue and replacing it with fibroblasts and collagen. The secretion by macrophages of fibroblast growth factor can lead to the formation of blood vessels and the development of fibrous tissue, or scarring at the site of chronic antigenic stimulation. This fibrosis is the result of chronic DTH reaction when the antigen causing the reaction cannot be eliminated quickly. Activated macrophages themselves can undergo morphological changes during the course of chronic DTH reactions. Cytoplasm and cytoplasmic organelles increase and the macrophages fuse to form mutinucleated giant cells, producing area of granulomatous tissue located around the antigen source.

1.5 Rationale and Goals for This Thesis

The use of alginate microcapsules to deliver recombinant gene products to mice has been well documented (Chang et al., 1993, Al-Hendy et al., 1995, Hortelano et al., 1996). In these cases, microcapsules were recovered from the mice at various times post-implantation, and the retrieved capsules were free of fibrotic overgrowth. In some previous studies, immunosuppressant therapy was administered from the time of microcapsule implantation to prevent immune rejection (Mazaheri et al., 1991). In the absence of immune suppression in these cases, a fibrotic reaction to the microcapsules resulted in early graft failure. More recently, therapeutic recombinant gene products have been delivered to mice from microencapsulated cells in the absence of immune suppressant drugs, and have resulted in clinical improvements in murine models of human disease (Al-Hendy et al., 1995).

Similar studies in dogs have demonstrated the ability for microencapsulated islets of Langerhans to deliver therapeutically significant levels of insulin to the circulation of diabetic dogs (Soon-Shiong et al., 1993). In these studies, however, cyclosporin A was administered concurrently with the microcapsule implantation in order to prevent their immune rejection. The same group also implanted microencapsulated islets of Langerhans into a human patient, resulting in his eventual independence from exogenous insulin. Again, this therapy required concurrent immunosuppressant therapy.

In the experiments to be discussed in this thesis, microcapsules were implanted into the peritoneal cavities of normal dogs. The microencapsulated cells delivered human growth hormone to the circulation of the dogs. Human growth hormone was used as a marker protein in this study because of its easy detection in dog plasma using a human growth hormone specific ELISA. The following analyses were performed: human growth hormone ELISA and anti-human growth hormone ELISA using plasma from the implanted dogs, analysis of retrieved microcapsules and histological analysis of tissue from the implanted animals. Three different types of microcapsules were used to immunoisolate the human growth hormone-producing cells, and these were compared

with respect to their relative suitability as implantable devices for the purpose of gene therapy. The three microcapsule types were also analyzed extensively *in vitro*, using a number of parameters for study. The efficacy of implantation of non-autologous recombinant cells enclosed in alginate microcapsules in the absence of immunosuppressant therapy has been well established in mice (Al-Hendy et al., 1995, Hortelano et al., 1996). The goal of this study was to explore the efficacy of the implantation of non-autologous microencapsulated cells into dogs without immunosuppression . Additionally, a new type of alginate microcapsule was formulated and characterized both *in vitro* and *in vivo* . Its suitability as an alternative type of alginate microcapsule for somatic cell gene therapy was also examined. The ultimate goal of this work was to attempt to bring the field of microencapsulation and implantation of biomaterials without the need for immunosuppression one step closer to human clinical trials.

2.0 Materials and Methods

The materials and methods used for the completion of the work in this thesis are explained in detail in the two manuscripts that comprise the results section.

RESULTS

This section is divided into two chapters, each consisting of a manuscript that is in the process of being submitted for publication. Each paper is preceded by a brief summary of my specific contributions to the paper.

3.0 Properties of Different Types of Alginate Microcapsules. Peirone, M.A., Ross, C., Brash, J., and Chang, P.L. *To be submitted.*

My specific contributions to Peirone et al., 1996, *to be submitted*

Different types of alginate microcapsules were compared with respect to their physical parameters and to the condition and behavior of their encapsulated cells. I transfected a canine renal epithelial cell line, MDCK, with a plasmid encoding the human growth hormone gene. These cells were enclosed in alginate-poly-L-lysine-alginate, barium-alginate, and barium-alginate-poly-L-lysine-alginate microcapsules. During the experiment I sampled the media containing the microencapsulated MDCK cells and assayed it for human growth hormone. I released the cells from the capsules at regular intervals to evaluate the viability and rate of cell proliferation. With the help of Dr.John Brash, I used a cone and plate apparatus developed in his laboratory to measure the relative mechanical integrity of the three types of microcapsules, as shown by their resistance to a well defined fluid shearing force. By photographing the microcapsules weekly, I was able to evaluate the growth patterns of the enclosed cells.

ABSTRACT

The microencapsulation of recombinant non-autologous cells in biocompatible membranes is a method of immunoisolating biomaterials. In the immunoprotective microcapsules, the same "universal" cell line can be implanted into different hosts without rejection of the enclosed tissue. Alginate-poly-L-lysine-alginate microcapsules are one such immunoisolating device that has been employed to enclose cells secreting gene products of interest. Barium-alginate beads have also been used to enclose islet cells for *in vitro* and *in vivo* studies. Here we introduce a new microcapsule formulation, the barium-alginate-poly-L-lysine-alginate microcapsule, a hybrid that represents a combination of the alginate-poly-L-lysine-alginate and barium-alginate types. All three capsules are compared with respect to parameters such as cell viability and proliferation, permeability, mechanical integrity and biocompatibility. The results indicate that the capsules made with higher concentrations of alginate and $BaCl_2$ as a cross-linking agent are stronger than the $CaCl_2$ cross-linked alginate-poly-L-lysine-alginate type. While characteristics such as viability, proliferation and secretion rates of enclosed cells are slightly higher in the alginate poly-L-lysine-alginate microcapsules relative to the barium-alginate capsules, the cells inside the latter types proliferated rapidly and maintained a viability >90%. Additionally, molecules of varying molecular weights diffused freely from all three types of capsules.

Introduction

Microencapsulation has proven an effective method of immunoprotecting biomaterials for implantation into recipients, without the need for immunosuppressive therapy. Materials such as cells, enzymes, hormones and bioadsorbants can be protected from the extracellular environment while allowing the bi-directional exchange of small molecules such as oxygen, nutrients, wastes and cell secretions. Microencapsulation involves the enclosure of these biomaterials in devices with micrometer dimensions, whose surface area to volume ratio allows for increased loading capacities when the microcapsules are implanted into a recipient, while restricting contact between enclosed materials and immune mediators.

Lim and Sun (1980), first used microcapsules made of alginate, cross-linked with a polymer membrane and coated with another layer of alginate, to enclose pancreatic islet cells. Since then alginate microcapsules have been used for a variety of applications, particularly for the encapsulation of pancreatic islets (Halle et al., 1992, Soon-Shiong et al, 1992, 1993 and 1994, Grohn et al., 1994), purified recombinant hormones (Johnson et al., 1996) and the encapsulation of recombinant cells for the delivery of therapeutic gene products such as β-glucuronidase (Bastedo et al., 1995) and murine (Al-Hendy et al., 1995) and human (Chang et al., 1993) growth hormone.

Alginate is a polysaccharide extracted from seaweed which is 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 (Otterlei et al., 1991). Gels form due to interchain chelation of divalent cations, such as Ca^{2+} and Ba^{2+} with G-blocks in the alginate (Otterlei et al., 1991, Martinsen et al., 1989). 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). The optimal parameters for the production of strong, appropriately permeable and non-immunogenic alginate microcapsules have been well characterized by many researchers since their first

use. For a microcapsule to perform effectively, it requires an appropriate semipermeable membrane which will provide the enclosed cells with an environment compatible with cell growth, while providing an optimal immunoisolating molecular cutoff. The materials used to formulate the capsules must be biocompatible after implantation by being non-mitogenic and able to completely surround the implanted tissue.

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, coated with poly-L-lysine and another layer of alginate, and cross-linked in a calcium chloride bath (**APA** capsules). The poly-L-lysine layer allows for the strict control of the molecular weight cutoff of the microcapsule membrane (Goosen et al., 1985). The alginate core of these capsules is liquefied using sodium citrate, leaving cells floating freely in the centre of the capsule. These APA capsules have been implanted into a number of animal models (Lim and Sun, 1980, Soon-Shiong et al., 1992), and have successfully delivered recombinant gene products for varying lengths of time *in vivo*. An alternate type of microcapsule, one using a higher concentration of alginate, a different cross-linking reagent and without the poly-L-lysine and second alginate layer has also been used *in vivo* (Zekorn et al., 1992, Klock et al., 1994, Soon-Shiong et al., 1994). These capsules, formulated using a 2% (w/v) alginate-cell solution extruded into a barium chloride solution, maintain their solid alginate core and are essentially barium-alginate (**BaAlginate**) beads.

In this *in vitro* study of different types of microcapsules, we included a new type of capsule which is essentially 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 were then 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, combined with the ability to more rigidly control the molecular weight cutoff of the microcapsule membrane.

The behavior of encapsulated cells will be compared among the three types of microcapsules described above. Encapsulated cell viability, proliferation and

permeability will be assessed. Additionally, the three capsule types will be compared with respect to their ability to withstand a fluid shearing force as an indication of their relative mechanical integrity.

Materials and Methods

Large Scale Preparation of Plasmid DNA

Bacteria were grown for eight hours 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 media with 50 μg/ml ampicillin. This was allowed to grow overnight under the previously described conditions. The bacterial DNA was extracted from the growing culture using 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. Culture media was α–minimal essential medium supplemented with 10% fetal bovine serum, 1% penicillin-streptomycin and 1% L- glutamine (Gibco, BRL). The untransfected MDCK cells, (derived from a normal adult female cocker spaniel in 1958 by Madin and Danby, ATCC, Rockville, M.D., CCL-34), a canine renal epithelial cell line, were supplied by Dr.Frank Graham, McMaster University, Hamilton, Canada. The 2A50 cells secreted the lysosomal enzymes β-hexosaminidase and β-glucuronidase, and were provided by Dr. W.S. Sly (Tse et al., 1996).

Transfection of MDCK cells

Dog MDCK cells were transfected, using calcium phosphate-mediated DNA precipitation (Graham and Van der Eb, 1973) with the plasmid pNMG3. This plasmid contains 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 (Chang et al., 1990). Additionally, the phosphotransferase gene in pNMG3 allowed for the selection of transduced clones using 3 μg of G418/ml of media. The surviving clones were assayed for hGH secretion. Secretion rates of the 24 highest producing clones were determined and the highest producing clone (H8) was found to be secreting 19 ± 1.2 ng/10^6 cells/hour and was chosen for expansion in 75 cm^2 tissue culture flasks. The 2A50 cells were created by transfecting mouse Rec- fibroblasts deficient in the mannose 6-phosphate receptor with the plasmid pMSXND-MβG encoding the cDNA for dihydrofolate reductase. The recombinant cells were amplified using methotrexate selection (Tse et al., 1996).

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 cold saline and mixed with the desired volume of 1.5% alginate (for APA capsules cross-linked with $CaCl_2$) or 2% alginate (for BaAlginate and BPA capsules cross-linked with $BaCl_2$) sterile filtered with first a 0.45 μm, and then a .22 μm syringe filter (Gelman Sciences, product #4184). Approximately 2 x 10^6 cells were added/ml of sterile filtered potassium alginate (Keltone LV Lot No. 17703A, supplied by Kelco, San Diego, CA), and the mixture drawn up into a sterile 20 cc syringe. The syringe 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) at a rate of 99.9cm^3/hr x 1/100 and dispersed with a concentric air stream of 1 mm diameter delivered at 4000 cm^3/min. The resulting capsules fell into 20 ml of cold 1.1% $CaCl_2$ or 20 mM $BaCl_2$, which served to cross-link the polysaccharide polymers.

Capsule Preparation

APA: Alginate-Poly-L-lysine-Alginate Capsules

The capsules were fabricated according to the procedure of Sun et al., (1988), with some modification. Briefly, after extrusion of the cell-1.5% alginate mixture into the cross-linking 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 droplet was then 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 un-polymerized polysaccharide in the core of the microcapsule was dissolved by washing for 6 min. in 0.55 mM sodium citrate. After two rinses in serum free medium, the capsules were transferred to complete medium and kept under the normal culture conditions.

BaAlginate: Barium Alginate Capsules

The capsules were extruded into a 20mM $BaCl_2$ bath for 90 seconds while the alginate cross-linked with the barium. After 90 seconds, 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 sec. Once extrusion had finished, the capsules were rinsed 6-8 times in 3 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 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 rinsed with 0.9% NaCl. 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 with two washes of 3 capsule volumes of 0.9% NaCl, followed by two rinses with 3 volumes of serum-free medium and kept under normal tissue culture conditions.

Assay for Secreted Gene Products

hGH was measured quantitatively using an enzyme linked immunosorbent assay (UBI-Magiwell hGH Quantitative kit, United Biotech Inc., CA.) according to the instructions supplied with the kit. Unless otherwise stated, the *in vitro* determination of rate of hGH secretion was performed on culture media containing either microencapsulated or unencapsulated pNMG3 transduced cells, sampled at specific time intervals. The media was removed from the capsules or cells and they were washed with sterile PBS before being re-fed with equilibrated media. Samples were drawn from the media at 0, 1 and 2 hours and replaced with an equivalent volume of equilibrated media. From standards of known hGH concentration, the hGH values in the sampled media were estimated based upon the absorbance at an optical density (O.D.) of 405 nm recorded with a microwell reader. β-glucuronidase activity was assayed by sampling the fresh, equilibrated media of cultured cells at timed intervals and determining the amounts of gene product at each time point. These readings were plotted against the time of sample collection and the linear rate of gene product production versus time was taken to be the secretion rate in units/h/10^6 cells (Tse et al., 1996).

Characterization of microencapsulated cells

I. Viability

A small volume of capsule suspension was drawn up into a Pasteur pipette and extruded onto the surface of a clean microscope slide. Excess media was removed with a Kimwipe. A drop of 0.4% Trypan blue stain (Gibco, catalogue #15250-061) was placed

on the capsules which were then crushed with a coverslip. Viability was determined by scoring the percentage of unstained cells.

II. Cell number per capsule

A volume of 100 µl of capsule suspension containing a known number of APA capsules was transferred to an eppendorf tube. The cells were released from the microcapsules by rupturing the capsules using a small pestle (Baxter, 749520-0000). Fifteen microlitres of the resulting suspension was transferred to a haemacytometer for counting. The count was repeated five times, and the average and standard deviation were calculated.

Similarly, 100 µl of BaAlginate or BPA capsules were transferred to an eppendorf tube and approximately 100 µl of 0.5M EDTA was added to weaken or break open the microcapsules. The capsules were then further broken down by grinding with a pestle. Fifteen microlitres of the resulting suspension were transferred to a haemacytometer for counting. The count was repeated five times and the average and standard deviation were calculated.

III. Microcapsule Shearing

Using a *cone and plate apparatus* designed by the biomedical engineering shop at McMaster (Karja, 1994), microcapsules were loaded into wells at a concentration of 500 µl of capsules in 1.5 ml of media. Plexiglass cones with an angle of 4° were lowered into the wells and spun with a rotor at 333 rpm for 15, 30 and 45 min, subjecting the microcapsules to a fluid shearing force. 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. The images were stored on a Bernuille disk using the Vidas 21 software. The percentage of capsules remaining intact were 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

All statistical analyses were carried out using Microsoft Excel™. A two-tailed student's T-test was performed ($p=0.05$).

RESULTS

Microcapsule Morphology

When compared morphologically, each capsule type displayed a distinctive appearance, characteristic of its individual formulation (figure 1, capsules at D1). The APA microcapsules, with a liquefied core of alginate had their enclosed cells floating freely in the centre of the capsule. As a result, within 24 hours of enclosure in the capsules, cells came together inside the capsule and grew in a clump, often gravitating towards the inside surface of the capsule wall. The surface of the capsule wall was clearly visible under light microscopy, presumably due to the presence of the poly-L-lysine and the second alginate coat. Because alginate core was liquefied with sodium citrate in the final washing step, the microcapsules swelled from 50% to 100% of their original size, 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 second alginate coat, were distinguished from the APA capsules by the lack of a distinctive surface when examined using dark field microscopy. The enclosed cells remained distributed throughout the interior of the capsule, growing in discreet clumps. Maintenance of the solid alginate core in this capsule type prevented swelling, resulting in a smaller microcapsule (500-700 μm) than the APA type.

The BPA capsule, being a hybrid of the aforementioned types shared qualities of each capsule. 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 second coat of alginate, however, allowed for the surface of the capsule wall to be easily visualized, as in the APA capsules. Maintenance of the solid alginate core as in the BaAlginate type prevented swelling, resulting in both capsule types being about the same size.

Encapsulated cell viability

In the case of all three capsule types, viability of the encapsulated MDCK cells remained high throughout the *in vitro* experiment, although there was a statistically significant difference in viability between the APA-encapsulated cells and the barium-alginate encapsulated cells ($p<0.05$). The highest cell viability was observed in the APA-encapsulated cells, which was slightly higher than the BaAlginate and BPA-encapsulated cells (fig. 2a). There was no significant difference in viability of cells between the BaAlginate and BPA microcapsules. In the cases of all the capsules, the cell viabilities remained >90% throughout the experiment.

Viability analysis was also carried out using 2A50 cells, a fibroblast cell line secreting murine β-glucuronidase. In this cell type, there was no significant difference in the encapsulated cell viability profile among the different capsule types (fig. 2b). The cells in each type of capsule showed a similar decline in viability after 28 days *in vitro.*

Encapsulated cell proliferation

Throughout the experiment, the encapsulated MDCK cells continued to divide and proliferate inside the microcapsules. As stated before, the cells grew either in a large clump (APA capsules) or in discreet clumps throughout the inside of the capsules (BaAlginate and BPA). In all of the capsule types a similar profile of the cell proliferation rates was observed. From days 1 to 7 post-encapsulation, the number of cells inside the capsules increased by 50 to 100%, followed by a similar increase between days 7 and 14 (figure 3a). On day 21 however, the cell proliferation appears to have plateaued and in all the capsule types the cell number remained essentially unchanged between days 14 and 21 post-encapsulation. By day 28, the number of cells again increased by about 100% in all three capsule types (figure 3a.). This result suggests that encapsulation of cells in any of the microcapsule types was consistent with cell proliferation.

The 2A50 cells enclosed in the APA capsules, showed a greater rate of proliferation over 28 days *in vitro* than did the BaAlginate or BPA capsules (figure 3b). In the APA capsules, there was an increase of nearly 200% in the number of encapsulated cells between days 7 and 14 post-encapsulation, while the number actually decreased nearly 20% from day 14 until the end of the experiment on day 28. In contrast, the cell number in the BaAlginate and BPA capsules increased only 18-33% respectively, between days 7 and 14 post-encapsulation. In the BaAlginate capsules, cell number increased 43% between days 14 and 21, before declining by 23% on day 28. The rate of cell proliferation in the BPA capsules was higher than in the BaAlginate capsules, with cell number increasing by 46% between days 14 and 21, before declining by 31% on day 28 (figure 3b).

Secretion of recombinant gene products from microencapsulated cells

Unlike the viability and proliferation profiles for the different types of capsules, the cell secretion rate of human growth hormone (hGH) over 4 weeks *in vitro* varied widely among the three types (figure 4a). The cells in the APA capsules showed significantly lower rates of hGH secretion on day 1 post-implantation than both the BaAlginate and BPA capsules ($p<0.05$). The rate of human growth hormone secretion from the APA capsules peaked at day 14 before declining again on day 28. There was no significant difference in hGH secretion rate between the barium-alginate microcapsule types, BaAlginate and BPA ($p>0.05$), on day 1 and 14 post-encapsulation, while the secretion from the BPA caspules was higher than from the BaAlginate capsules by the end of the experiment on day 28. The cells in the BaAlginate capsules started with their highest secretion on day 1 post-encapsulation, before decreasing steadily throughout the duration of the experiment. The cells in the BPA capsules showed a secretion rate pattern similar to that observed in the APA cells, with a peak on day 14 (figure 4a.). Despite the variability among the different capsule types, a molecule the size of human growth hormone (MW 22 000) was able to diffuse freely from all of the microcapsules.

The secretion profile of mouse β-glucuronidase, however, was very similar among the three capsule types containing the 2A50 cells, with no significant difference in secretion profile among the three capsule types ($p>0.05$). The secretion rate from all of the capsules declined by 59-75% between days 7 and 14 post-encapsulation. From day 14 until the end of the experiment, the secretion rate remained relatively stable, showing no more significant declines (figure 4b). The similarity in β-glucuronidase secretion profiles among the different capsule types showed that a molecule the size of this enzyme (MW 300 000) was also able to diffuse freely from all of the microcapsule types.

Mechanical strength of different microcapsules

In order to assess the effects that different alginate concentrations and coatings had on the mechanical stability of the three types of capsules, each was subjected to fluid shearing forces for the same duration of time. Figure 5 demonstrates that the shearing forces generated by the cone and plate apparatus allowed for the observation of a distinct difference in the relative mechanical integrity of the three types of microcapsules. When the APA capsules were sheared for only 15 minutes, the percentage of the capsules remaining intact in the sample had decreased by 7.7%. After 45 minutes of shearing, the percentage decreased to 22.7%. In contrast, the BPA and BaAlginate capsules with 2% (w/v) alginate, had a decrease in the number of intact capsules following 15 minutes of shearing of only 0.2-1.4% respectively. After 45 minutes of shearing, neither capsule type sustained any further significant damage to capsule integrity (figure 5).

Discussion

In these *in vitro* experiments, we observed that the performance of microcapsules as immunoisolation devices for encapsulating recombinant cells showed distinct differences depending on their composition.

In the APA capsules, the liquefied alginate core allowed the enclosed cells to grow together in a clump at the periphery of the capsule. The clumping of the cells was likely due to the fact that the MDCK cells are an adherent cell type. 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 was washed first with $CaCl_2$ and then with the subsequent washing solutions (de Vos et al., 1994). It has been observed that during the production of APA microcapsules that when the K^+ in the alginate is exchanged for Ca^{2+}, the CaAlginate bead expanded and the enclosed cells moved to the periphery of the bead. Coating the CaAlginate bead in PLL results in shrinkage due to the cross-linkage of the alginate, and subsequent liquefaction of the capsule core occurs leaving the cells remaining located near the periphery of the capsule. This peripheral localization of cells would be observed less often in the solid-core $BaCl_2$ cross-linked capsules, possibly because the cells remain embedded in the alginate centre. In the case of the BaAlginate and BPA capsules, the cells grew in several discreet clumps in the capsules, never coming together into one large cell clump as in the APA capsules. In the case of the BPA capsules which also have a poly-L-lysine coat, the cells remained separated due to the maintenance of the solid alginate core, unlike the freely floating cells in the centre of the APA capsules (fig. 1).

By liquefying the alginate core of the APA capsules, this capsule type grew by 50-100% of its original size immediately after extrusion from the needle as a result of swelling. The resulting capsule size 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 were originally mixed with the alginate to make all of the different microcapsule types, the larger APA capsules would be less efficient at cell loading. 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. The implantation of large volumes of capsules may be disadvantageous when implanting into a small animal, or even human subject.

The viability of the MDCK cells inside the APA capsules was higher than in either the BaAlginate or BPA capsules. The reason for this may have been the toxicity of the $BaCl_2$, to which the cells inside the BaAlginate and BPA capsules are exposed until they are rinsed with NaCl as part of a later washing step (Park and Khang, 1995). However, even in the case of the BaAlginate and BPA capsules made using $BaCl_2$, the viability of the enclosed MDCK cells remained above 90% throughout the experiment. No significant difference in cell viability was observed between the BaAlginate and BPA-encapsulated cells. In the 2A50 cells, viability also remained relatively high throughout the first three weeks post-encapsulation before declining on day 28. No significant difference in cell viability among the three microcapsule types was observed. The decrease in cell viability observed between days 21 and 28 post-implantation may have been attributable to the fact that the 2A50 cells are extremely sensitive to overconfluence. The high nutrient demand in a crowded capsule could result in a higher rate of apoptosis among the cells near the centre of the microcapsule. The relative cell viabilities among the three types of capsules did not differ significantly throughout the experiment.

MDCK cells enclosed in APA microcapsules showed a slightly higher cell proliferation rate than did the cells in the BaAlginate or BPA capsules. The cell proliferation rates between the two barium-alginate microcapsules, however, did not differ significantly. In all of the capsules, the cell number increased by 50-100% during each week, with a temporary plateau in cell proliferation between days 14 and 21 post-encapsulation. Such a pause in cell proliferation inside of microcapsules was not a unique finding (Tai and Sun, 1993) and was not associated with a decrease in cell viability during that period. The slightly lower rate of cell proliferation inside the BaAlginate and BPA microcapsules may be attributable to the maintenance of the solid

alginate core in these capsule types. While the cells floating freely inside the liquefied core of the APA capsules had no physical constraints on their growth, the cells in the barium-alginate capsules were embedded in the alginate, and thus might be slightly more limited in their ability to expand within the available space.

The 2A50 cells, however, displayed a dissimilar cell proliferation pattern among the different microcapsules. The cell number increased rapidly in the APA capsules between days 7 and 14 post-encapsulation, before declining slightly towards the end of the experiment. In the barium cross-linked capsules the cell number increased much less rapidly until day 21 post-implantation before decreasing slightly until day 28. In the case of the 2A50 cells, this decrease in cell proliferation between days 21 and 28 post-encapsulation appeared to be associated with a corresponding decrease in the cell viability during that time.

The data concerning the release of hGH from the encapsulated MDCK-H8 cells showed a different secretion "profile" from each type of microcapsule. The capsules with the poly-L-lysine and outer alginate coats, APA and BPA, showed a peak in hGH delivery on day 14 post-encapsulation, with lower secretion rates on both day 1 and 28. The BaAlginate capsules started with the highest secretion rate on day 1 post-encapsulation followed by a gradual decline in hGH release from that type of capsule until day 28. The reasons for the differences in hGH secretion between the APA and the barium-alginate types of capsules are not known. It is clear though, that all three capsule types have sufficiently large pore size to allow for the diffusion of nutrients into the capsules, as evidenced by the high cell viability, while allowing the diffusion of the 22 kD hGH out of the capsules. Studies comparing the three types of 2A50 cell-containing capsules with respect to their permeability to murine β-glucuronidase showed that this 300 kD molecular weight enzyme also diffused freely from each type of capsule. The secretion rate profile did not differ significantly among the APA, BaAlginate and BPA capsule types.

The cone and plate apparatus used to measure the relative mechanical strengths of the APA, BaAlginate and BPA microcapsules highlighted the most distinctive difference among the capsule types. The fluid shearing forces that each capsule type was subjected

to for varying lengths of time showed a distinct strength difference between the capsules made from differing alginate concentrations. Under the same conditions of fluid shear, 18.4%-22.3% of the APA capsule broke after 45 minutes of shearing. In contrast, only 1.1%-1.3% of BaAlginte capsules and 0.7%-1.8% of BPA capsules broke in the same amount of time. It has been documented elsewhere that alginate beads made from the highest alginate concentration are the most stable (Martinsen et al., 1989). 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 that use $BaCl_2$ as a cross-linking agent (Klock et al., 1994). The increased durability of the BaAlginate and BPA capsules compared to the 1.5%-alginate APA type, would make the former capsule types more suitable candidates for long-term implantation studies.

This *in vitro* study highlights the efficacy of microencapsulation of cells as a benign method for cell entrapment. The three types of microcapsules studied showed little difference with respect to encapsulated cell viability, proliferation or permeability. However, there was a marked difference in the relative strength of the different capsules determined by the concentration of alginate and the cross-linking reagents used. Higher alginate concentrations, and reagents such as $BaCl_2$, which have a high affinity for alginate produced more stable microcapsules that were better able to withstand damaging mechanical forces. A comparison of these three types of microcapsules *in vivo* would determine their relative suitability for applications such as somatic cell gene therapy (Peirone et al., 1996).

Figure 1. Morphology of the three types of microcapsules. MDCK cells, encapsulated at an initial cell density of 2 x 10^6 cells/ml of alginate were photographed inside of each microcapsule type on day 1 post-encapsulation, and the proliferation of the MDCK cells inside the capsules over 28 days *in vitro* is shown. The surface of the APA and BPA microcapsules can be visually distinguished from the BaAlginate capsules by the presence of the poly-L-lysine and second alginate layers (see white arrows). In the absence of this layer, the surface of the BaAlginate cannot be seen using the dark field photomicroscope. Microcapsules in photographs are magnified approximately 1000x.

Figure 1

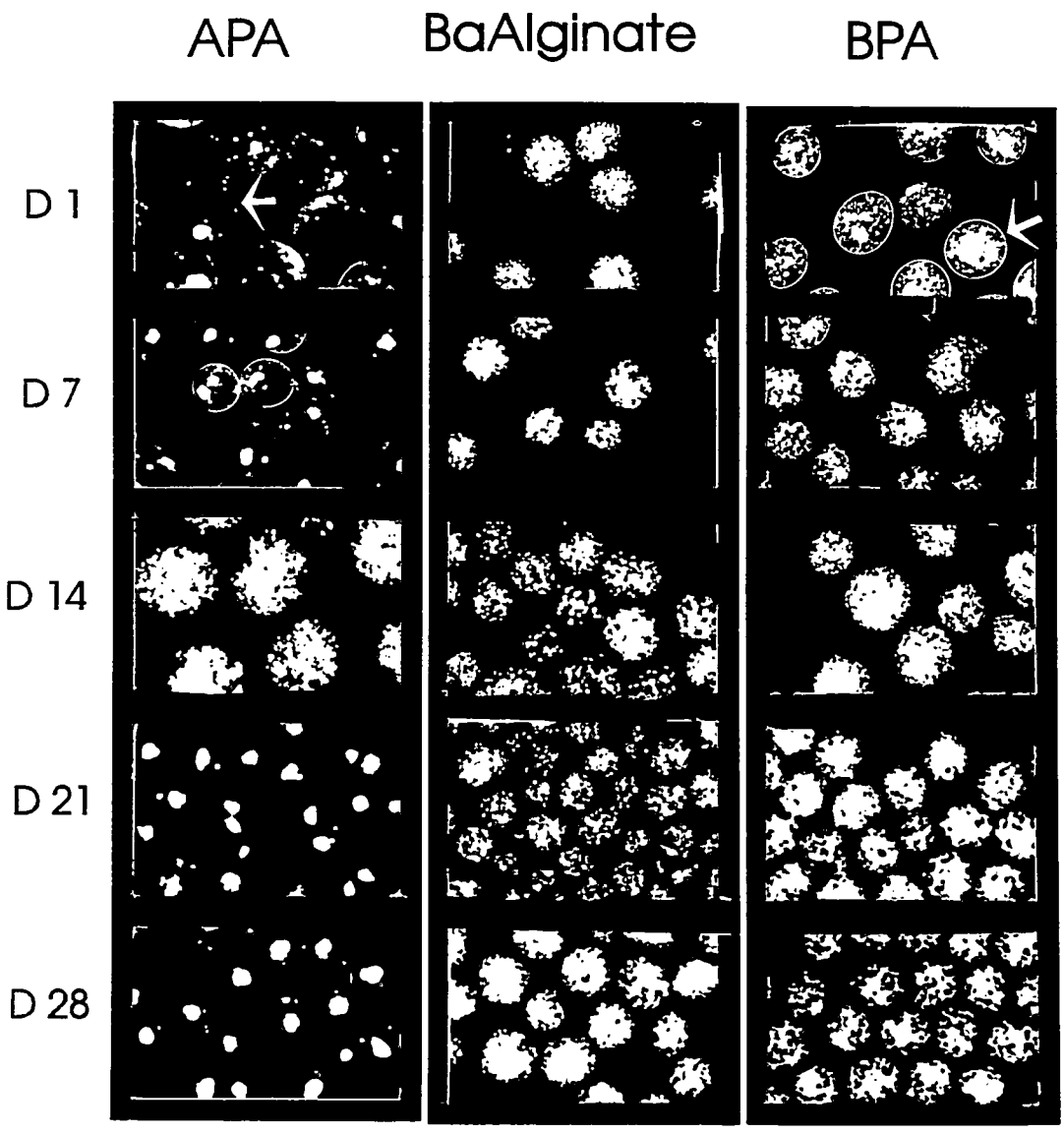


Figure 2. Viability of microencapsulated cells. The viability of the encapsulated cells was measured each week for 4 weeks by crushing the microcapsules and releasing the cells, which were then scored by trypan blue exclusion staining as in Materials and Methods. (a) Viability of the MDCK cells in the APA microcapsules was significantly greater than in the BaAlginate or BPA microcapsules at each time point measured ($p<0.05$, using 2-tailed student's T-test). There was no significant difference in viability between the cells in BaAlginate and BPA capsules. (b) In the case of the 2A50 cell type, there was no significant difference in cell viability among the three microcapsule types. Data represent the average of 5 measurements $\pm$ SD.

figure 2a

Encapsulated Cell Viabilities

MDCK Cells

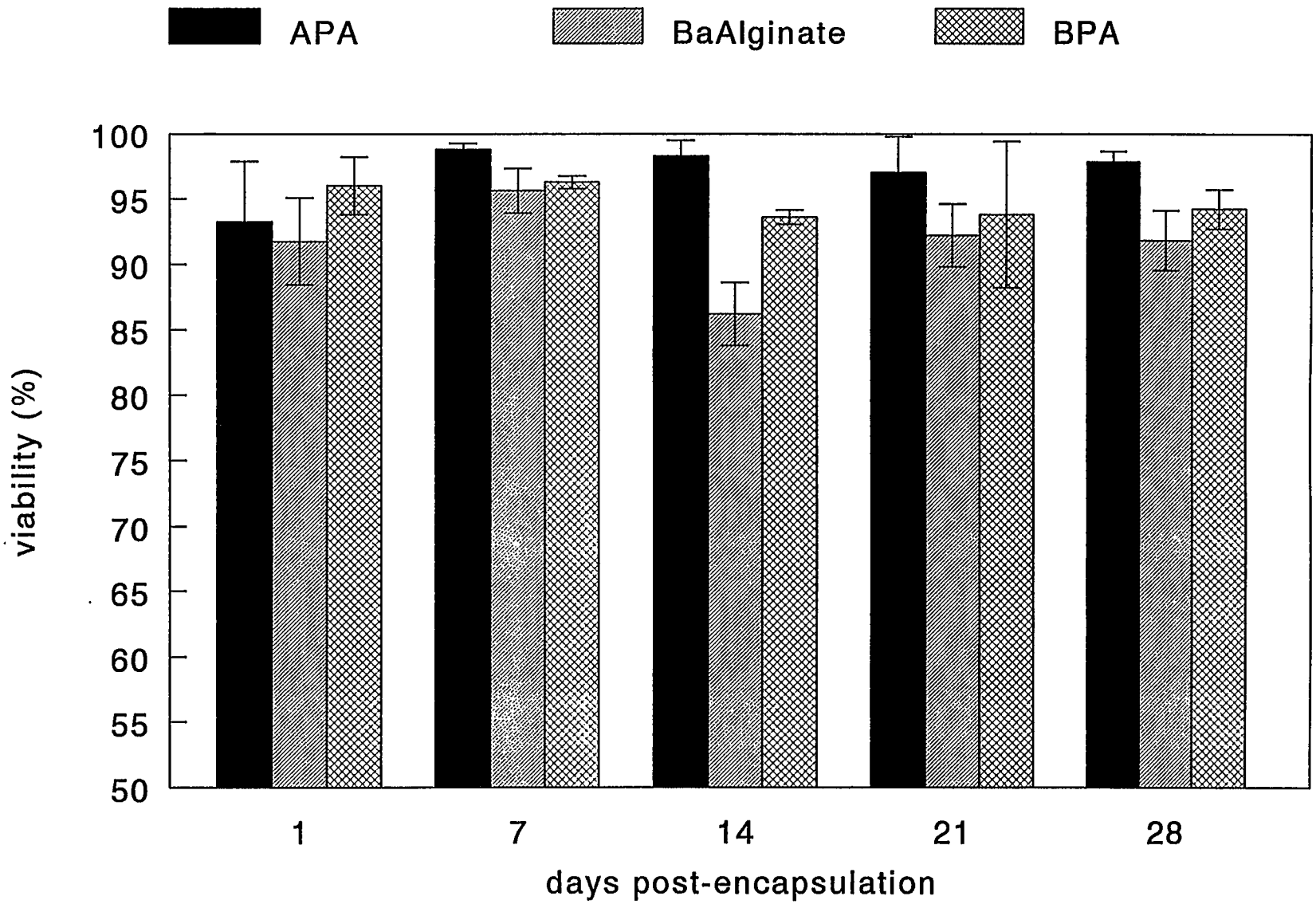


figure 2b

Encapsulated Cell Viabilities

2A50 Cells

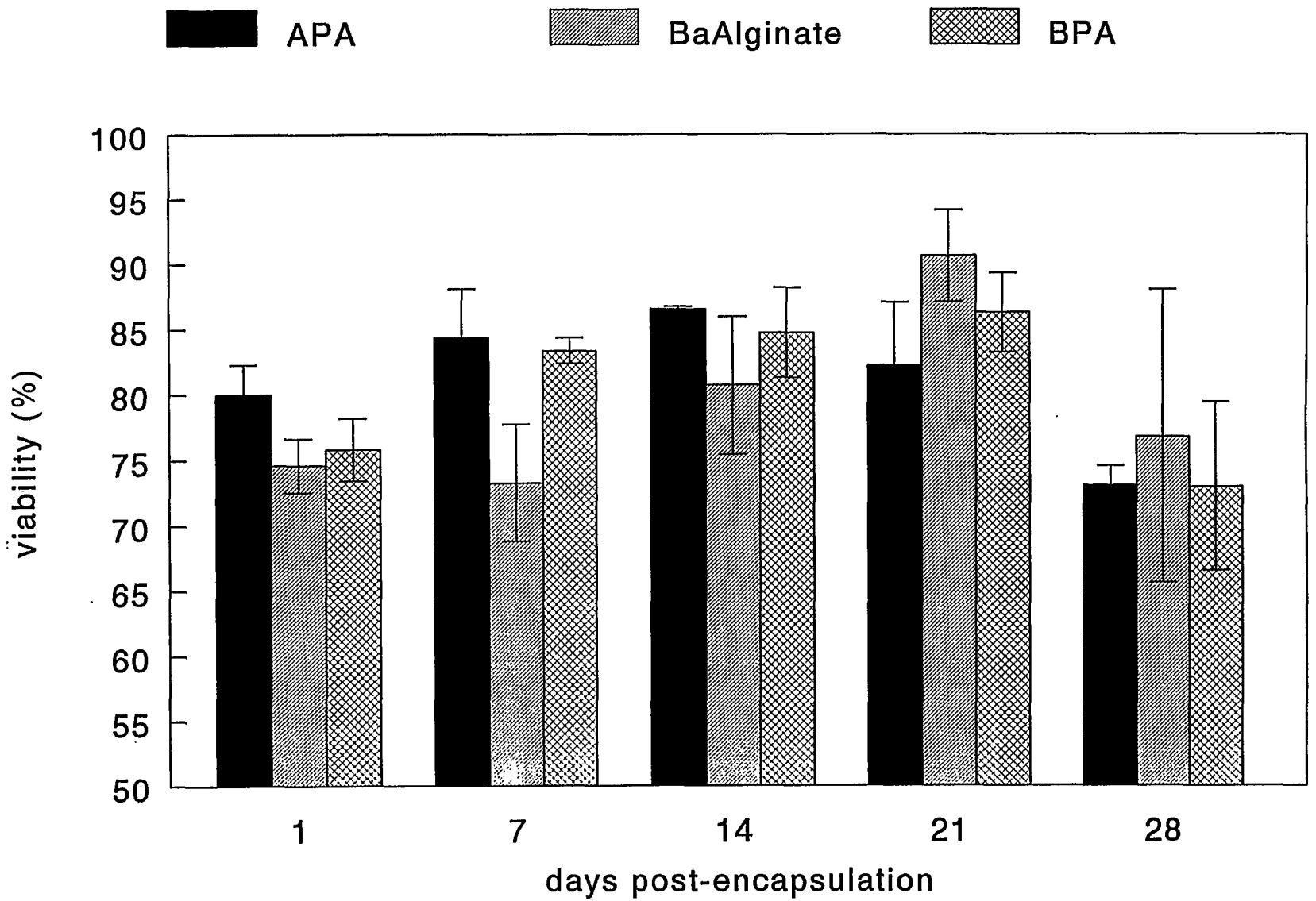


Figure 3. Proliferation of recombinant cells within microcapsules. (a) The cell proliferation profiles of MDCK cells inside each type of microcapsule was calculated each week for 4 weeks (see fig. 1) by releasing cells from the microcapsules and counting them using a haemacytometer, as in the Materials and Methods section. The MDCK cells proliferated more rapidly inside the APA capsules than in the BaAlginate and BPA capsules. There was no significant difference in cell proliferation between the two types of barium-alginate microcapsules (b). The cell proliferation profiles for the 2A50 cells is also shown. Cells proliferated most rapidly inside the APA capsules, followed by cells inside the BPA capsules, and finally proliferated most slowly inside the BaAlginate capsules. Data represent the average of 5 measurements $\pm$ SD.

figure 3a

Encapsulated Cell Proliferation

MDCK Cells

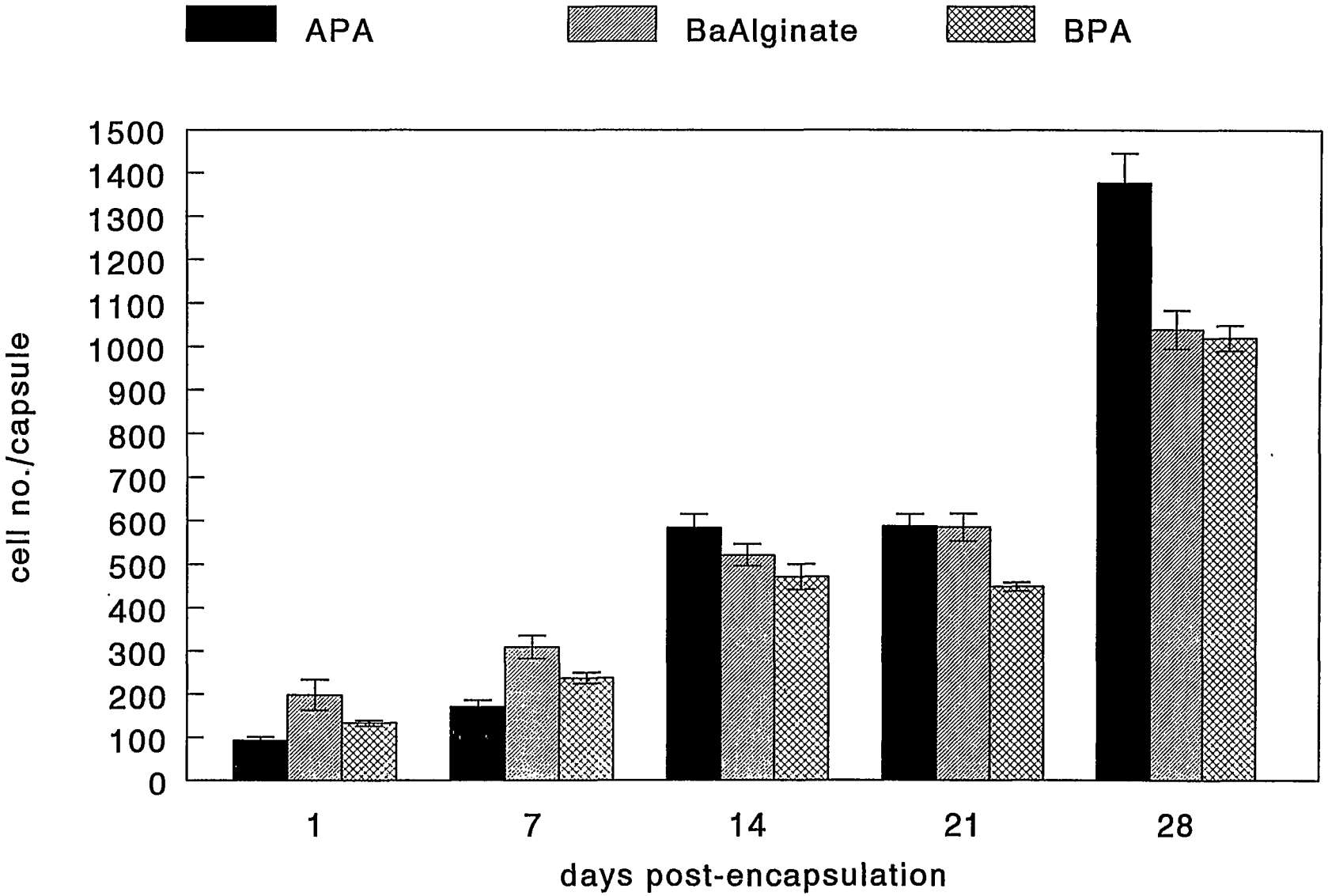


figure 3b

Encapsulated Cell Proliferation

2A50 Cells

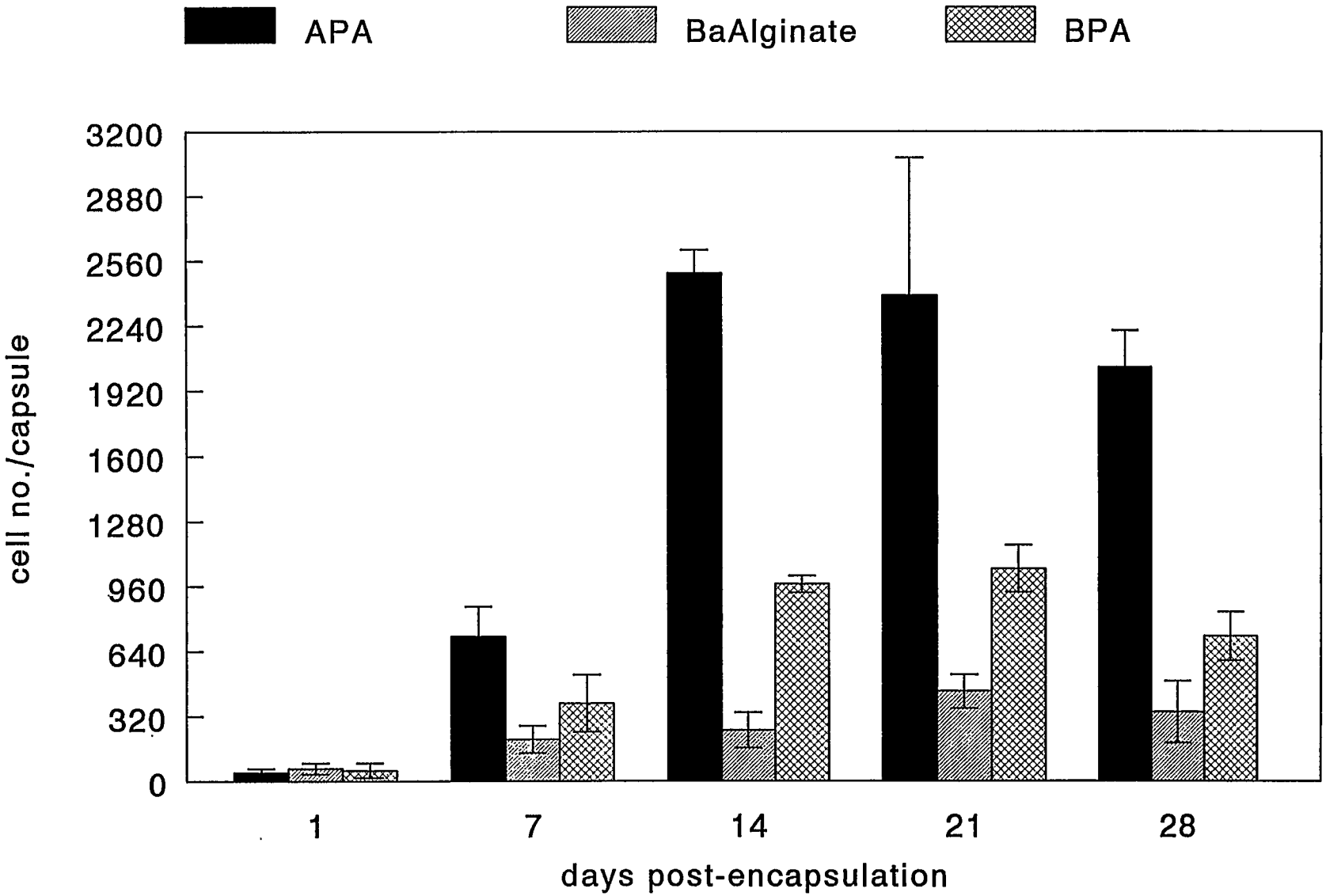


Figure 4. Secretion by microencapsulated recombinant cells. (a) On days 1 and 14 post-encapsulation, there was a significant difference in the rates of secretion of hGH between the APA and barium-alginate microcapsule types, but no significant difference in secretion between the barium-alginate types. On day 28, there was no significant difference in secretion between the APA and BPA capsule types, but the rate of secretion form the BaAlginate capsules was significantly lower than from the other two types. Secretion of human growth hormone from both the APA- and BPA-encapsulated cells peaked on day 14, while the secretion from the BaAlginate-encapsulated cells declined slowly throughout the experiment. (b) There was no significant difference in secretion of β-glucuronidase from cells inside of each of the three microcapsule types. Data represent the average of 4 measurements $\pm$ SD.

figure 4a

Encapsulated Cell Secretion Rates

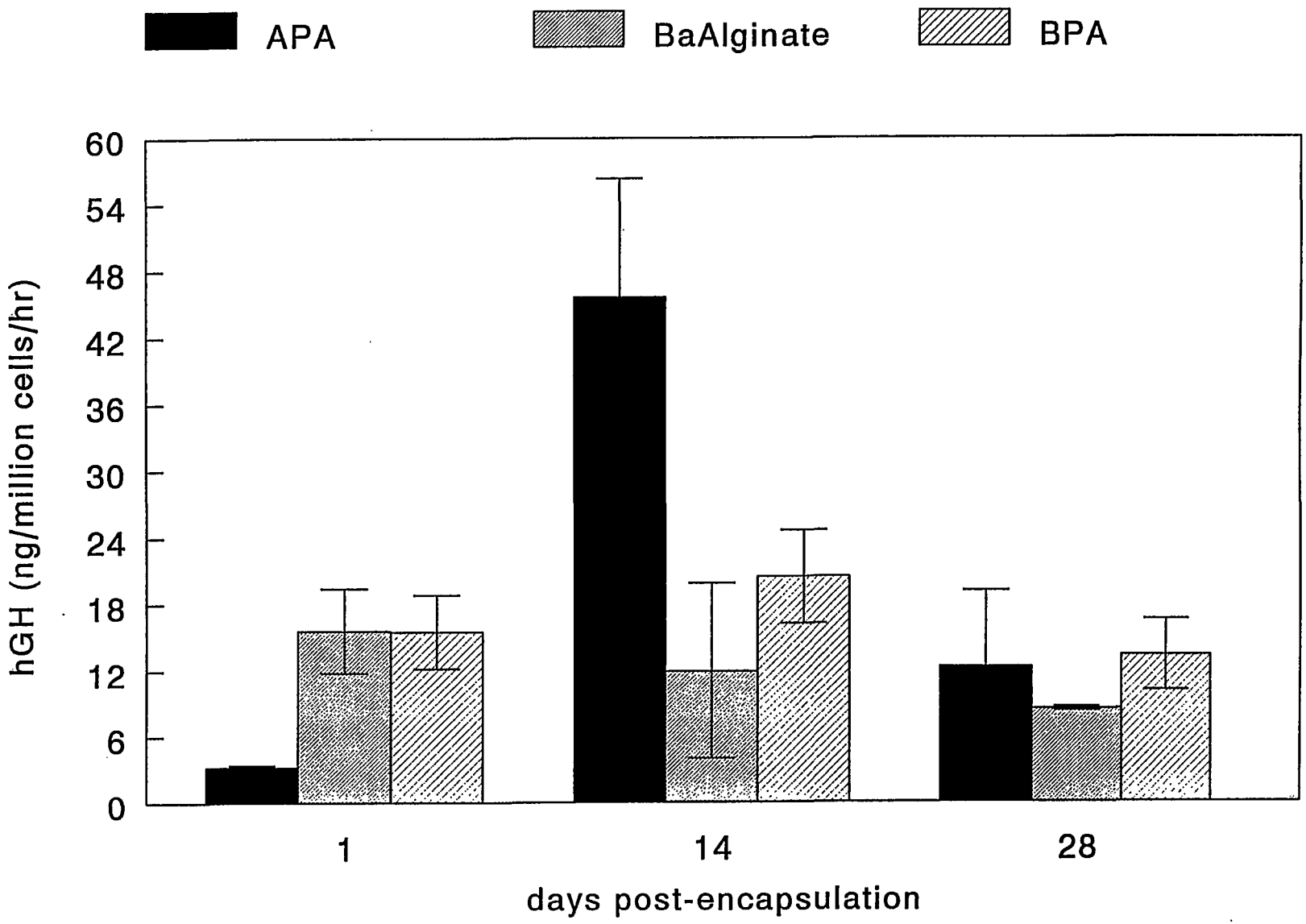


figure 4b

Encapsulated Cell Secretion Rates

2A50 Cells

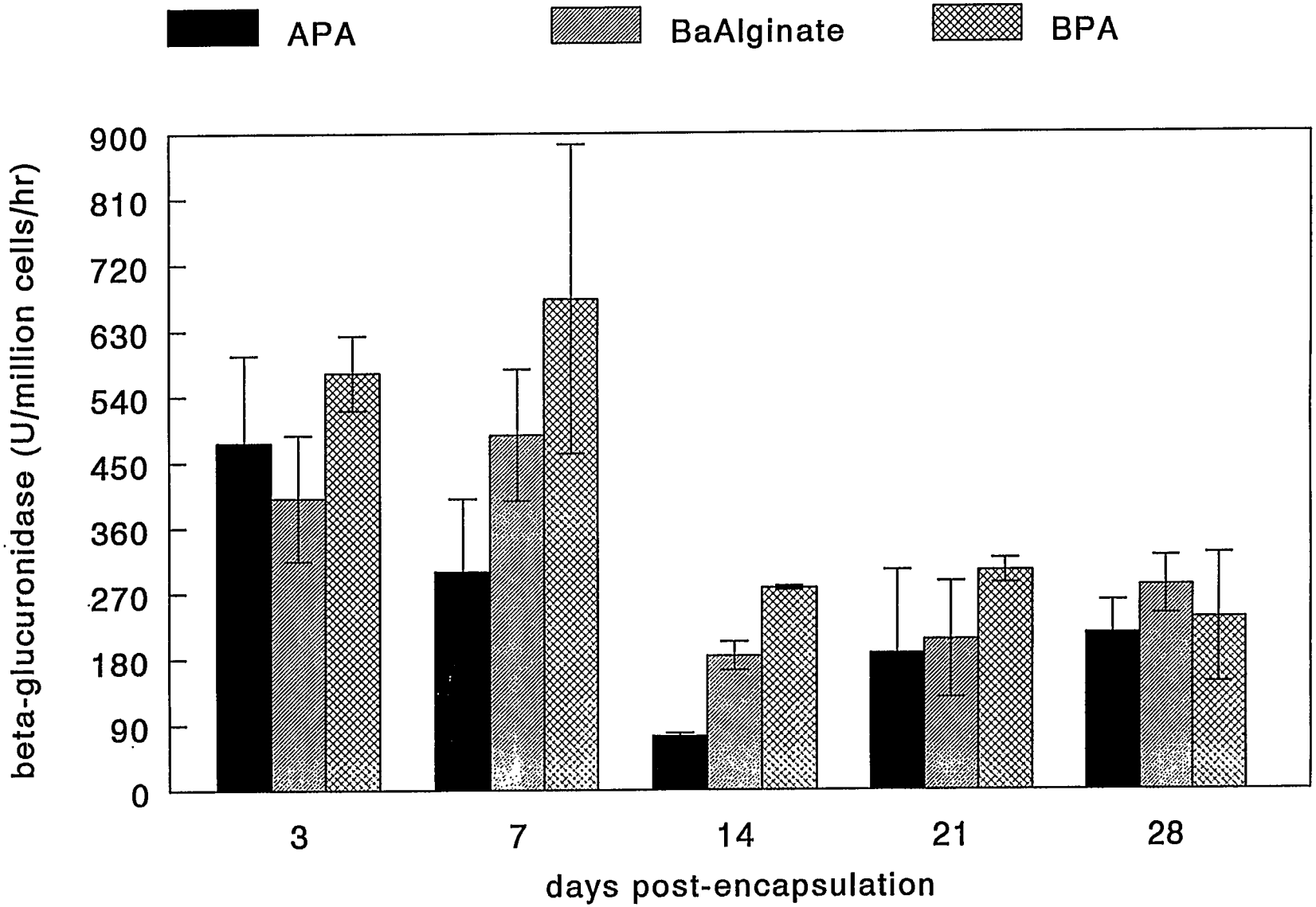
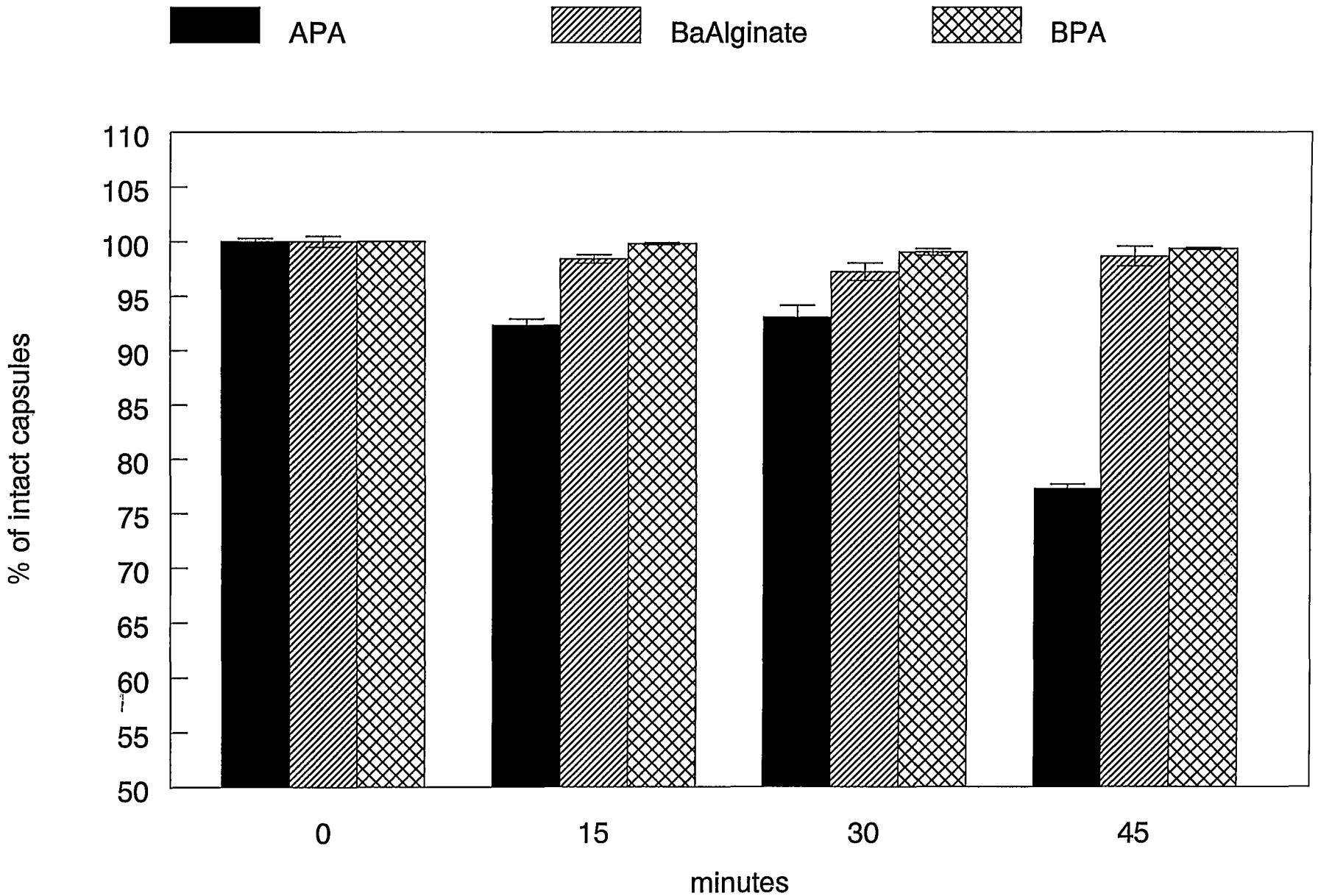


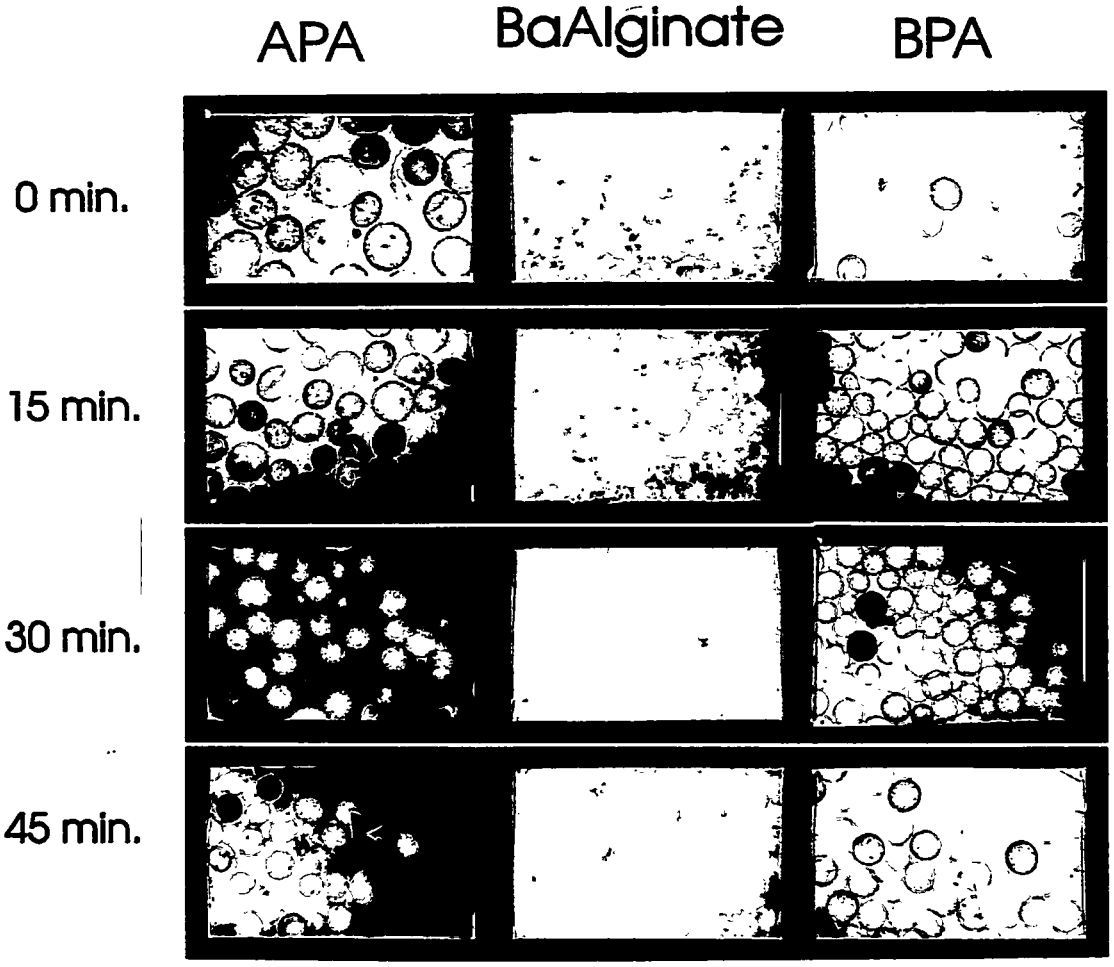
Figure 5. Shearing of microcapsules using the cone and plate apparatus. (a) APA microcapsules showed significant breakage after being subjected to a fluid shearing force for 0, 15, 30 and 45 min. The barium-alginate microcapsule types remained virtually undamamged throughout the shearing experiment. (b) Morphological changes in the capsules after shearing for various times was recorded using a Sony CDD video camera and attached to a Zeiss light microscope, as described in Materials and Methods. Black arrow in APA capsule sample at 45 min. shows a microcapsule broken by the fluid shearing force. Microcapsules in photographs are magnified approximately 800x. Data represents an average of three measurements $\pm$ SD.

figure 5a

Microcapsule Shearing



igure 5b.



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4.0 Delivery of Recombinant Human Growth Hormone to Dogs Using Microencapsulated Cells. Peirone, M.A., Delaney, K., and Chang, P.L. *To be submitted*

My specific contributions to Peirone et al., 1996, *to be submitted*

Non-autologous somatic gene therapy involves the delivery of recombinant gene products by a universal cell line enclosed in immunoprotective, perm-selective microcapsules. A canine renal epithelial cell line was transfected with the human growth hormone cDNA. Once I had transfected an MDCK cell line, I enclosed it in alginate microcapsules of various formulations which permitted the free diffusion of the growth hormone out of the capsule. With the help of Dr. K. Delaney, the microcapsules were implanted intraperitoneally into dogs. During the experiment, I collected blood samples from the dogs at regular intervals and assayed them for human growth hormone as well as human growth hormone specific antibodies. The microcapsules that I retrieved periodically were examined with respect to secretion rate, cell growth and viability of the enclosed cells. Additionally, with the help of Dr. J. Brash the mechanical stability of the retrieved microcapsules was compared to *in vitro* capsules to evaluate the effects of intraperitoneal implantation.

ABSTRACT

The ideal method for gene therapy using somatic cells would be to take cells from the patient, engineer them to secrete a desired product and reintroduce them into the patient, avoiding any potential immune rejection. However, if a universal cell line can be created that delivers the gene product of interest, it could be enclosed in immunoprotective alginate microcapsules and implanted into a number of patients, saving both time and considerable cost. The clinical efficacy of this approach to somatic gene therapy has been demonstrated by the implantation of microencapsulated recombinant cells to correct mutant phenotypes in murine models of human diseases such as lysosomal storage disease (Bastedo et al., 1995) and dwarfism (Al-Hendy et al., 1995). To explore the feasibility of this approach to large animal models, we created a canine MDCK cell line secreting over 19 ng of human growth hormone (hGH)/10^6 cells/hr through immunoisolating alginate microcapsules. When implanted into normal dogs, a peak of >20 ng human growth hormone could be detected in the plasma between days 1 and 14 post-implantation by immunoassay. Disappearance of the human growth hormone from the circulation of the dog corresponded with the appearance and increase of anti-human growth hormone antibodies, suggesting prolonged secretion. Additionally, alginate microcapsules made with different alginate concentrations and cross-linking reagents were compared. It was found that solid spheres made of 2% (w/v) alginate and cross-linked with $BaCl_2$ lasted for almost 8 weeks longer *in vivo* than hollow, $CaCl_2$ cross-linked capsules made with 1.5% (w/v) alginate.

INTRODUCTION

With the number of clinical trials of somatic gene therapy protocols growing, cost-effectiveness and efficacy of a treatment are nearly as important as technical issues such as the creation of a stable, high producing recombinant cell lines as well as long-term expression. If the use of immunosuppressive agents is to be avoided in human clinical trials of gene therapy, autologous human cells must be harvested from each individual patient before engineering and reintroduction to avoid graft rejection. Not only is this strategy difficult, considering the technical challenges of culturing harvested tissue, but certain commonly used human cell types such as fibroblasts will not grow indefinitely in culture (Rohme, 1981). In addition to being labour-intensive, this approach is likely to be cost prohibitive.

An alternative approach to somatic gene therapy is to develop a genetically-modified universal cell line, which can deliver the desired therapeutic gene product *in vivo* to different recipients (Chang, 1995). Once the immortalized cell line has been engineered to secrete the desired gene product, it can be stored frozen, and thawed to expand in culture as needed. To prevent immune rejection of the universal cell line after implantation, the cells can be enclosed in immuno-protective microcapsules that are permeable to the gene product being delivered but not to the host's high molecular weight immune mediators (>150kD). In essence this approach uses cells secreting engineered gene products as "drugs" that can be administered to a number of different patients regardless of the patient's histocompatibility tissue type. This approach addresses the high costs associated with creating patient-specific recombinant cells for implantation.

In 1964, Chang first proposed the idea of enclosing biological materials into perm-selective membranes as a way of immuno-isolating the enclosed tissue. Materials such as alginate, a polysaccharide extract of brown algae (Smidsrod, 1974) have been used to enclose cells and make them biocompatible. We have demonstrated the ability to deliver recombinant gene products both *in vitro* and *in vivo* using cells enclosed in perm-selective alginate-poly-L-lysine alginate microcapsules. High levels of biologically

active human factor IX were secreted by encapsulated Ltk$^-$ mouse fibroblasts (Lui et al., 1993) which maintained high viability and continued to proliferate inside microcapsules for over three weeks *in vitro*. The same cell line transfected to secrete human growth hormone was also able to deliver that gene product to the circulation of rodents for over 100 day post-implantation (Chang et al., 1993). The efficacy of this gene delivery system for the treatment of animal models of human disease has also been demonstrated. Microencapsulated myoblasts secreting murine growth hormone were implanted into Snell dwarf mice, resulting in an enhancement in growth (Al-Hendy et al., 1995). Additionally, microencapsulated fibroblasts secreting murine β-glucuronidase were implanted into gus^{mps}/gus^{mps} mice with lysosomal storage disease, resulting in partial correction of the mutant phenotype (Bastedo, 1995).

The first of the capsule types, calcium alginate-poly-L-lysine-alginate microcapsules (**APA**) was formulated using 1.5% (w/v) potassium alginate, cross-linked in calcium chloride bath. Poly-L-lysine and another layer of alginate were added and the centre core of alginate was liquefied using sodium citrate, leaving the enclosed cells floating freely in the centre of the capsule. The diffusion limit for this capsule has been calculated to be ~60-70 kD (King et al, 1987) allowing for the free diffusion of the 22 kD human growth hormone. This capsule type has been used to deliver a variety of products such as insulin from pancreatic islets (Lim and Sun, 1980, Halle et al., 1992, Soon-Shiong et al., 1992) and human (Chang et al., 1993) and murine (Al-Hendy et al., 1995) growth hormone, among others. When this type of microcapsule failed to remain intact in an *in vivo* pilot study in the dog (unpublished data), new microcapsule formulations were tested as more appropriate delivery systems for this large animal model.

The second capsule type was made with 2% (w/v) potassium alginate and cross-linking with barium chloride (**BaAlginate**). The alginate core remains solid leaving the cells embedded in the alginate (Ma et al., 1994). Additionally, there is no poly-L-lysine or second alginate coat added. The diffusion limit for this type of capsule has been reported to be >100kD, allowing the permeability of molecules with high molecular weights (Horcher et al., 1994). A molecule the size of human growth hormone can be released without impediment. Preliminary studies using this capsule type in our lab have also

demonstrated the ability of human factor IX (MW 57 000) and mouse β-glucuronidase (MW 300 000) to be secreted from the BaAlginate microcapsules (personal communication, G.Hortelano, C.Ross). This type of microcapsule has also been used for the delivery of insulin from microencapsulated pancreatic islets (Horcher et al., 1994, Zekorn et al., 1992).

The third type of microcapsule used in this study represented a hybrid of the two previously described capsules. The barium alginate-poly-L-lysine-alginate (**BPA**) capsules were formed using 2% (w/v) potassium alginate extruded into a barium alginate bath. These capsules then received a layer of poly-L-lysine and a second layer of alginate. The alginate core remained solid in this type of capsule as well, keeping the enclosed cells embedded in the alginate. The benefits of this BPA "hybrid" capsule was the added stability of the more concentrated alginate core combined with the more controlled membrane permeability afforded by the poly-L-lysine layer. Barium is used as a divalent cation to cross-link with the alginate in the BaAlginate and BPA capsules due to its higher affinity for alginate compared with calcium (Klock et al., 1994).

To determine the efficacy of this technology for large animal models, we implanted microcapsules containing cells delivering recombinant human growth hormone intraperitoneally into phenotypically normal dogs. In an effort to prolong the survival of the microcapsules *in vivo*, different formulations of microcapsules were implanted. These capsules were evaluated with respect to their relative mechanical stability, and ability to allow for the diffusion of the growth hormone from the microcapsules while preventing rejection of the enclosed cells by the recipient's immune system. By implanting these capsules into the dog model, we were also able to address the technical challenges associated with the progression of this technology from the small animal rodent model towards the stage of human clinical trials.

MATERIALS AND METHODS

Animals

All animals used were obtained through the Central Animal Facility at McMaster University in the Health Sciences Centre. The two dogs used for anti-hGH antibody production were pound-source dogs of mixed breed, each with a mass of about 30 kg. The animals used for the large-scale dog implantation study were 8-12 kg pure-bred, phenotypically normal Beagles, purpose bred and supplied by the University of Guelph.

Large Scale Preparation of Plasmid DNA

Bacteria were grown for eight hours in a 15 ml centrifuge tube containing 5 ml of Luria Broth 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 media with 50 μg/ml ampicillin. This was allowed to grow overnight under the previously described conditions. The bacterial DNA was extracted from the growing culture using the Mini-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. Culture media was α– minimal essential medium supplemented with 10% fetal bovine serum, 1% penicillin-streptomycin and 1% L- glutamine (Gibco, BRL). The untransfected MDCK cells were supplied by Dr.Frank Graham, McMaster University, Hamilton, Canada. The MDCK cell line was derived from a normal adult female cocker spaniel in 1958 by S.H. Madin and N.B. Danby. It

has been determined that these epithelioid cells multiply about 15-fold in 1 week (ATCC, Rockville, M.D., CCL-34).

Transfection of MDCK cells

Dog MDCK cells were transfected, using phosphate-mediated DNA precipitation (Graham and Van der Eb, 1973) with the plasmid pNMG3. This plasmid contains 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 (Chang et al., 1990). Additionally, the phosphotransferase gene in pNMG3 allowed for the selection of transduced clones using 3 μg of G418/ml media. The surviving clones were assayed for hGH secretion. Secretion rates of the 24 highest producing clones were determined and the highest producing clone (H8) was found to be secreting 19 ± 1.2 ng/10^6 cells/hour and was chosen for expansion in 75 cm^2 tissue culture flasks.

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 with 0.125% trypsin, re-suspended in 500 μl cold saline and mixed with the desired volume of 1.5% alginate (for APA capsules cross-linked with $CaCl_2$) or 2% alginate (for BaAlginate and BPA capsules cross-linked with $BaCl_2$) sterile filtered with first a 0.45 μm, and then a .22 μm syringe filter (Gelman Sciences, product #4184). Approximately 2 x 10^6 cells were added per ml of sterile filtered potassium alginate (Keltone LV Lot No. 17703A, supplied by Kelco Co., San Diego, CA), and the mixture drawn up into a sterile 20 cc syringe. The syringe 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) at a rate of 99.9cm^3/hr x 1/100 and dispersed with a concentric air stream of 1 mm diameter delivered at 4000 cm^3/min. The resulting capsules were

collected into 20 ml of cold 1.1% $CaCl_2$ or 20 mM $BaCl_2$, which served to cross-link the polysaccharide polymers.

Capsule Preparation

APA: Alginate-Poly-L-lysine-Alginate Capsules

The capsules were fabricated according to the procedures of Sun et al., 1988, as modified by Lim et al., 1993. Briefly, after extrusion of the cell-1.5% alginate mixture into the cross-linking 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 droplet was then further cross-linked with 0.05% poly-L-lysine (PLL, MW 22 000 from Sigma Chemical Co.) 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 un-polymerized polysaccharide in the core of the microcapsule was dissolved by washing for 6 min. in 0.55 mM sodium citrate. After two rinses in serum free medium, the capsules were transferred to complete medium and kept under the normal culture conditions.

BaAlginate: Barium Alginate Capsules

The capsules were extruded into a 20 mM $BaCl_2$ bath for 90 seconds while the alginate cross-linkeded with the barium. After 90 seconds, 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 sec. Once extrusion had finished, the capsules were rinsed 6-8 times in 3 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 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

collected into 20 ml of cold 1.1% $CaCl_2$ or 20 mM $BaCl_2$, which served to cross-link the polysaccharide polymers.

Capsule Preparation

APA: Alginate-Poly-L-lysine-Alginate Capsules

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BPA: Barium-Poly-L-lysine Alginate Capsules

The capsules were prepared as above, 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 rinsed with 0.9% NaCl. 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 with two washes of 0.9% NaCl equivalent to 3 volumes of packed capsules, followed by two rinses with 3 volumes of serum-free medium and kept under normal tissue culture conditions.

Characterization of cells recovered from microcapsules in vitro

I. Viability

A small volume of capsule suspension was drawn up into a Pasteur pipette and extruded onto the surface of a clean microscope slide. The accompanying media was drawn from the capsules using by capillary action using a Kimwipe. A drop of 0.4% Trypan blue stain (Gibco, catalogue #15250-061) was placed on the capsules which were then crushed with a coverslip. Cell viability in the sample was determined by scoring the percentage of cells in the sample that excluded the trypan blue stain.

II. Cell number per capsule

A volume of 100 µl of capsule suspension containing a known number of APA capsules was transferred to an eppendorf tube. The cells were released from the microcapsules by rupturing the capsules using a small pestle (Baxter, 749520-0000). Fifteen microlitres of the resulting suspension was transferred to a haemacytometer for counting. The count was repeated five times, and the average and standard deviation were calculated.

Similarly, a volume of BaAlginate or BPA capsules were transferred to a tube and approximately 100 µl of 0.5M EDTA was added to weaken or break open the microcapsules. The capsules were then further broken down by grinding with a pestle. 15 µl of the resulting suspension were transferred to a haemacytometer for counting. The count was repeated five times and the average and standard deviation were calculated.

III. Microcapsule Shearing

Using a cone and plate apparatus designed by the biomedical engineering shop at McMaster University (Karja, 1994), microcapsules were loaded into wells at a concentration of 500 µl of capsules in 1.5 ml of media. Plexiglass cones with an angle of 4° were lowered into the wells and spun with a rotor at 333 rpm for 15, 30 and 45 min, subjecting the microcapsules to a fluid shearing force equivalent to $\gamma = 498$ (where g = cone rotation rate (rads/sec)/cone angle (rads)). A sample of the microcapsules were taken at each time point, photographed using a Sony CDD colour video camera attached to a Zeiss light microscope and the images were stored on a Bernuille disk using the Vidas 21 program. The percentage of capsules remaining intact were 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 as the percentage of capsules remaining intact with increasing duration of shear.

Assay for Secreted Recombinant Gene Products

Human growth hormone was measured quantitatively using an enzyme linked immunosorbent assay (UBI-Magiwell hGH Quantitative kit, United Biotech Inc., CA.) according to the instructions supplied with the kit. Unless otherwise stated, the *in vitro* determination of hGH was performed on culture media containing the pNMG3 transduced cells, sampled at specific time intervals. Briefly, the media was removed from the cells which were then washed with sterile PBS before being re-fed with equilibrated media. Samples were drawn from the media and replaced with an equivalent volume of equilibrated media. From standards of known hGH concentration, the hGH values in the sampled media were estimated based upon the absorbance readings at an O.D. of 450 nm recorded with a microwell reader.

Immunization of Dogs With Human Growth Hormone

In order to provide a positive control for the anti-human growth hormone antibody ELISA, two pound source mixed-breed dogs were injected sub-cutaneously with a total of 1ml of the antigen. Five hundred micrograms of recombinant human growth hormone (rhGH, brand name Humatrope, supplied by Eli Lilly Indianapolis, Indiana) in 0.85% saline (0.5 mg/ml) was mixed mixed 1:1 (v/v) with Freund's Complete Adjuvant (FCA). Four 0.25 cc injections were given on either side of the dorsal surface of the dogs' neck, in accordance with the Guidelines for the Use of Immunization Adjuvants, Central Animal Facility, McMaster University. Booster injections using 0.5 mg/ml of rhGH suspension mixed 1:1 with Freunds Incomplete Adjuvant (FIA) were administered 3 and 8 weeks after the first injection, followed by 0.5 mg/ml of rhGH suspension mixed 1:1 with PBS on week 11. Twelve weeks after the initial injection, 100 ml of blood was taken from each dog *via* a butterfly needle inserted into the jugular vein following shaving the area and wiping it with an alcohol swab. The blood was collected into 50 ml centrifuge tubes containing 10 % (v/v) sodium citrate as an anticoagulant. The blood samples were centrifuged at 3000 g for 10 min. at 4°C and the plasma was collected and stored at -70°C.

Microcapsule Implantation

Capsules were removed from the incubator and washed 10 to 12 times with 3 volumes of sterile PBS warmed to 37°C. Once all visible traces of media were removed, the PBS was removed and the capsules remained on ice until needed. The bladder was expressed by applying manual external pressure. Following clipping the hair from the abdomen, the animals were prepared for aseptic surgery. In all implantations, a 2 cm skin incision was made down to the linea alba, caudal to the umbilicus using a sterile scalpel blade. The linea alba was perforated using the scalpel blade. An 11 guage French catheter with the stilette removed was inserted into the peritoneal cavity. Microcapsules were drawn up into a sterile 60cc syringe and the syringe attached to the cannula. The

capsules were injected into the peritoneal cavity at a rate of ~0.5 ml/sec., using the cannula to direct the flow of the capsules into the dorsal gutters lateral to the emptied bladder. Once the capsules were infused the cannula was removed and the peritoneal wall was closed using 2.0 vycril sutures. The skin incision was closed using skin staples and the animals were removed to recovery.

Blood Collection

The area of the front leg over the cephalic vein was shaved using an animal clipper, and wiped with an alcohol swab. Using a 20 guage needle, 5 ml of blood was drawn into a sterile 10 cc syringe. After collection, the blood was transferred to a 10 ml heparinized centrifuge tube and centrifuged at 3000g for 10 minutes at 4°C. The collected plasma was divided into 100 µl aliquots and stored in a -20°C freezer.

Anti-hGH Antibody ELISA

Plasma samples from dogs were diluted in a series from 1:75 to 1:9600 with 5% skim milk powder in PBS/T ("blotto"). Samples were loaded into a 96 well plate coated with recombinant human growth hormone (Humatrope, supplied by Eli Lilly and Company, Indianapolis , In.) in 0.1N sodium bicarbonate buffer at pH 9.6. As a positive control for canine anti-hGH antibodies, plasma was also run from a dog immunized against hGH by a series of injections of the purified hormone. After rinsing with the wells with PBS/T, rabbit anti-dog IgG conjugated to alkaline phosphatase (Sigma Cat. #A-0793) was loaded, incubated for 1 hour and then removed. Alkaline phosphatase developing solution was added and allowed to react for 12 min. The absorbance of the wells was measured using a Microwell microplate reader at an O.D. of 405 nm.

Clearance of Human Growth Hormone from Dog Plasma

Two dogs implanted with human growth hormone-secreting microcapsules and two dogs never having been exposed to human growth hormone (hGH) were each injected intravenously with 3 mg/ml of recombinant human growth hormone in phosphate buffered saline (PBS). Serial blood samples were taken at 1, 5, 10, 15, 30 and 60 minutes post-injection, and the samples were assayed for hGH by ELISA as described above.

Western Blotting

Both transfected and untransfected canine MDCK cells were harvested (5×10^6 cells) after 72 hours in culture in serum free media, resuspended in 100 µl of lysis buffer, and the suspension was freeze-thawed three times to lyse the cells. The lysate was centrifuged for 30 minutes at 14000 rpm and the supernatant was removed and stored frozen. Proteins from the culture medium from these cells was precipitated using 10% (w/v) trichloroacetic acid, incubated at 4°C for 1 hour and centrifuged at 14000 rpm for 5 minutes. The resulting protein pellet was washed with acetone and dried using a vacuum centrifuge. Purified recombinant human growth hormone (Humatrope, provided by Eli Lilly, Indianapolis, In.). Protein samples were electrophoresed on a 12.5 % acrylamide sodium dodecyl sulfate-polyacrylamide gel (SDS-PAGE) for approximately 1 hour and transferred to a nitrocellulose membrane (Bio-Rad, Richmond, Ca.) for 1 hour using a transfer apparatus. The membrane was blocked for 2 hours using 5% blotto in PBS/T (500 mg/ml skim milk powder in phosphate buffered saline/Tween 20). Plasma from the experimental animals was diluted 1:300 in 5% blotto, and was used as a primary antibody source to react with the nitrocellulose membrane for 1 hour. The plasma/blotto was washed off and a 1:3000 dilution of rabbit anti-dog IgG conjugated to alkaline phosphatase was added and allowed to react for 1 hour. The colour was developed using nitroblue tetrazolium and 5-bromo-4chloro-3indolyl phosphate.

Microcapsule Retrieval Procedure

The animal was prepped for aseptic surgery as for the implantation procedure, and all equipment used in the procedure was sterile. A 2 cm-long incision was made caudal to the umbilicus down to the linea alba. The linea alba was perforated using the scalpel blade and a 16 guage flexible feeding tube was advanced into the peritoneal cavity, towards the dorsal gutters on either side of the emptied bladder. The end of the feeding tube was attached via a three way stopcock, to a 60 cc syringe and to a 500 ml bag of normal saline. In volumes of 250 ml at a time, the saline was flushed into the abdominal cavity, the stopcock opened to the syringe, and the fluid withdrawn at a rate of 0.5 ml/sec., while the external sides of the abdomen were gently massaged to ensure that the microcapsules were floating freely. The feeding tube was repositioned as necessary to free it from the omentum or other tissue clogging the opening of the tube. The retrieved capsules were transferred to a sterile 50 ml centrifuge tube and the linea alba perforation closed using 2.0 vycril sutures. The skin incision was closed using skin staples and the animal removed to recovery.

Reculture of Cells from Retrieved Microcapsules

Where possible, a sample of 50 µl of microcapsules was placed in a sterile 35 mm tissue culture dish and overlaid with a sterile 22 x 22 mm coverslip. Gentle pressure was applied with sterile forceps used to grind the capsules on the bottom of the dish to release the encapsulated cells. The sterile coverslip used to crush the capsules was transferred to another 35 mm tissue culture dish, and both dishes were fed with regular growth media supplemented with 60 mg/ml G418 per ml of media. The cells that grew on the dish were sampled to determine their hGH secretion rate as described previously.

Tissue Sectioning and Staining

Tissues taken from the animals were fixed in 10% formalin overnight before being trimmed into small sections and placed in a cassette. The cassettes were then paraffin embedded and sectioned using a Reichert OmU2 microtome. The tissue sections were fixed to a glass microscope slide and stained with Haematoxylin and Eosin.

RESULTS

hGH secreted from alginate microcapsules of varying composition *in vivo*

A total of 11 dogs were implanted with microcapsules containing hGH-secreting MDCK cells (Table 1). Three dogs received alginate-poly-L-lysine-alginate (APA) capsules, three received barium alginate (BaAlg) capsules, and three received barium-alginate-poly-L-lysine-alginate (BPA) capsules. One group of two dogs was implanted with unencapsulated MDCK cells to evaluate the immuno-isolating properties of the microcapsules. The plasma was collected from the dogs in each group, and tested with a human growth hormone specific ELISA.

Alginate-poly-L-lysine-alginate (APA) Capsules (figure 1a)

On average, 100 ml of microencapsulated MDCK cells were implanted into each dog. The encapsulated cells had a viability of 97.5 $\pm$ 1%. There were 150 $\pm$ 9 cells/capsule and 980 $\pm$ 93.8 capsules per ml (packed capsule volume). The hGH was detectable in all of the dogs by day 1 post-implantation. The average peak in delivery of the hormone to the plasma was 7.3 $\pm$ 0.9 ng hGH/ ml of plasma, occurring between days 1 and 5 post-implantation, before disappearing entirely from the circulation between days 7 and 10. From day 10 until the cessation of the experiment on day 60 post-implantation, human growth hormone could not be detected in the circulation of the dogs.

On day 14 post-implantation, a peritoneal lavage was performed on one of the implanted dogs to recover microcapsules from the abdomen. In the dogs with the APA capsules, there were no microcapsules retrieved in the peritoneal lavage, despite an initial infusion volume of over 100 ml of microencapsulated cells. A laparotomy to open the peritoneal cavity for visual inspection also proved unsuccessful at identifying capsules floating freely in the abdomen. Upon gross examination of the peritoneal organs no adhesions of microcapsules to the exterior organ surfaces was observed.

Barium Alginate (BaAlginate) Capsules (figure 1b)

On average, 120 ± 10 ml of microcapsules were implanted into each dog. The encapsulated cells had an average viability of 93.75 ± 1.3%. There were 84.1 ± 11 cells per capsule and 7615 ± 370 capsules per ml (packed capsule volume). The hGH was detectable in all of the dogs by day 1 post-implantation. The average peak in delivery to the plasma was 20.2 ± 3.4 ng hGH/ml of plasma, with the highest level being 24 ng/ml and the lowest 18 ng/ml. As in the dogs with the APA capsules, the hGH disappears entirely from the circulation, but not until after day 14 post-implantation.

On day 14 post-implantation, a peritoneal lavage was performed on dog 5 and a sample of the implanted microcapsules was recovered. The number of cells in the retrieved capsules had increased more than six-fold and the viability of the enclosed cells remained high, at >92%. When recultured, the encapsulated cells continued to secrete 2.04 ± 0.3 ng/10^6 cells/hr of hGH into the media . The microcapsules were intact and clear of any adhesive cells, and there was no evidence of any pathology on the peritoneal organs on gross examination. A second peritoneal lavage was performed on the same dog on day 42 post-implantation, but no microcapsules were recovered at that time. A laparotomy to open the peritoneal cavity for inspection allowed for the visualization of the microcapsules that remained in the abdomen. Capsules were found scattered and embedded throughout the omentum, which showed signs of an inflammatory immune response against the capsules.

Barium-alginate-poly-L-lysine-alginate (BPA) Capsules (figure 1c)

On average, 110 ± 10 ml of microcapsules were implanted into each dog. The encapsulated cells had an average viability of 93.25 ± 3.3 %. There were 141.98 ± 54.3 cells per capsule and 6480 ± 556 capsules per ml (packed capsule volume). The hGH was detectable in the circulation of each dog by day 1 post-implantation. An average peak delivery of 17.3 ± 9.8 ng hGH/ml of plasma occurred at day 7 post-implantation with the highest level being 23.5 ng/ml and the lowest being 5.8 ng/ml. As seen in the

dogs receiving BaAlginate capsules, by day 14 the hGH had disappeared from the circulation.

On day 14 post-implantation, a peritoneal lavage was performed and a sample of the microcapsules was recovered. The encapsulated cells had continued to grow inside the capsules *in vivo* to a total of 401 cells/capsule, and maintained a viability of ~92%. When recultured, the encapsulated cells continued to secrete 7.9 $\pm$ 1.2 ng/10^6 cells/hr of hGH from the microcapsules, a level roughly equivalent to that observed at the time of implantation. The microcapsules were intact and clear of adhesive cells on their surface, and there was no evidence of pathology of the peritoneal organs on gross (non-microscopic) examination. A second peritoneal lavage was performed on day 42 post-implantation, and the recovered capsules continued to float freely in the abdomen and appeared clear and free of adhesive cells on their surface. At this time, the number of cells per capsule had decreased to 100 cells/capsule, a decrease of 75 % from the level that was observed in the day 14 post-implantation recovered sample (table 2). Additionally, the encapsulated cell viability had decreased slightly to ~89%. However, when recultured and tested for hGH secretion by the encapsulated cells into the media, there appeared to be no more secretion from the recovered capsules. Because of the small volume of capsules recovered, there were insufficient numbers upon which to perform the release and regrowth of encapsulated cells.

Unencapsulated Cells (figure 1d)

In the control dogs that received unencapsulated MDCK cells secreting human growth hormone, the hGH was detected in the circulation by day 1 post-implantation at levels less than 0.5 ng/ml of plasma. The growth hormone was not detectable in the circulation of the dogs after day 3 post-implantation.

Anti-hGH Antibodies Detected in the Circulation of the Implanted Dogs

APA Capsules (figure 2a)

In dogs that received APA microcapsules, antibodies specific to human growth hormone could be detected in the circulation beginning at day 10 post-implantation. The antibody titre increased to a peak of 1:17000 between days 14 and 21, after which it decreased rapidly to just above background by day 49 post-implantation.

BaAlginate Capsules (figure 2b)

In the dogs receiving BaAlginate microcapsules, anti-hGH specific antibodies appeared in the circulation starting at day 10 post-implantation, increasing to a peak of 1:5000 at day 21, before declining gradually to levels just above background between days 49 and 60 post-implantation. Dog #6 was euthanized on day 28 post-implantation for examination of its abdominal organs.

BPA Capsules (figure 2c)

As in the other two groups of dogs receiving microencapsulated cells, anti-hGH specific antibodies first appeared in the circulation at day 10 post-implantation. The antibody titre increased to a peak of over 1:44 000 at day 21, before declining to levels just above background by day 56 post-implantation. Dog #9 was euthanized on day 35 post-implantation for examination of its abdominal organs.

Unencapsulated Cells (figure 2d)

In the dogs receiving unencapsulated recombinant MDCK cells, antibodies appeared in the circulation first on day 10 post-implantation before reaching a peak of

1:476 on day 14. Anti-hGH antibody titre decreased rapidly after this time to levels just above background by day 35 post-implantation.

To confirm that the antibodies detected by the hGH-specific antibody ELISA in the circulation of the implanted dogs were in fact specific to human growth hormone and not to some other component of the implant, western blots were performed using plasma from animals in each of the four groups. It was found that an antigen-antibody complex signal appeared at MW ~22 kD on the Western blot only in the lanes containing media from cultured recombinant cells secreting hGH and lanes containing purified human growth hormone. There was no signal in the lanes containing alginate. Additionally, cell lysate did not appear to be responsible for eliciting any of the immune response (fig. 3).

The disappearance of the hGH from the circulation of all of the dogs that occurred between days 14-21 post-implantation appeared not to be due to the failure of the encapsulated cells to continue to secrete their gene product, but may in fact have been due to the hGH being cleared from the circulation shortly after its release form the capsules. To confirm this hypothesis, a plasma clearance study was performed using dogs implanted with microcapsules containing hGH-secreting MDCK cells and naive dogs, who had never received capsules and had never been exposed to human growth hormone. In this study, the subjects were injected with 3 mg of purified human growth hormone into the cephalic vein, and serial blood samples were taken and assayed for the presence of the hormone in the circulation. A peak of >19 ng hGH/ ml of plasma was observed at 1 minute post-injection in the naive dogs, and this level declined steadily to a level of slightly >1 ng hGH/ ml of plasma after 60 minutes. In the encapsulated subjects, the human growth hormone never appeared in the circulation at levels significantly above background readings (figure 4). This result confirmed that the disappearance of the human growth hormone from the circulation of the dogs implanted with recombinant microencapsulated cells was due to the presence of neutralizing antibodies, which peak at day 14, corresponding to the disappearance of the hormone from the circulation. This finding was confirmed when samples of both the BaAlginate and BPA microcapsules retrieved on day 14 post-implantation continued to secrete human growth hormone.

Retrieval of Implanted Microcapsules

Periodically throughout the implantations, microcapsules were retrieved from the animals by peritoneal lavage. The retrieved capsules were washed with PBS and recultured using normal growth media. The media was sampled at timed intervals to determine the secretion rate of the encapsulated cells. Where possible, the capsules were crushed in a 35 mm tissue culture dish, re-fed with media and the released cells allowed to grow. These cultured cells were also assayed to determine hGH secretion rate (Table 2). By fixing the retrieved microcapsules in formalyn and embedding them in 5% (w/v) gelatin, it was also possible to examine the surface of the microcapsules for fibrotic overgrowth.

APA Capsules

A microcapsule retrieval by peritoneal lavage was attempted on one of the dogs implanted with APA capsules, but it was discovered that by day 14 post-implantation, no intact capsules or capsule fragments could be retrieved from the peritoneal cavity. Laparotomy performed on the implanted dog failed to yield any capsules, which appeared to have been destroyed some time between days 1 and 14 post-implantation. A retrieval performed at an earlier time point in one of the other implanted dogs did yield some retrieved capsules. On day 7 post-implantation, peritoneal lavage allowed for the recovery of ~10% of the original capsule infusion volume. The encapsulated cells maintained a high viability (>97%) and had continued to proliferate *in vivo*. When recultured and assayed for hGH secretion, these capsules continued to secrete the hormone into the media at levels nearly equivalent to the 19 ± 1.2 ng/10^6 cells/hr at the time of implantation.

BaAlginate Capsules

On day 14 post-implantation, barium alginate capsules were retrieved by peritoneal lavage, washed and recultured in growth media. It was found that the encapsulated cells were still secreting hGH despite its disappearance from the plasma of the implanted dogs. Media samples from the recultured microcapsules were assayed, showing that the encapsulated cells were secreting 2.04 $\pm$ 0.3 ng of hGH/10^6 cells/hr, a decrease from the 19 $\pm$ 1.2 ng/10^6 cells/hr at the time of implantation. The surface of the microcapsules at this time point was clear of any adhesive cells or other signs of fibrotic reaction.

Another retrieval, performed at day 42 post-implantation failed to recover any microcapsules from the peritoneal cavity of the same dog. Laparotomy allowed for the visualization of the peritoneal organs, upon which it was discovered that the capsules that should have been floating freely in the peritoneum had become trapped in the omentum, which was inflamed, suggesting an inflammatory reaction against the microcapsules.

When a post-mortem was performed on all three of the dogs with the BaAlginate capsules on day 60 post-implantation, it was discovered that a small volume of the originally implanted capsules still remained embedded in the slightly inflamed omentum, but the surrounding abdominal organs appeared normal upon gross examination. The number of capsules remaining in the omentum at this time, however, had decreased to ~2% of the originally implanted volume, considerably fewer than were observed at the time of the abdominal examination performed on day 42 post-implantation.

BPA Capsules

As with the BaAlginate capsules, a sample of the BPA capsules was retrieved on day 14 post-implantation, re-cultured, and the media sampled at timed intervals. The hGH secretion rate of the encapsulated cells in the day 14 sample was similar to that at implantation. Media samples from the recultured capsules were assayed, showing that the encapsulated cells were secreting 7.9 $\pm$ 1.2 ng/10^6 cells/hr. This secretion rate had

decreased from the 19 $\pm$ 1.2 ng/10^6 cells/hr being secreted at the time of implantation. Like the capsules retrieved at day 14 from BaAlginate-implanted dogs, the surface of these capsules was clear and free of adhesive cells or other signs of fibrotic reaction.

A second peritoneal lavage performed on the same dog on day 42 post-implantation yielded another sample of the BPA capsules which were recultured and the media sampled as before. Despite the appearance of the microcapsules, which were free of adherent cells that might impede the release of hGH into the media, there was no hGH signal detectable above background levels in the day 42 sample. Microscopically, the surface of these capsules remained clear and free of adhesive cells. Due to the small sample of capsules retrieved at this time, it was impossible to release the cells from the microcapsules and test the naked cells for hGH secretion, as had been done in the sample retrieved on day 14 post-implantation.

When a post-mortem examination was performed on day 60 post-implantation on all three of the dogs implanted with the BPA capsules , there were capsules trapped throughout the omentum causing it to appear red and inflamed. Aside from this mild omentitis, the other abdominal organs appeared free of any pathology on gross examination.

Histopathology of Peritoneal Organs

In all of the 9 dogs implanted with the different types of microcapsules, some degree of pathology was observed in certain areas common to all dogs when autopsies were performed on day 60 post-implantation (figure 5). The omentum of the dogs receiving microcapsules was inflamed as a result of the microcapsules floating freely in the peritoneal cavity eventually becoming trapped in this large structure during their 60 days *in vivo*. Additionally, the human growth hormone being released by the microcapsules drained from the peritoneal cavity *via* the mesenteric lymph nodes. As a result of the hGH being a foreign protein, there was also varying degrees of hypertrophy of these lymphatic tissues that was common to all of the experimental dogs.

The lesions in the omentum and MLN were milder in the dogs receiving APA capsules and unencapsulated cells.

Dogs with APA Capsules

In the dogs receiving this type of microcapsule, there was a mild localized granulomatous peritonitis in the omentum, which presumably trapped the APA microcapsules before they were destroyed. Additionally, there was evidence of lymphoid follicular hyperplasia and mild lymphadenitis in the mesenteric lymph nodes draining the peritoneal cavity.

Dogs with BaAlginate Capsules

In the dogs receiving this type of microcapsule, the same pathology was observed as in the dogs with APA capsules. Lymphoid hyperplasia and lymphadenitis as well as granulomatous peritonitis and a foreign body reaction was observed in the omentum of these dogs, but was more severe than in the dogs receiving the APA capsules or unencapsulated cells.

Dogs with BPA Capsules

The same pathology observed in the dogs with BaAlginate capsules was found in these dogs. The severity of the lesions in these dogs was also greater than in the dogs receiving APA capsules or unencapsulated cells.

Dogs with Unencapsulated Cells

In these dogs as well, there was mild pathology observed in the omentum. These lesions were milder than those in the dogs receiving microcapsules due presumably to the fact that there were no capsules to become trapped in the structure and elicit a more severe foreign body reaction. However, the lymphoid hyperplasia and mild lymphadenitis that was seen in the dogs with microcapsules was present in these dogs in only a very mild form.

Since the APA capsule type did not last long enough *in vivo* to be retrieved during the first peritoneal lavage on day 14 post-implantation, its ability to withstand the fluid shearing forces of the cone and plate apparatus could not be assessed. After 14 days *in vivo*, the retrieved BaAlginate microcapsules had all been broken after 15 minutes of fluid shear. In contrast, after 14 days *in vivo*, 70% of the retrieved BPA capsules remained intact after 15 minutes of shearing in the cone and plate apparatus. None of the capsules remained intact after 30 minutes of shear, suggesting a decrease in the mechanical integrity of the capsules resulting from their time in the peritoneal cavity of the dogs.

Discussion

Microencapsulation of biomaterials is an effective method for the delivery of missing gene products to a number of different patients requiring the same treatment. Renal epithelial cells secreting recombinant human growth hormone were encapsulated in a number of different types of microcapsules and implanted into the peritoneal cavity of normal dogs. The types of capsules that were used differed in characteristics such as the concentration of alginate, and the presence or absence of a solid alginate core or a poly-L-lysine layer.

The delivery of hGH to the dogs *in vivo* was detected using a hGH-specific ELISA. In all of the dogs implanted, human growth hormone was detectable in the circulation by day 1 post-implantation, but was no longer detectable by day 14 post-implantation if not earlier, regardless of the type of microcapsule implanted. Despite the eventual disappearance of the hGH form the circulation of the dogs, the peak amounts of human growth hormone delivered by each type of capsule were within human physiological range (Holl et al., 1991, Despopolous and Silbernagl, 1991). The appearance of the human growth hormone in the circulation is probably due to the drainage of peritoneal fluid through the retroperitoneal or posterior lymph sacs into the systemic circulation (Tortora and Grabowski, 1993). In this way, the hGH that is secreted from the microcapsules quickly enters the bloodstream of the implanted dog, where it has a half life of ~20 minutes (DeCherney and Pernoll, 1994).

Despite the high structural (antiparallel 4-α-helical bundle) and sequence homology (80%) of human growth hormone and canine growth hormone (Lovejoy et al., 1993), a humoral immune response to the recombinant human growth hormone being secreted from the microencapsulated cells still occurred. Anti-hGH specific antibody titre was monitored in the implanted dogs by anti-hGH antibody ELISA throughout the experiment. It was observed that a decrease in plasma levels of hGH corresponded exactly with the appearance and increase of these antibodies in the circulation. Plasma clearance studies using dogs with implanted hGH-secreting microcapsules, and naive

dogs without microencapsulated cells demonstrated that the antibodies appearing in the circulation at day 14 post-implantation were neutralizing, and quickly cleared the hGH from the circulation immediately after its release from the microcapsules. Similar increases in plasma binding of human growth hormone in antigen-antibody complexes was observed by van Herpen, which led to the exclusion of human growth hormone therapy as a treatment for canine growth hormone deficiency (van Herpen et al., 1994).

The specificity of these antibodies to the human growth hormone were confirmed by running a number of the components comprising the microcapsule implants: alginate, culture media from the encapsulated cells, cell lysate, and purified human growth hormone, on Western blots using implanted dog plasma as the primary antibody source. Aside form a weak signal to the fetal bovine serum (FBS) in the encapsulated cells growth media, the only IgG antibodies detected on the western blots were to human growth hormone in the samples. The antigen antibody complex attributed to the FBS, with a size of about 80 kD was believed to be present due to a component of the cell culture media and not to some component of the microcapsule implant, as it also appeared in the negative control media that was taken from non-recombinant cells. Similar signals have been observed in western blots of plasma taken from mice implanted with hGH secreting Ltk^- cells (Chang et al., 1993).

Histopathalogical examination of the dogs implanted with either encapsulated or unencapsulated cells demonstrates that the microencapsulation process is, as yet, not completely biocompatible. The granulomatous omentitis observed in the dogs receiving microencapsulated cells is suggestive of a foreign body reaction to the microcapsules. The inability to detect antibodies against the alginate used in the microcapsules, suggests that this foreign body reaction is suggestive of a cellular rather than humoral immune response. The weak antibody signal observed against fetal bovine serum in the cell media probably resulted from serum that was absorbed by the microcapsules while in culture prior to implantation. Despite the thorough washing in PBS prior to implantation, some of the fetal bovine serum still remains, absorbed into the microcapsule walls. In some cases, a weak signal was also observed against cells that were either released from ruptured capsules at implantation or cells debris being released from cells following

apoptosis. The lymphoid hyperplasia and mild lymphadenitis observed in the mesenteric lymph nodes (MLN) suggests that the human growth hormone that was being released by the encapsulated cells was entering the systemic circulation from the peritoneal cavity *via* this route. The fact that the same pathology was observed in the mesenteric lymph nodes of both the experimental and control dogs suggests that this pathology at least may be due to the release of the hGH, a foreign protein, or to cellular debris from inside the microcapsules and not necessarily only attributable to the presence of the microcapsules in the peritoneal cavity. The less severe lesions observed in the omentum and MLN of the dogs receiving APA capsules was most likely attributable to the early destruction of this microcapsule type *in vivo*, and thus the early removal of the source of antigenic challenge.

Another challenge associated with the implantation of microcapsules into the peritoneal cavity is the inability to recover all of the implanted material. Abdominal pericentesis and lavage were performed on the dogs to retrieve the implanted microcapsules (Perry and Strate, 1972). Once the capsules had been retrieved from the abdominal cavity, it was then possible to examine the enclosed cells. In all cases, the encapsulated cells yielded from the retrievals, both early in the experiment and near its end, continued to have a high viability (>80%), the capsules retrieved 6 weeks and 8 weeks post-implantation no longer appeared to be secreting human growth hormone. Possible explanations for this phenomenon may include encapsulated cells death (disproved in this case), gene inactivation of the recombinant cells, or loss of the transgene from the encapsulated cell line. The MDCK-H8 cells used in this experiment were transfected with pNMG3, a plasmid which contains the neomycin resistance gene. During selection, transfectant clones were treated with G418, a neomycin analogue. It was found that culturing these transfectant cells through numerous passages in the absence of G418 selection, actually led to a loss of the plasmid from the cells. This finding was confirmed with a Southern blot that showed the presence of the plasmid in cells kept in G418-enriched growth media over >30 passages, and its absence from the same cells kept in non-selection media (unpublished data). It is believed that this

long periods *in vivo*. While most peritoneal organs remained free of pathology as a result of microencapsulated autologous cell implants, the omentum has been found to be inflamed consistently throughout the studies involving dog implantations. As reported by de Vos in a review of the application of microencapsulation in islet transplantation, capsules retrieved form the peritoneum of implanted animals often show varying degrees of fibrotic overgrowth (de Vos et al., 1993). While it is possible that improperly produced microcapsules stimulate the immune system due to incomplete isolation of the enclosed materials, later studies by de Vos confirmed that even empty microcapsules retrieved form the peritoneum of diabetic rats showed varying degrees of fibrotic overgrowth. This fibrosis results from stimulation of macrophages by some antigenic component of the microcapsules. The stimulated macrophages release cytokines such as IFN-γ, which generate tissue factors resulting in neutrophil-rich local inflammation. These cytokines and growth factors released by activated macrophages lead to the destruction of tissues and to fibroblast proliferation and collagen production. This type of chronic delayed type hypersensitivity reaction (DTH) caused by the microcapsules and the products that they release, leads to the eventual replacement of differentiated tissues by fibrous tissue (fibrosis). This process usually occurs in the omentum, which traps the microcapsules floating freely in the peritoneal cavity.

The alginate used to make the microcapsules is composed of regions of β-D-mannuronic (M) and α-L-guluronic (G) acid, interspersed with mixed M-G regions. In studies examining the ability of alginates, specifically M, G and M-G blocks, to stimulate cytokine production by monocytes, the M-blocks have been identified as the major culprit in the stimulation of cytokine production (Otterlei at al, 1991, Soon-Shiong et al., 1993). It has been found that alginate gels comprised of greater than 50% mannuronic acid residues were far more stimulatory of cytokine release than alginate comprised of less than 40% mannuronic acid residues. With the alginates used in this experiment, mannuronic acid accounted for >60% of the alginate composition (personal communication by Kelco product support division), strongly implicating it in the development of fibrotic overgrowth on retrieved capsules and of the inflammatory response found in the omentum of the implanted dogs. The fact that the number of

microencapsulated cells that could be retrieved from the peritoneum decreased with increasing time *in vivo*, and those removed from the omentum showed considerable fibrotic overgrowth, is highly suggestive of this DTH process playing a contributory part in the eventual failure of the non-autologous cell implants.

In conclusion, the data show that microencapsulated cells are an effective vehicle for the delivery of recombinant gene products to large animal models such as the dog. The solid alginate core microcapsules, BaAlginate and BPA, last longer *in vivo* than the APA capsules whose alginate core is liquefied. However, when subjected to well-defined fluid shearing forces, microcapsules retrieved from the peritoneum of implanted dogs show evidence of weakening. This phenomenon is demonstrated by a decrease in the percentage of intact capsules with increasing duration of shearing. The inability to retrieve intact microcapsules from dogs implanted with APA capsules is evidence of the superior capsule strength provided by the increased alginate concentration and solid alginate core of the BaAlginate and BPA capsules. The increasing concentration of alginate used to make the capsules does not interfere with the delivery of human growth hormone. Neutralizing anti-hGH antibodies appearing after day 10 post-implantation cleared the hormone form the circulation immediately after its release form the capsules, but rising titre of these antibodies suggested its continued release. While long term efficacy of this technology needs further evaluation, the potential for microencapsulation of biomaterials as a gene therapy protocol is encouraging. The development of this protocol for use in large animal models such as the dog, has allowed many of the difficulties associated with the progression towards human clinical trials to be addressed.

ACKNOWLEDGMENTS

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Table 1. Dogs receiving microcapsules. All implantations were done using the surgical procedures outlined in the Materials and Methods section. Where possible, dogs receiving the same type of microcapsule were age and sex matched. N/A represents control dogs that received unencapsulated hGH-secreting MDCK cells. Encapsulated cell counts were performed on the day of encapsulation.

Table 1

EXPERIMENTAL ANIMALS

capsule type	dog #	weight (kg)	sex	age (mo.)	capsule vol. (ml)	capsule number	capsule diam (μm)	# cells
APA	1	11.6	F	11	100	98 000	700-900	12 x 10e7
APA	2	11	F	12	100	98 000	700-900	12 x 10e7
APA	3	6	F	11	100	147 200	700-900	11 x 10e7
BaAlginate	4	9.5	F	12	80	609 200	500-700	5.2 x 10e7
BaAlginate	5	9.5	F	12	80	609 200	500-700	5.2 x 10e7
BaAlginate	6	19.3	M	7	140	191 520	500-700	15 x 10e7
BPA	7	6.8	F	11	110	712 800	500-700	10 x 10e7
BPA	8	8.1	F	13	110	712 800	500-700	10 x 10e7
BPA	9	11.8	M	14	110	368 100	500-700	5.3 x 10e7
naked cell	10	10	F	10	N/A	N/A	N/A	7.3 x 10e7
naked cell	11	9.5	F	11	N/A	N/A	N/A	7.3 x 10e7

Figure 1. Human growth hormone delivered to dog plasma. Human growth hormone-secreting MDCK cells were encapsulated in (a) APA microcapsules, (b) BaAlginate microcapsules, (c) BPA microcapsules. Two control dogs (d) were implanted with unencapsulated recombinant cells. Human growth hormone secretion into dog plasma was detected by ELISA.

figure 1a

APA Capsules

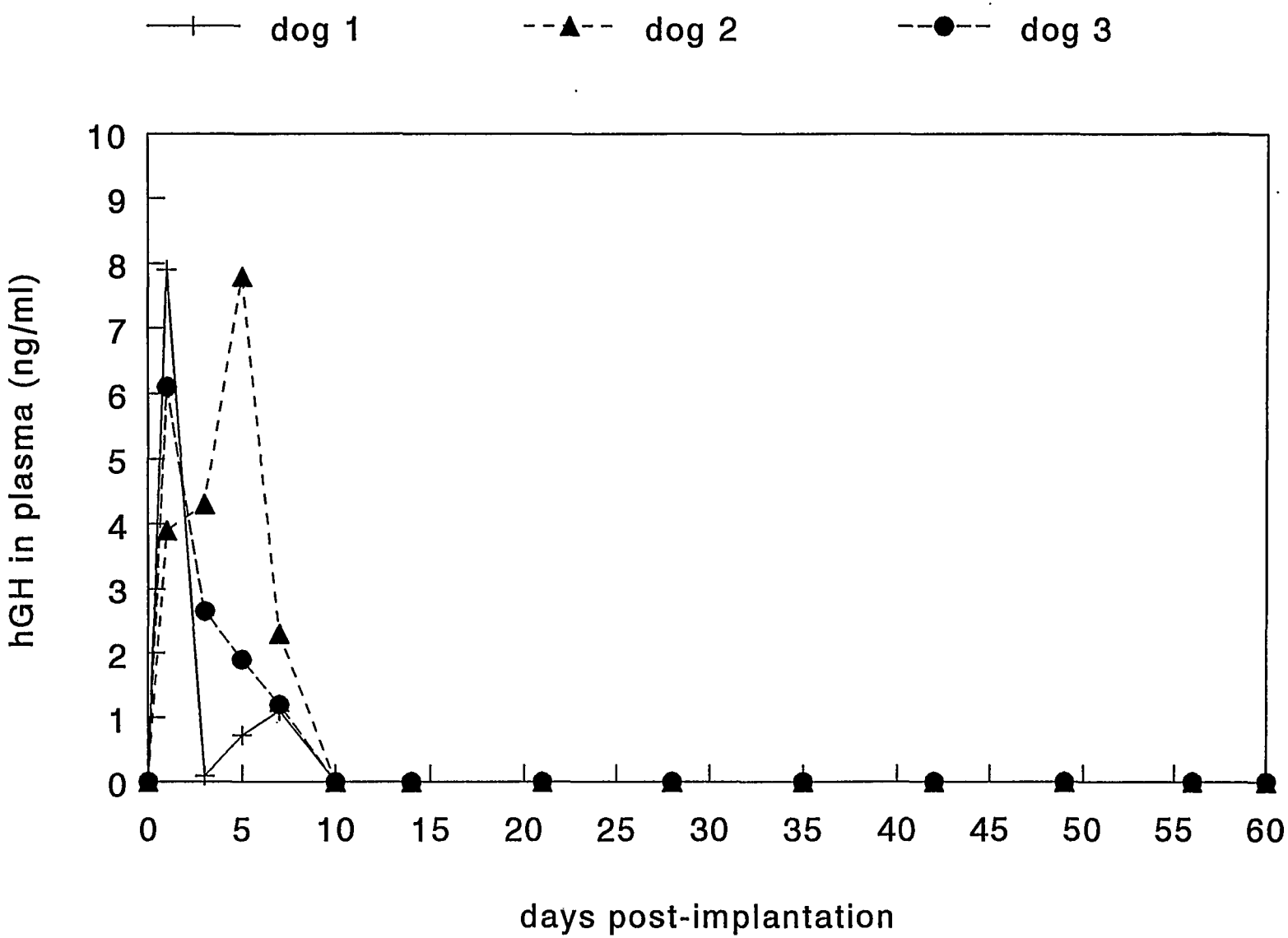


figure 1b.

BaAlginate Capsules

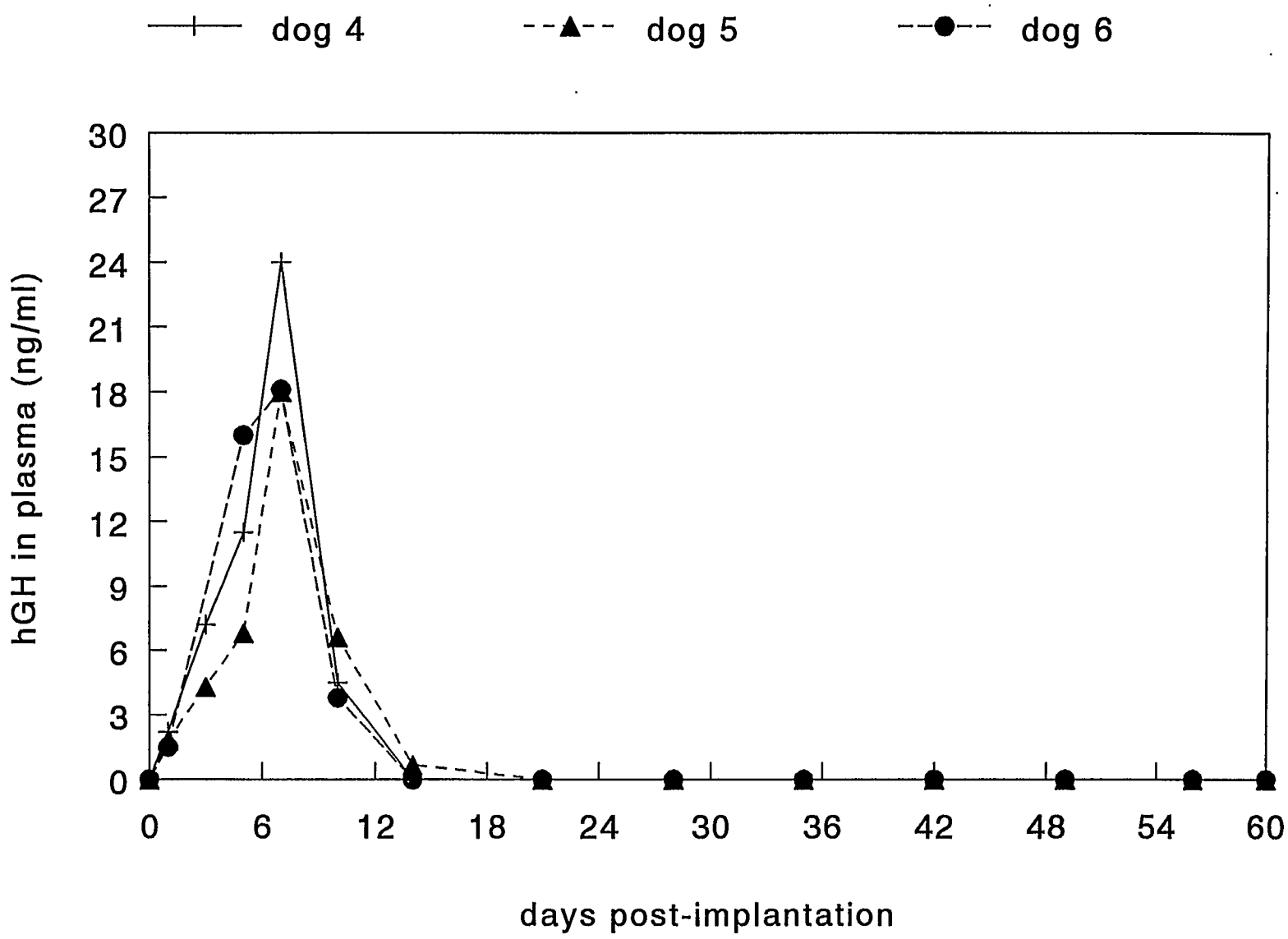


figure 1c.

BPA Capsules

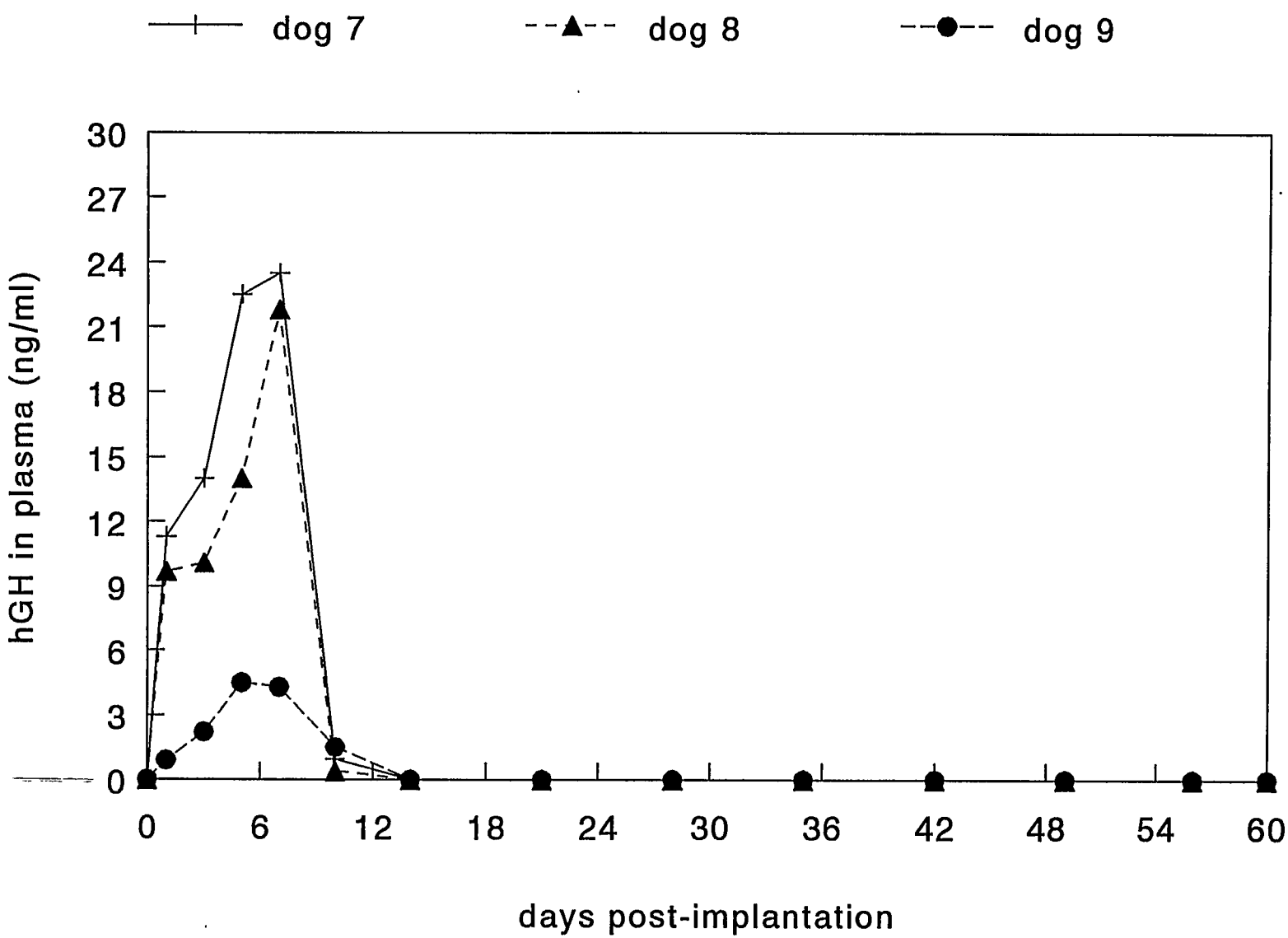


figure 1d.

Unencapsulated Cells

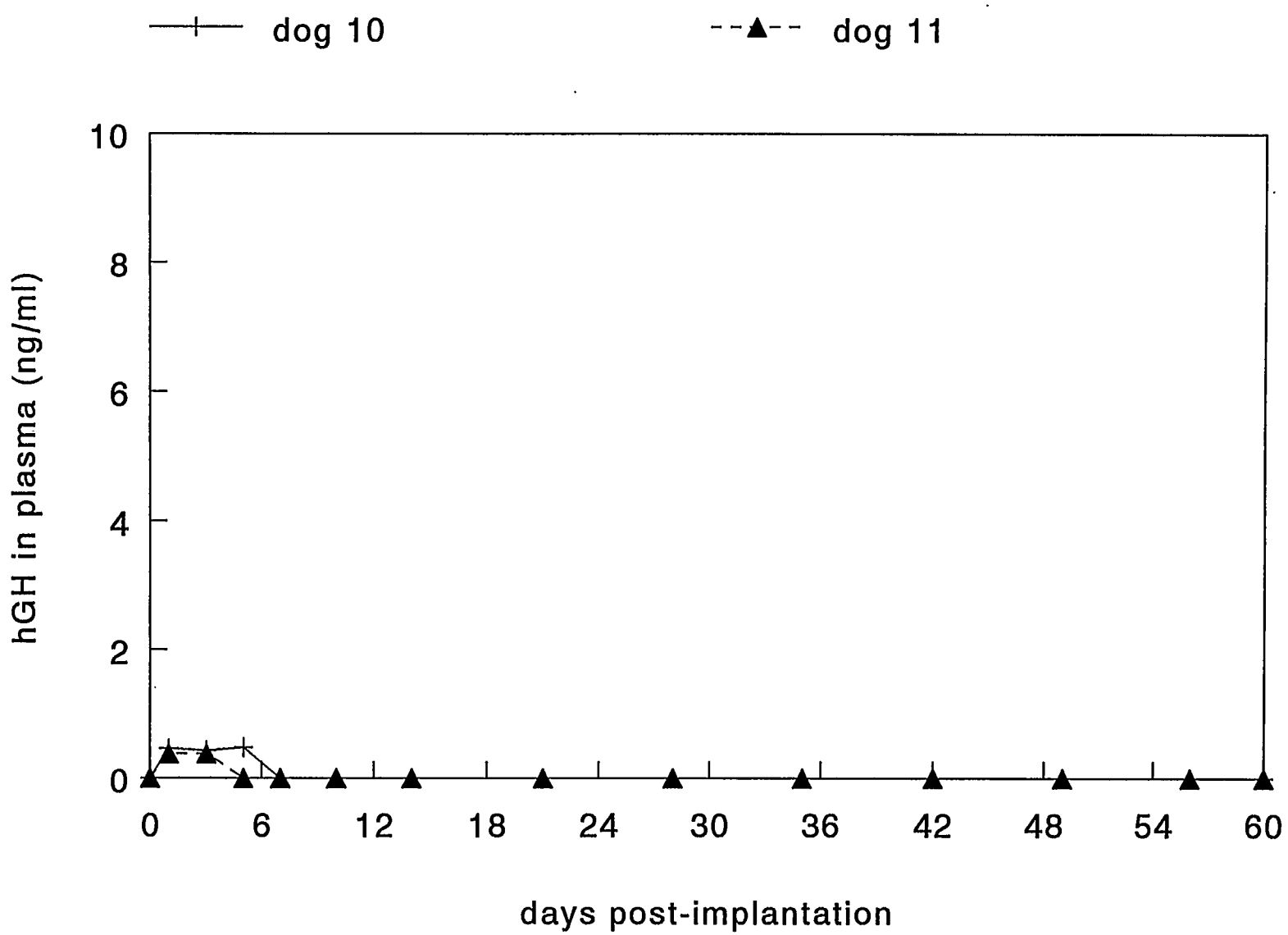


Figure 2. Development of anti-human growth hormone antibodies in the implanted dogs. Antibodies against human growth hormone were detected in the dog plasma by anti-hGH specific ELISA. Antibody titre is representative of the dilution of plasma required to give an absorbance reading of 0.65 O.D. at 405 nm. A dilution series of plasma from a dog immunized with purified human growth hormone was included in all assays as a positive control. Antibody titres are shown for dogs implanted with (a) APA capsules, (b) BaAlginate capsules, (c) BPA capsules, and (d) control dogs implanted with unencapsulated recombinant cells.

figure 2a

APA Capsules

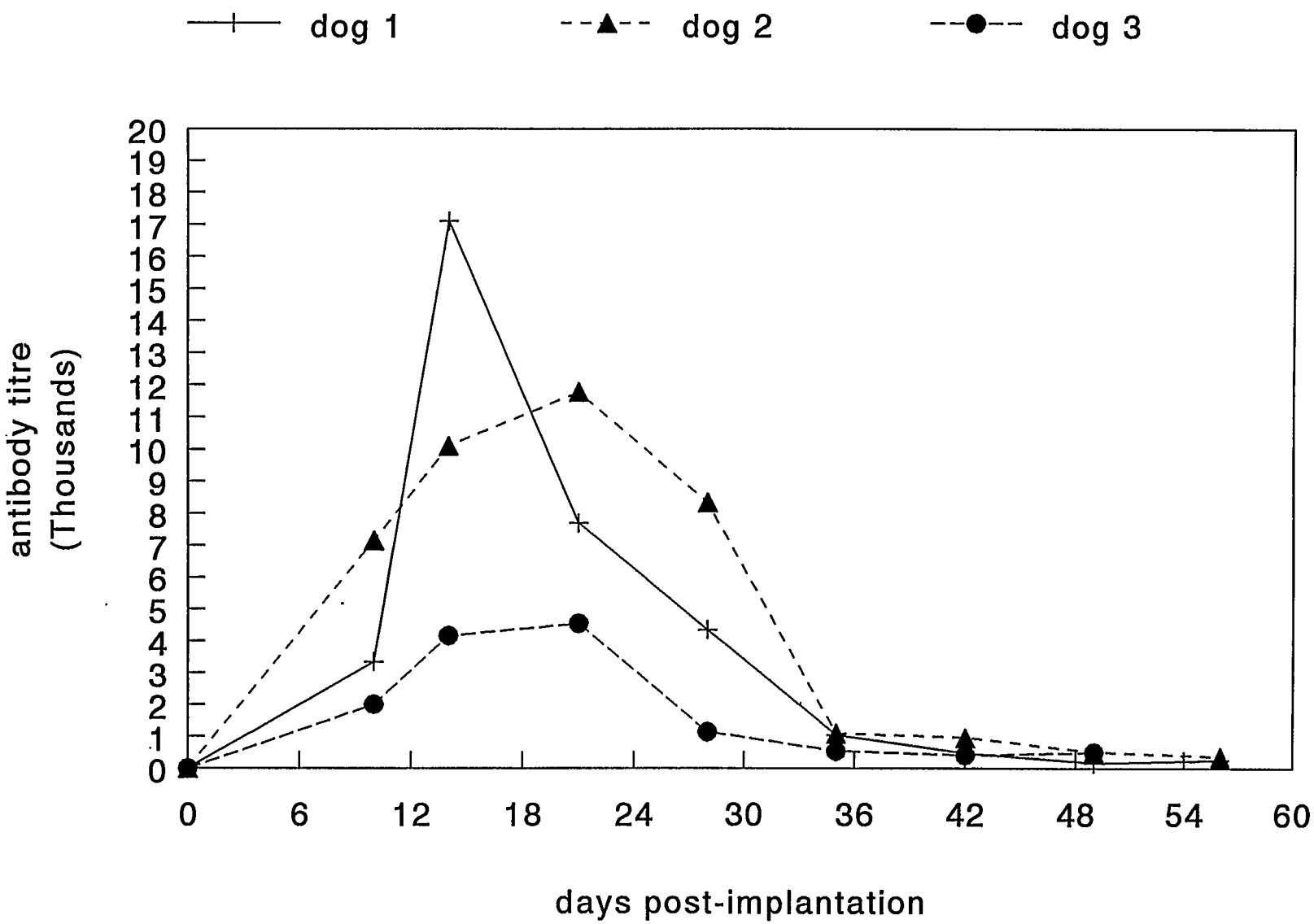


figure 2b

BaAlginate Capsules

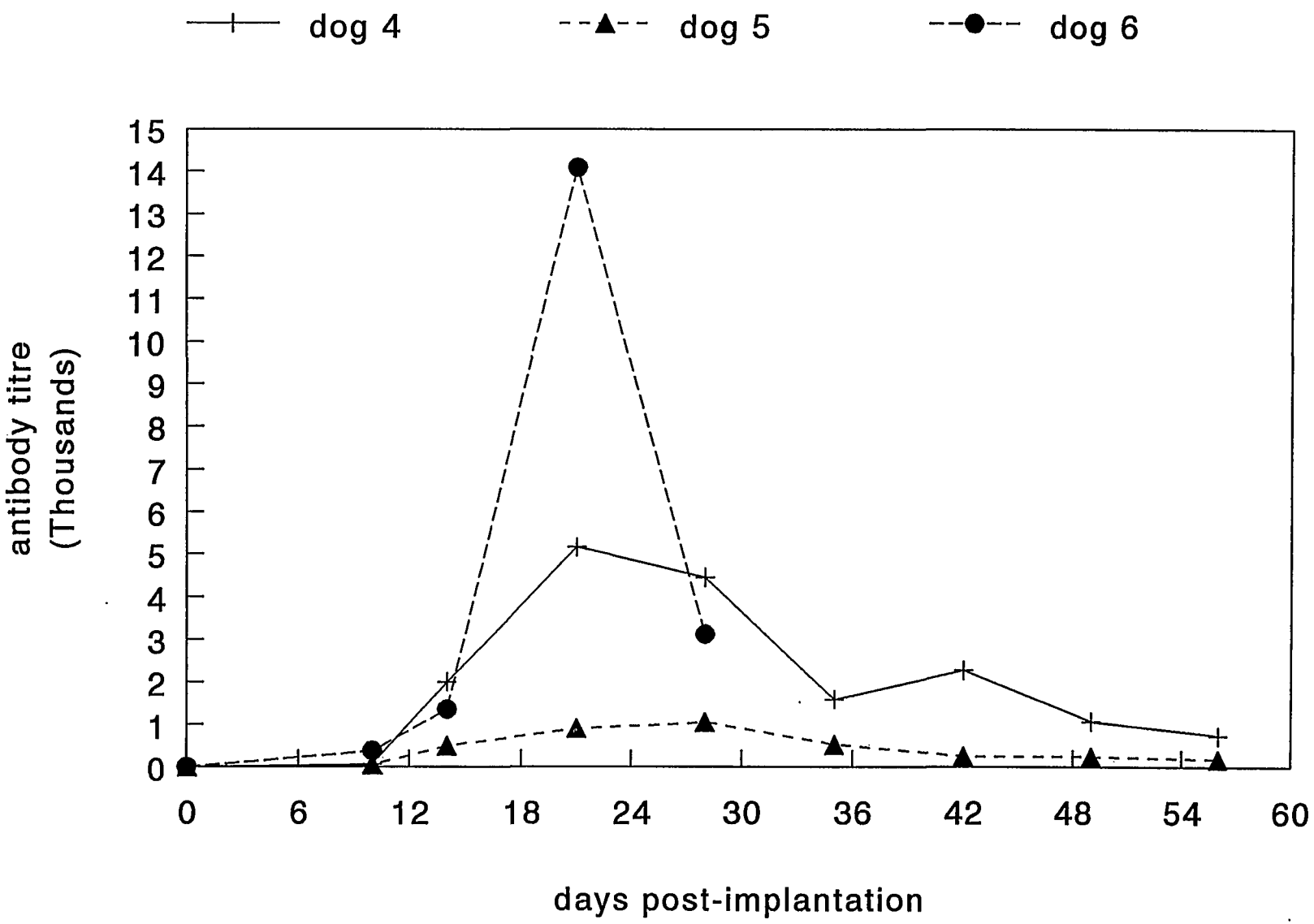


figure 2c

BPA Capsules

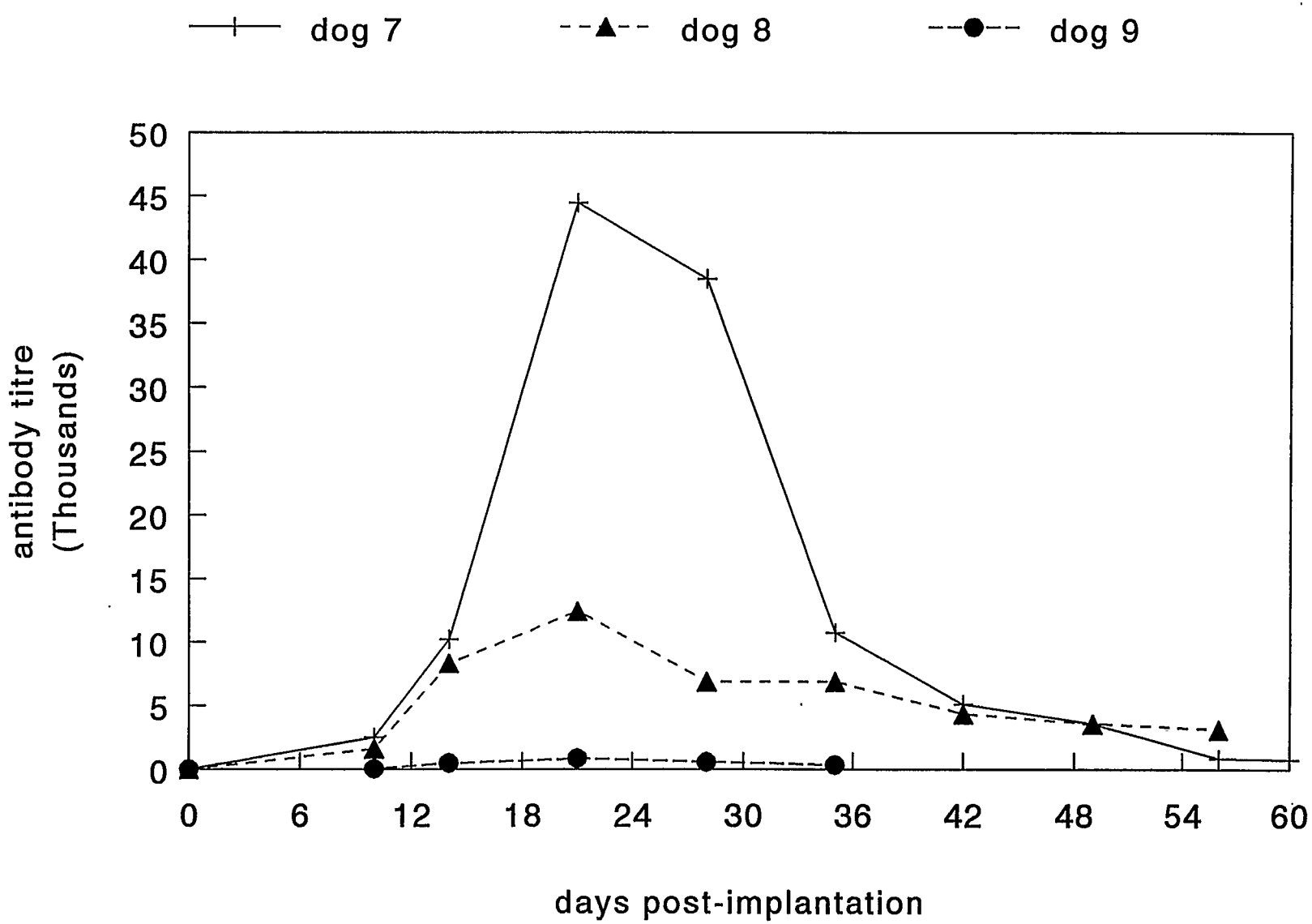


figure 2d

Naked Cells

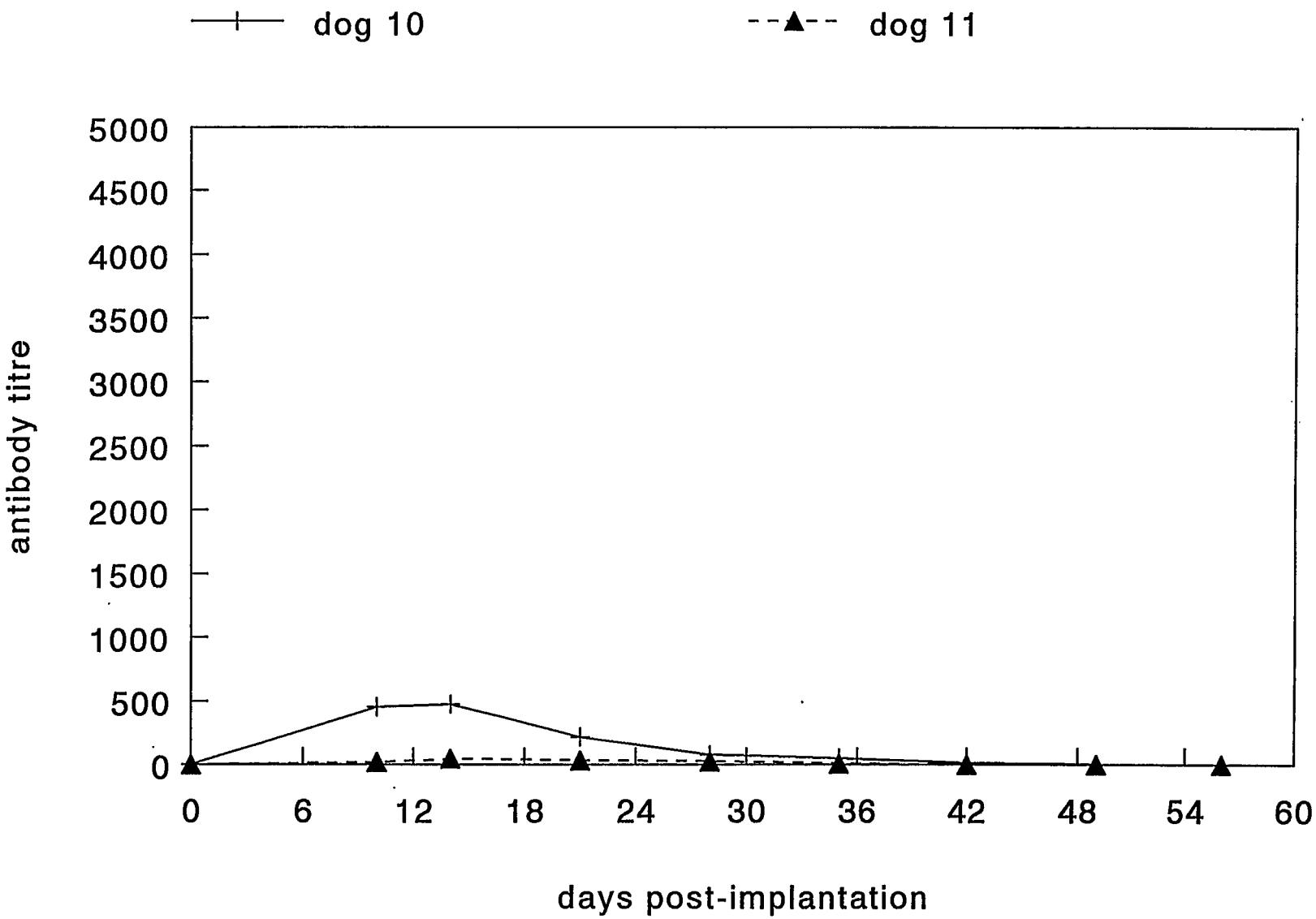


Figure 3. Presence of specific anti-human growth hormone antibodies in the plasma of implanted dogs. Purified human growth hormone (250 ng), serum free media (36 ng) and cell lysate from non-recombinant (untrans) and recombinant (trans) cells cultured for 72 hours, and alginate was electrophoresed on an SDS-PAGE gel. Once separated, these samples were transferred to nitrocellulose and blotted using 1:300 diluted plasma from dogs implanted for 28 days with human growth hormone-secreting microencapsulated or unencapsulated MDCK cells. Anti-hGH antibodies bound to the hGH were detected at about 22kD with rabbit anti-dog IgG-alkaline phosphatase as described in the Materials and Methods section. An antibody signal appearing between 80 and 116.5 kD MW in the media lanes can probably attributed to fetal bovine serum present in the culture media in which the cells and capsules were kept prior to implantation. Colour was developed using chromogenic substrate. Markers (M) are expressed in kD.

Figure 3

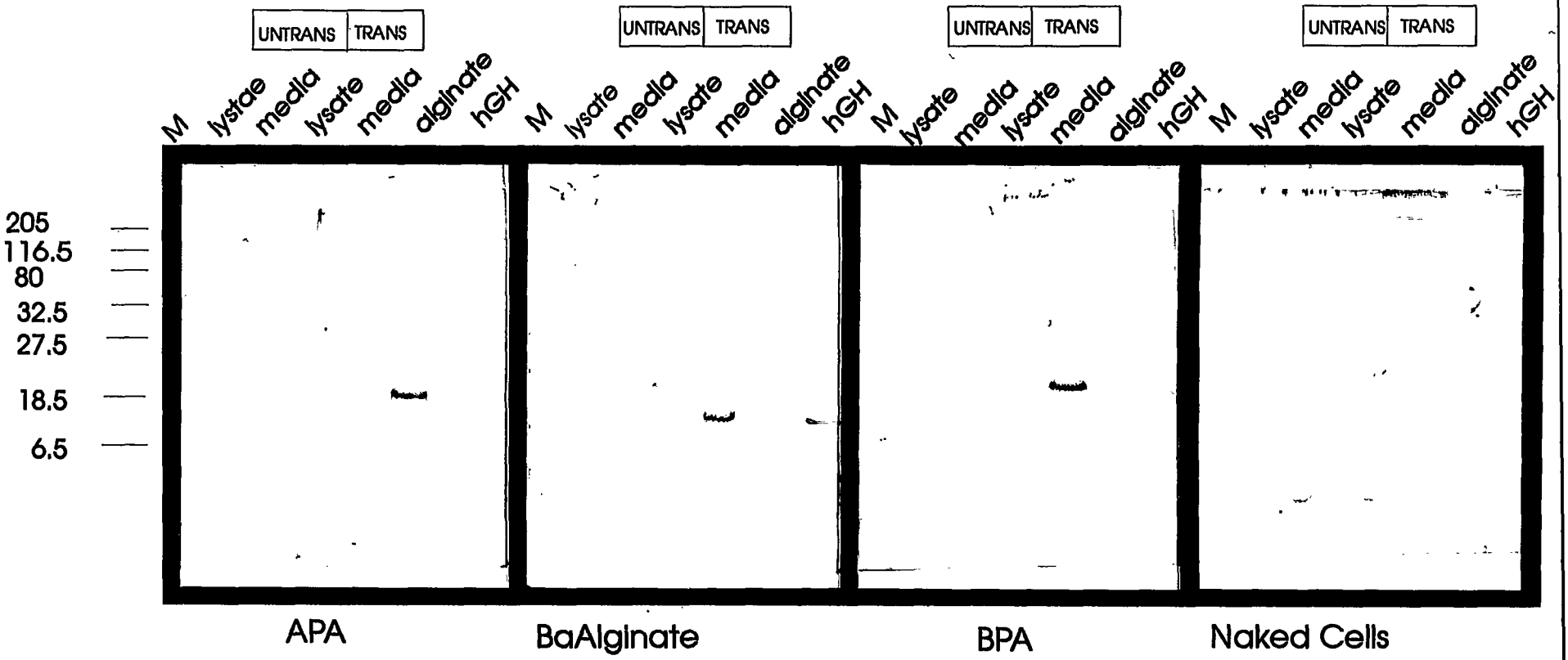

UNTRANS
TRANS
M
lysate
media
lysate
media
alginate
hGH
205
116.5
80
32.5
27.5
18.5
6.5
APA
BaAlginate
BPA
Naked Cells

Figure 4. Increased plasma clearance rate of human growth hormone from dogs with encapsulated hGH-secreting cells. Arrow represents the time of intravenous injection of 3 mg of purified hGH into two dogs implanted 21 days earlier with microencapsulated hGH-secreting MDCK cells (● , ■) or two naive dogs as controls (□ , ○). All of the dogs were fully grown adults (>12 months of age), the encapsulated dogs weighed 19.1 kg (male) and 8 kg (female) respectively and the naive dogs weighed 17.3 kg (male), and 15.5 kg (female) respectively. Blood samples were drawn at 1, 5, 20, 25, 30 and 60 minutes post-injection. ELISA was performed on samples to determine the presence of hGH in the plasma. Data represents averages of four measurements $\pm$ SD.

figure 4

hGH Clearance from Dog

Plasma

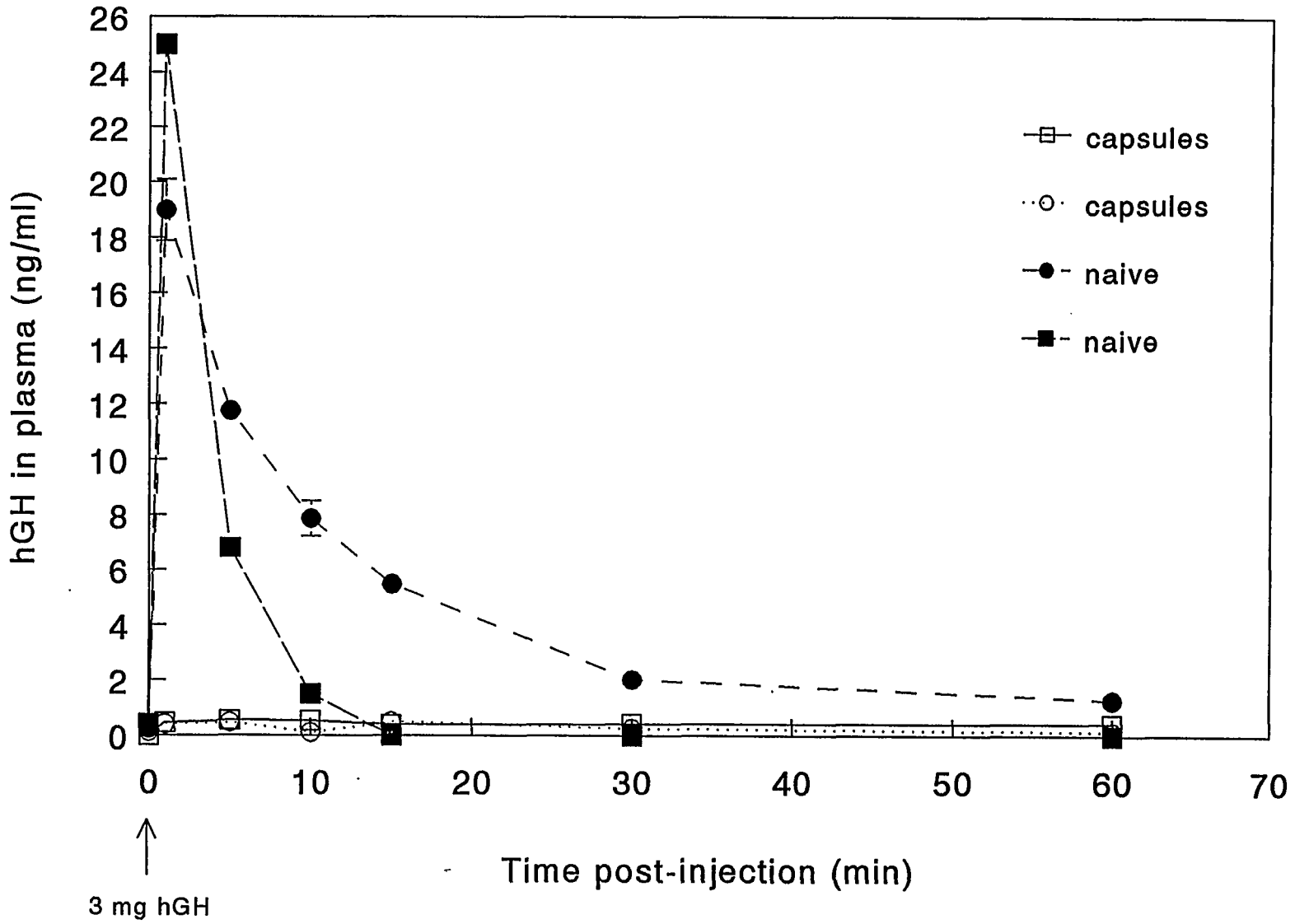


Table 2. Characteristics of microcapsules retrieved at various times post-implantation. Microcapsules were retrieved from the peritoneal cavities of implanted dogs by peritoneal lavage. Once the capsules had been washed and placed in tissue culture media, their condition was visually recorded using dark-field photomicroscopy. The enclosed cells were counted and viability was calculated as described in Materials and Methods. Where possible, cells released from the microcapsules were regrown *in vitro* in the presence of G418 and assayed for growth hormone secretion. (*) represents microcapsules surgically resected from the omentum of the implanted dogs. N/A represents the inability to perform a particular measurement on the retrieved microcapsules or their enclosed cells. Quadruplicate samples were used to make each of the determinations, and numbers are expressed as averages $\pm$ SD. The photographs above show the condition of the microcapsules at each retrieval. The microcapsules in the photographs are magnified approximately 1000x.

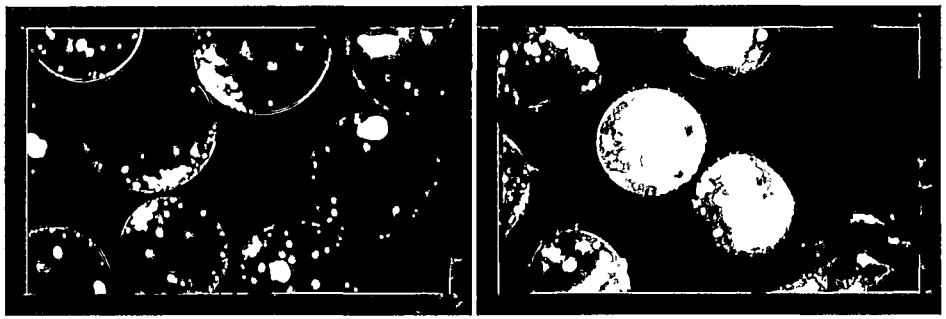
Table 2

CHARACTERISTICS OF RETRIEVED MICROCAPSULES

capsule type	days post-implant	viability (%)	cell#/cap	secretion from microcapsules (ng/10e6 cells/hr)	secretion from recultured cells (ng/10e6 cells/hr)
APA	7	80.4 ± 7.7	393 ± 12.9	4.17 ± 1.1	9.08 ± 0.3
BaAlginate	14	92.3 ± 2.2	652 ± 40	2.04 ± 0.3	5.63 ± 0.5
	42	N/A	N/A	N/A	N/A
	*60	76.7 ± 8.8	156 ± 19.2	N/A	N/A
BPA	14	92.3 ± 5.1	401± 22.5	7.9 ± 1.2	8.89 ± 0.4
	42	89.3 ± 3.4	100 ± 8.8	0	N/A
	*60	N/A	N/A	0	N/A

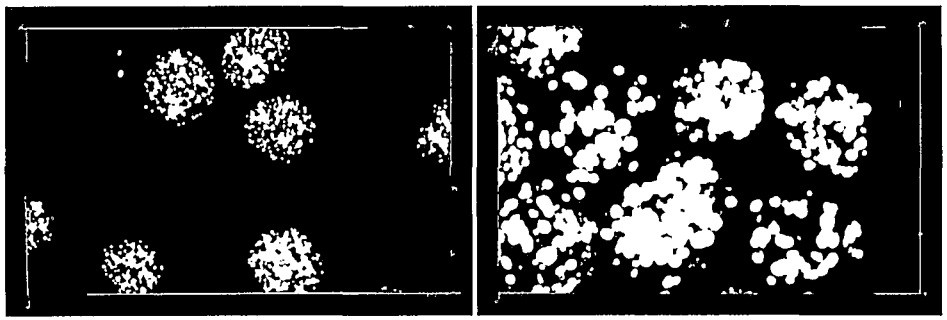
Table 2

APA



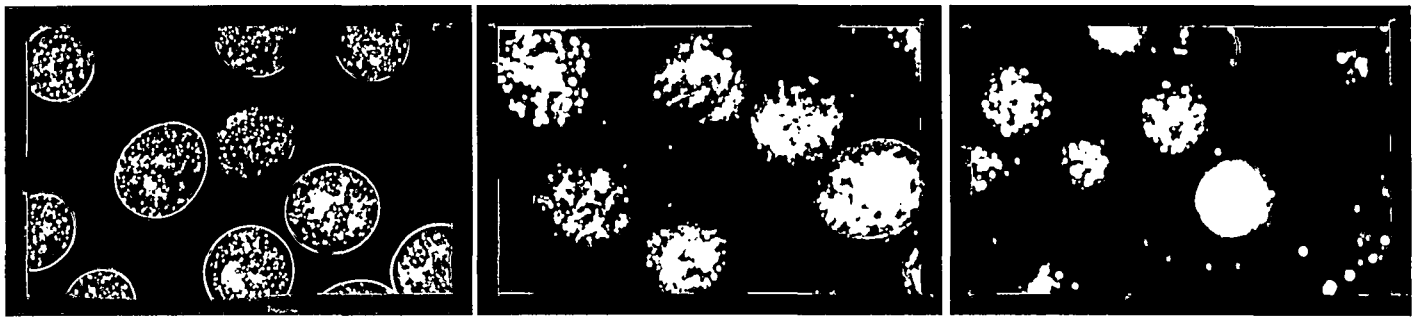
PRE-IMPLANTATION D 7

BaAlginate



PRE-IMPLANTATION D 14

BPA



PRE-IMPLANTATION D14 D 42

Figure 5. Histopathology of omentum (a,b,c,d) and mesenteric lymph nodes (MLN) (e,f,g,h) removed from dogs implanted intraperitoneally with APA (a,e), BaAlginate (b,f), BPA (c,g) and naked (unencapsulated) cells (d,h) for 61 days.

APA: there is (a) localized moderate infiltration of the omentum (arrow) by mononuclear leukocytes and (e) lymphoid follicular hyperplasia and infiltration of the sinusoids by mononuclear leukocytes in the MLN (asterix)

BaAlginate: (b) a large pale eosinophilic structure (asterix) interpreted as a BaAlginate microcapsule is circumscribed by an intense diffuse granulomatous inflammatory response (G) rich in epithelioid macrophages. Corresponding MLN (f) has sinusoidal infiltration by epithelioid macrophages.

BPA: in the omentum (c) there are large areas of microcapsule material (arrows) circumscribed by granulomatous infiltration. One capsule (asterix) appears to be intensely infiltrated by leukocytes. Corresponding MLN (g) shows the same pathology as in the dogs with the BaAlginate microcapsules.

Unencapsulated (Naked) Cells: the omentum (d) contains small areas of mild leukocyte infiltration (arrow) while the MLN (h) appears normal.

Each of the tissue sections is magnified 160x.

Figure 5.

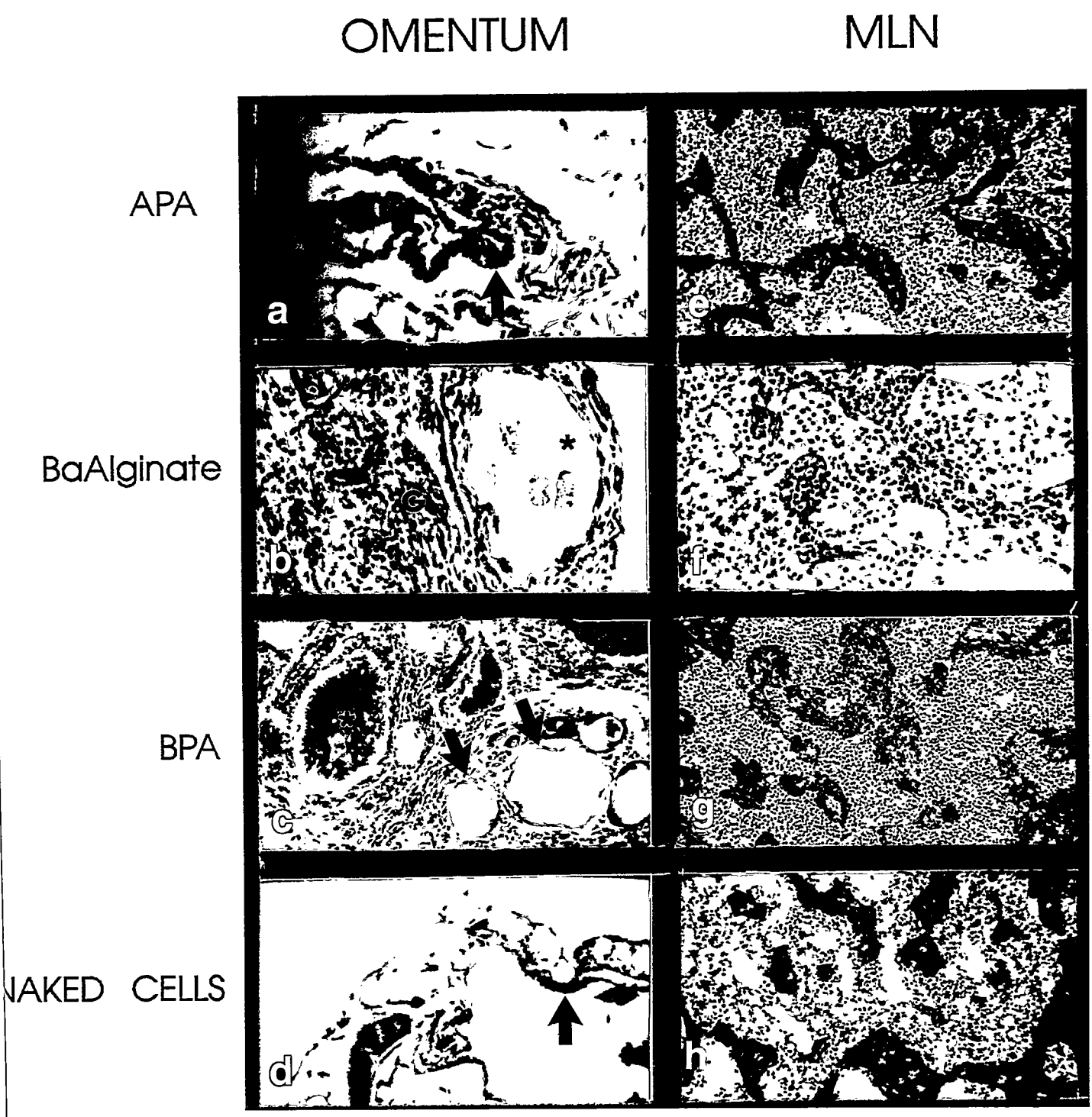
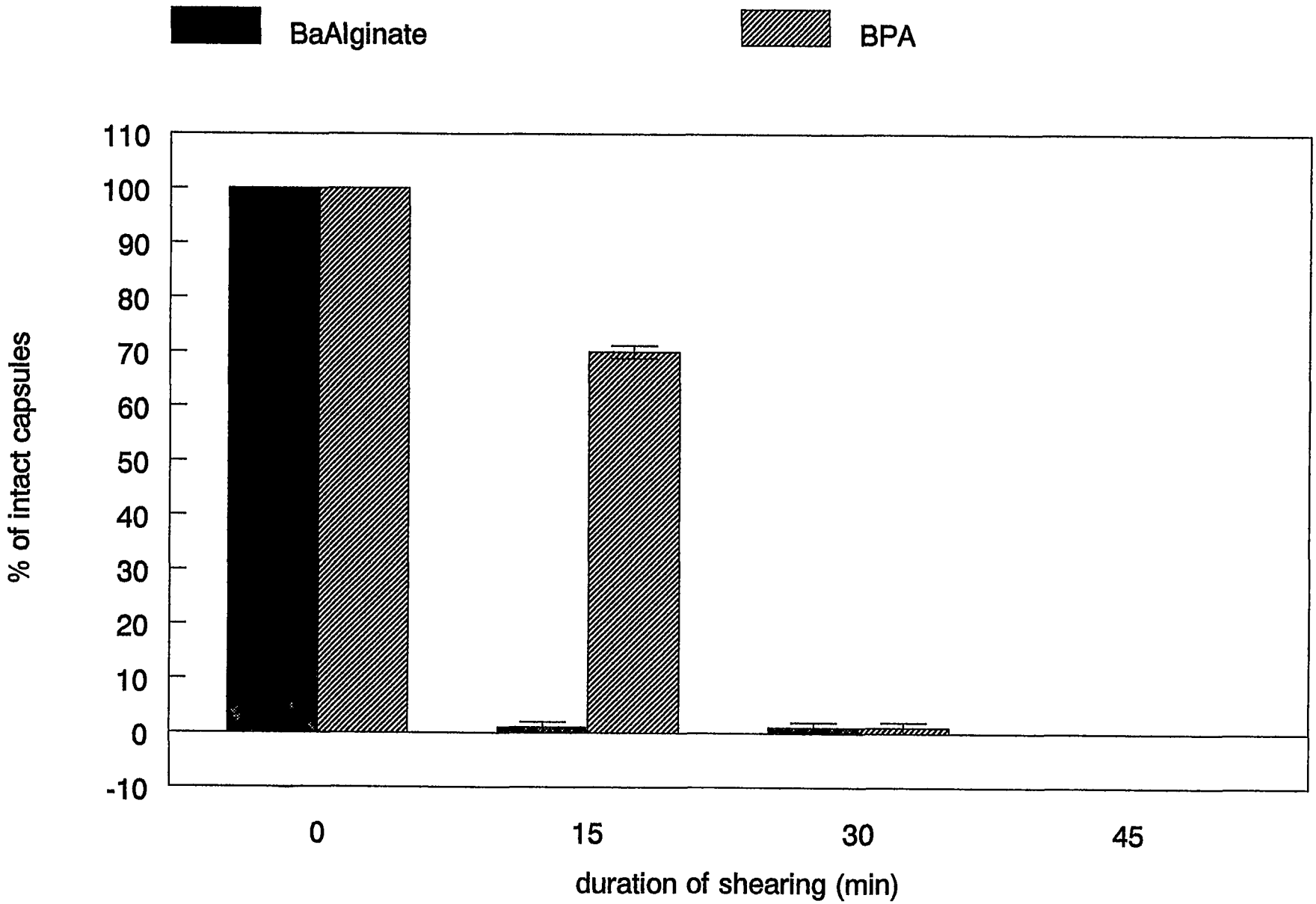


Figure 6. Shearing of microcapsules retrieved on day 14 post-implantation. Microcapsules retrieved from the peritoneal cavity were subjected to a well-defined fluid shearing force using a cone and plate apparatus, as described in Materials and Methods. After 15, 30 and 45 minutes of shearing, the mechanical integrity and appearance of the retrieved capsules was calculated by determining the percentage of capsules unbroken after the shearing interval. Counts were performed in triplicate and data are expressed as average $\pm$ SD.

figure 6

Shearing of Retrieved Microcapsules

Day 14 Post-Implantation



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DISCUSSION

Microencapsulated recombinant non-autologous cells were effective at delivering human growth hormone to dogs. Destruction of the enclosed cells by the hosts immune system was prevented by the immunoprotective, permselective microcapsule membrane. Human growth hormone was detectable in the circulation of the treated animals using ELISA, and its disappearance corresponded with an increase in the titre of anti-human growth hormone antibodies. While there were no clinical consequences associated with the implantation of the microcapsules, there was some mild intra-abdominal pathology, particularly in the omentum and the mesenteric lymph nodes. Alginate-poly-L-lysine-alginate (APA) microcapsules with a liquefied alginate core were less able to withstand a fluid shearing force than were barium-alginate (BaAlginate) and barium-poly-L-lysine-alginate (BPA), solid-alginate core capsules made with a higher concentration of alginate. As expected from this observation, the APA microcapsules were destroyed shortly after implantation into the dog, while the BaAlginate and BPA capsules persisted for much longer times *in vivo*. Despite the different physical properties of the capsules, the encapsulated cell viability and proliferation rates of the enclosed cells were high in all of the microcapsule types. In the discussion that follows, issues such as the immunologic reaction to the microcapsules, and the modifications affecting the structural integrity of the different microcapsule types will be discussed.

Hydrogel microcapsules have been used to enclose biomaterials such as pancreatic islet cells for the treatment of diabetes (O'Shea and Sun, 1986, Fan et al., 1990, Soon-Shiong et al., 1994) and recombinant cells for the treatment of neurodegenerative disorders, (Sagot et al., 1995, Aebischer et al., 1996), mucopolysaccharide disorders (Bastedo et al., 1994) and growth hormone deficiencies (Al-Hendy et al., 1995) among others.

The formation of microcapsules can be achieved in many ways. High voltage electrostatic pulses can be used to generate very small capsules (300 μm in diameter) (Halle et al., 1992), as can a submerged jet apparatus that shears the microcapsule from

an extrusion needle as it passes through an air/liquid interface (~900 μm in diameter) (Sefton et al., 1992). The protocol used to formulate the microcapsules used in this study was performed as described elsewhere, with some modifications (Chang et al., 1994). A droplet generator (syringe pump) was used to generate cell-containing alginate beads with a diameter of 400-700 μm. A concentric air stream passed over the extrusion needle ensuring microcapsules of this size range were produced, and the size of the beads was modified by adjusting the velocity of the concentric air flow. Increasing the air velocity, while resulting in microcapsules with smaller diameters, also leads to fragmentation of the alginate droplets once a certain velocity is exceeded, resulting in the loss of cell-alginate material (de Vos et al., 1993). For intraperitoneal implantations in mice, small volumes of microcapsules were required (<20 ml, Hortelano et al., 1996), and could be made in a relatively short time. A modified syringe pump was used to extrude the alginate-cell mixture from a single syringe through a small bore (27 guage) needle into the divalent cation solution ($CaCl_2$ or $BaCl_2$). Because of the back-pressure generated by the high viscosity of the alginate, the extrusion of 10 ml of alginate usually required 25-35 minutes to complete. For implantation in the dog, however, as much as 150-200 ml of alginate-cell mixture was extruded through the needle to produce the minimum of 100 ml of microcapsules, an appropriate implantation volume for the dog. As such, it was necessary to increase the capacity of the syringe pump to extrude larger volumes of alginate in order to increase the scale of production of the microcapsules. Towards this end, a syringe pump was modified to allow for the use of more than one syringe in series. The new device allowed for the use of three syringes, up to 60 cc in size to run in series, and could withstand a maximum of 1800 mm Hg of back pressure (manufacturers specifications). These modifications permitted the capsule production to be increased from 40-50 ml of microcapsules per day to more than 150 ml of microcapsules per day (personal observations, data not shown). Such increases were required to logistically implant large volumes of microcapsules into a number of large animal models. Due to the viscosity of the alginate mixture used to formulate the microcapsules, one 20cc syringe at a time was used, and could be emptied into the cation solution in less than 20 minutes. This modification allowed for the prevention of needle clogging, which was a

common problem with the less powerful syringe pump, and when more than one syringe was run in series.

The alginate-poly-L-lysine-alginate microcapsules used in this study were produced using a poly-L-lysine with a molecular weight of 22,000. The swelling observed following the liquefaction of the alginate core during the final washing step was a product of the distance that the poly-L-lysine molecules penetrated into the alginate, as explained in the introduction. The relatively low G-block composition of the alginate used to form these capsules (<40%), however, may have resulted in a capsule weaker than a capsule with the optimal (>70%) G-block composition (Martinsen et al., 1989). The M-block to G-block ratio of the alginate used in this study may also have had an affect on the antigenicity of the microcapsules used in this study, as will be explained later.

When implanted into small animal models such as rodents, alginate-poly-L-lysine-alginate (APA) microcapsules containing allogeneic materials remained intact from many months to years (O'Shea et al., 1964, Chang et al., 1993, Soon-Shiong et al., 1994). The APA microcapsules had an estimated pore size of ~60 to 100 kD (King et al., 1987, Ma et al., 1994) and allowed for the diffusion of molecules such as human growth hormone (22 kD), human factor IX (57 kD). Larger gene products such as mouse β-glucuronidase (300 kD) were also able to diffuse freely from the microcapsules (Chang et al., 1993, Hortelano et al., 1996).

In order that the microencapsulation protocol could be scaled up to use in larger animals such as the dog, beagles were chosen as the experimental animals in this study. Contraindications for use as experimental dogs include a long hair coat, large body size as well as large variations in body size within the breed (Anderson, 1987). The advantages to using beagles for this experiment were its small to medium size, moderate hair length and adaptability to living in groups. A minimal amount of restraint was required to work with these dogs due to their good nature, a trait that was selected for in their breeding history by culling ill-tempered dogs. While there is a degree of body size variation within the breed, the average weight of the adult dogs was only 9-10 kg.

Soon-Shiong et al., in 1994, reported the successful reversal of spontaneous diabetes in dogs following the injection of allogeneic pancreatic islets enclosed in alginate-poly-L-lysine alginate microcapsules. The microcapsules were made with an alginate high in guluronic acid and were implanted concurrent with low dose cyclosporin administration to prevent cytokine stimulation. Of the seven dogs receiving the microencapsulated islet allografts, all remained euglycemic for an average of 105 days post-implantation. As expected, unencapsulated islets were rejected within 1 week of implantation. In our initial pilot experiment examining the feasibility of implanting microencapsulated non-autologous cells into dogs in the absence of immunosuppressants like cyclosporin, the APA microcapsules survived for only 7-10 days before being destroyed. Peritoneal lavage was attempted to retrieve a sample of the microcapsules at 7 days post-implantation in the pilot dog, resulting in the retrieval of 11 ml of microcapsules, many of which were overgrown with fibrosis. A repeated attempt at retrieval was made on day 14 post-implantation, which failed to recover any microcapsules. Examination of the peritoneal cavity after laparotomy indicated that no intact microcapsules remained in the abdomen. These results were in agreement with the findings of Mazaheri et al., in 1991, who reported survival of APA microcapsules containing allogeneic islets of only 14 days in the peritoneum of rats in the absence of immunosuppressant therapy. Upon examination, these capsules too were surrounded by a thick cellular infiltrate composed of macrophages and other inflammatory cells.

To address the issue of microcapsule integrity *in vivo*, different microcapsule types were compared with respect to their ability to remain structurally intact when implanted into dogs. One of the changes made to the microcapsules was the use of a higher concentration of alginate in their production. Studies have reported that gel strength increases in proportion to the square of alginate concentration. (Martinsen et al., 1989). While the APA capsules were made using 1.5% (w/v) potassium alginate, a 2% (w/v) solution was used to make the other two types of microcapsules implanted into the beagles. While an alginate concentration of >1.2 % (w/v) is required for the formation of spherical alginate droplets without surface irregularities or "tails" (Goosen et al., 1985), it is also difficult to form perfectly spherical beads using alginate concentrations greater

than 2% (w/v) (Ma et al., 1994). The 2% alginate gel beads made for this study had a thicker capsule membrane, which increased to ~6.6 μm from ~4.6 μm in the 1.5% APA capsules, but the pore size of this bead was still sufficient to allow the diffusion from the capsule of molecules such as human growth hormone (22,000 MW), human factor IX (57,000 MW, G.Hortelano, unpublished observation) and mouse β-glucuronidase (300,000 MW). While molecules the size of immunoglobulins (150,000 MW) may not be completely prevented from diffusing into this type of microcapsule, diffusion studies have shown that the diffusion coefficient of a molecule of 149 kD in size was 20-times lower than the diffusion coefficient for a molecule 71 kD in size (Zekorn et al., 1992). This suggests that some barrier to the entry of molecules with large molecular weight is provided by the microcapsule membrane. The maintenance of a solid core in the barium-alginate types of microcapsules resulted in improved strength and mechanical integrity, as evidenced by their ability to better withstand a well-defined fluid shearing force, compared with the APA capsules.

Another modification made to the microcapsules used for the implantation study in the dog was the use of barium chloride ($BaCl_2$) as the divalent cation solution for gelling the alginate beads. Alginate forms a more stable gel if divalent cations with a higher affinity for the alginate than Ca^{2+} has, are used. The rigidity of the resulting gel increases with the affinity for the cation, with the affinity for $Pb^{2+} > Cu^{2+} > Cd^{2+} > Ba^{2+} > Sr^{2+} > Ca^{2+}$ (Smidsrod and Skjak-Braek, 1990). Because of the toxicity associated with many of the ions, only Ca^{2+}, Sr^{2+} and Ba^{2+} can be used for the encapsulation of living cells or tissue. The higher affinity of alginate for barium, makes an alginate cross-linked with $BaCl_2$ more stable in acidic and neutral solutions containing chelating agents as well as in the presence of phosphates (Klock et al., 1994). Studies comparing the behavior of calcium-alginate gel and barium-alginate gel in phosphate-buffered media, showed that the phosphates in the media dissolved the calcium-alginate gel within 24 hours, while the barium-alginate gel remained intact for over 80 hours (Park and Khang, 1995). Additionally, when chemically purified, many commercial alginates demonstrated a reduction in mitogenicity when complexed to Ba^{2+} ions compared to cross-linking with Ca^{2+} ions (Klock et al., 1994).

A third type of microcapsule used for implantation in the dogs was a hybrid capsule, which incorporated properties of both the alginate-poly-L-lysine (APA) and the barium-alginate microcapsules (BaAlginate). This microcapsule was also made from 2% (w/v) alginate and the solid alginate core was maintained. The modification that distinguished this microcapsule type from the BaAlginate capsule, was the addition of a poly-L-lysine coat, followed by another layer of alginate. With the addition of the PLL, the membrane diffusion rate of the microcapsule was controlled as in the APA capsules. This type of microcapsule had never before been described in the literature, so its behavior *in vivo* had not been documented prior to this study.

The behavior of the cells enclosed in each type of capsule was examined *in vitro*. It was found that while there was a slight difference between the viability of the cells encapsulated in the calcium-alginate (the APA) microcapsules and those encapsulated in the barium-alginate (BaAlginate and BPA) capsules, there was no significant difference between the two types of barium-alginate capsules. Cells inside the APA microcapsules had a consistently higher viability throughout the experiment than did the cells inside the barium-cross-linked microcapsules. This phenomenon may be attributable to the fact that the $BaCl_2$ solution used to gel the alginate is a toxic compound (Park and Khang, 1995). Once the barium alginate forms a complex with the alginate, however, it is no longer toxic to living tissues. Similarly a difference is observed among the microcapsule types with respect to their cell proliferation profiles. In general, the cell proliferation rate inside the APA microcapsules was slightly higher than that inside the BPA capsule type, while no difference was observed between the barium-alginate capsule types themselves. A slight suppression of cell proliferation inside the barium-alginate capsules may have been due to the maintenance of their solid alginate core. In contrast to the APA capsules, in which the cells float freely in the liquefied alginate, cells in the BaAlginate and BPA capsules are embedded in the alginate. This may account for the slightly slower cell proliferation rate observed in the solid-core capsules. The same trend applied to the secretion of hGH from each capsule type, due possibly to the difference in the capsule wall thickness between the APA and barium-alginate microcapsules (Ma et al., 1994).

The most obvious difference observed among the three capsule types was their relative abilities to remain intact under conditions of fluid shearing, using a cone and plate apparatus (see appendix D). While a significant proportion of the APA microcapsules were broken when subjected to well-defined fluid shearing forces *in vitro*, the barium cross-linked, solid-core capsules remained virtually undamaged. These differences in mechanical integrity among the capsule types may be attributed to the higher affinity of Ba^{2+} for alginate relative to Ca^{2+} (Smidsrod and Skjak-Braek, 1990, Park and Khang, 1995). Additionally, the solid alginate sphere was more difficult to break open than the hollow alginate "bubble".

The implantation procedure used to deliver the microcapsules to the abdomen of the dogs was a modification of a procedure used previously (Al-Hendy et al., 1995, Hortelano et al., 1996). Direct injection of microcapsules into the abdomen of the mouse in these studies resulted in sustained survival of the microcapsules for long periods *in vivo*. In the dog however, a highly vascularized omentum overlays the abdominal organs, and trapped the microcapsules as they are injected through the abdominal wall into the abdomen (see appendix C). The omentum is a fat-ladened peritoneal fold that extends, apron-like, from the greater curvature of the stomach to the transverse colon (Moore, 1992). In early pilot studies involving the injection of APA microcapsules through the linea alba connecting the abdominal muscle bundles, the omentum was seen to be filled with microcapsules, and appeared red and inflamed as shortly as 7 days post-implantation. To address this problem, the implantation protocol was modified with the use of a longer peritoneal lavage catheter to inject the microcapsules directly into the lumbar fauca, the regions adjacent to the bladder (see appendix A). In the short term, this prevented the physical aggravation of the omentum occurring immediately after the microcapsule infusion which was responsible for its immediate inflammation in the pilot experiment.

Aside from interference by the omentum in the dog, the peritoneal cavity was chosen as the implantation site for the microcapsules. As the human growth hormone was released by the microencapsulated MDCK cells, it joined the peritoneal serous fluid and probably entered the systemic circulation *via* drainage through the retroperitoneal or

posterior lymph sacs (Tortora and Grabowski, 1993). The recombinant MDCK cells were secreting 19 $\pm$ 1.2 ng of hGH/10^6 cells/hr at the time of encapsulation. Human growth hormone was delivered to the systemic circulation of the dogs with alginate-poly-L-lysine-alginate (APA) microcapsules for 7 days before its presence could no longer be detected by human growth hormone ELISA on day 10 post-implantation. A peak delivery of 7.26 $\pm$ 0.9 ng of hGH/ml of plasma was observed between days 1 and 5 post-implantation. In the dogs receiving the barium-alginate (BaAlginate) capsules, an average peak delivery of 20.2 $\pm$ 3.4 ng of hGH/ml of plasma was observed on day 7 post-implantation, before it could no longer be detected by hGH-specific ELISA on day 14 post-implantation. Similarly, a peak delivery of 17.3 $\pm$ 9.8 ng/ml of hGH was delivered to the circulation of the dogs receiving barium-alginate-poly-L-lysine-alginate (BPA) capsules. As in the dog receiving BaAlginate capsules, the human growth hormone could not be detected by ELISA on day 14 post-implantation. In contrast, human growth hormone delivered by unencapsulated MDCK cells reached a peak in the circulation of less than 0.5 ng of hGH/ ml of plasma between days 1 and 5 post-implantation. By day 7 post-implantation, human growth hormone could no longer be detected by ELISA.

These data demonstrate the ability of the microencapsulated human growth-hormone secreting cells to deliver as much as 2 times the human physiological levels of human growth hormone to the circulation of the dog. Serum hGH concentrations in normal individuals are <5 ng/ml when the subject is at rest or during periods of fasting, and can reach a peak of 10 ng/ml during exercise (Despopolous and Silbernagl, 1991). Unencapsulated cells, without the immunoprotective coat provided by the microcapsules, delivered less than 10 % of physiological levels of human growth hormone for a short time, before it disappeared entirely from the circulation. The control dogs receiving unencapsulated cells in this study were implanted with the same number of recombinant cells as the dogs in the microcapsule treatment group and under the same surgical conditions. Thus it appears that the higher, sustained delivery of human growth hormone to the dogs was due to the presence of the microcapsules, protecting the cells from being destroyed *in vivo*.

In an attempt to deliver human growth to mice by Selden et al., in 1987, transkaryotic implantation was used as the gene therapy strategy. In the transkaryotic implantation study, isolation, transfection and reimplantation of primary or secondary cells resulted in sustained human growth hormone expression only with the concurrent administration of immunosuppressant agents. In mice receiving the transkaryotic cell implants with immunosuppression, ascites formed in the peritoneal cavity, and large collections of the transfected cells were found as plaques on many peritoneal surfaces. In later studies, using transkaryotic implantation of xenogeneic primary rabbit fibroblasts, human growth hormone was delivered to immunodeficient nude mice for the lifetime of the experimental animals (Heartlein et al., 1994). Ascites and other peritoneal pathology was not observed, due presumably to the lack of an immune response against the xenogeneic implants in the immunodeficient mice. Pathology such as ascites and plaque formation on the abdominal organs were not observed with the use of the microencapsulated cells in this study, and no immunosuppressant therapy was required to allow the allogeneic cells to deliver hGH to the circulation of the dogs.

Despite the high structural and sequence homology between the antiparallel 4-α-helical bundles of canine and human growth hormone, an antibody response to the xenogeneic human growth hormone was observed in the dog (Lovejoy et al., 1993, van Herpen et al., 1994).

In the dogs receiving the APA encapsulated cells, anti-human growth hormone antibodies appeared in the circulation by day 10 post-implantation before reaching a peak titre on day 14-21. After this time, the antibody titre decreased to just above background levels between days 35 and 42 post-implantation. In the dogs receiving the BaAlg capsules, antibodies also appeared by day 10 post-implantation and reached a peak later, between days 21 and 28. The antibody titre declined more slowly in these animals than in the group receiving the APA capsules, declining to just above background levels between days 42 and 56 post-implantation. In the dogs receiving the BPA microcapsules, the kinetics of the antibody titre in the blood was similar to the dogs in the other two treatment groups, but the antibodies seemed to disappear from the circulation more slowly. Antibodies appeared in the circulation of the BPA dogs by day 10 post-

implantation and reached a peak on day 21. However, unlike the titre in the APA and BaAlginate dogs, the level of anti-hGH antibodies declined more slowly and reached background levels only after 60 days post-implantation. In the control dogs, a very low titre of anti-hGH antibodies appeared in the circulation on day 10 post-implantation and plateaued between days 7 and 14. The antibody titre declined to just above background levels after day 21 post-implantation. These data indicate that the kinetics of the anti-human growth hormone antibody titre in the dogs was correlated with the delivery of hGH to the circulation (Prader et al., 1964). The observed pattern demonstrated a typical antibody response to an antigen, with rising antibody titre during exposure to the antigen, followed by a slowly decreasing titre following cessation of the antigenic stimulation.

Controls of non-immune canine serum were run on each of the anti-human growth hormone antibody ELISAs to ensure that there was no cross-reactivity to other components of the dog plasma. In the cases of all of the dogs implanted with the microencapsulated hGH-secreting cells, the disappearance of the human growth hormone from the circulation corresponded with an increase of anti-hGH specific antibody titre. In a study evaluating the effectiveness of sub-cutaneous injections of human growth hormone in dogs with dwarfism showed a similar pattern of antibody formation (van Herpen et al., 1994). In all of the dogs treated, human growth hormone was found to be bound to plasma proteins starting about day 14 post-injection, in a pattern consistent with the presence of antibodies against human growth hormone.

The disappearance of the human growth hormone from the circulation of the beagles implanted with the hGH-secreting microcapsules in this study is believed to have been caused by its rapid removal from the circulation by neutralizing anti-hGH antibodies. Because of the xenogeneity of the gene product being delivered to animals in this experiment, an antibody response against this hormone was expected (Chang et al., 1993, Hortelano et al., 1996). The presence of antibodies in the plasma of the dogs directed specifically against human growth hormone was confirmed by Western blotting using rabbit anti-dog IgG.

To determine if the circulating anti-hGH antibodies in the experimental dogs accelerated the clearance of the recombinant hGH from the circulation, a clearance study

was performed. When recombinant human growth hormone was injected into pound-source dogs with no prior exposure to hGH, it disappeared slowly from the circulation to levels just above the background by 30 minutes post-injection. The same injection was administered to dogs implanted with microcapsules for 21 days, when hGH was no longer detectable in their plasma. In these immunized dogs, human growth hormone was detectable in the plasma only at levels slightly above the background reading given by non-immune plasma. These results suggest that the clearance of human growth hormone was accelerated in the dogs implanted with hGH-secreting microencapsulated cells, due to the presence of antibodies in the plasma. In addition to the IgG antibodies identified by the antibody ELISA and western blots in the plasma of the dogs with microcapsules, it is possible that IgE antibodies may also have formed. Immediately following the injection of the recombinant human growth hormone into these dogs, an anaphylactic reaction developed, characterized by hypotonia, bladder and bowel incontinence, and dyspnea. This response necessitated the administration of oxygen and anti-histamines to the dogs, which recovered fully. Anaphylactic reactions due to the development of IgE are observed in 13% of human patients treated with biosynthetic human growth hormone (Prader et al., 1964), and may have occurred in the dogs implanted with hGH-secreting microcapsules.

In order to evaluate the behavior of the three types of microcapsules *in vivo*, capsule samples were retrieved periodically from the implanted dogs by peritoneal lavage. On day 7 post-implantation microcapsules were retrieved from one of the dogs implanted with APA capsules. Only 10-15% of the original infusion volume could be recovered form the peritoneum.. The APA capsules that were retrieved had fibrotic overgrowths and the viability of the enclosed cells had decreased by nearly 20% from pre-implantation levels. When recultured, the retrieved microcapsules continued to secreted human growth hormone into the media at about 33% of the pre-implantation levels. When a peritoneal lavage was performed on day 14 post-implantation, it failed to yield any microcapsules. Examination of the peritoneal cavities of the dogs implanted with the APA capsules showed no evidence of intact capsules. Similar results were found when allogeneic islets encapsulated in APA capsules were implanted into diabetic

BB/W rats in the absence of immunosuppressive therapy (Mazaheri et al., 1991). Microcapsules retrieved from these rats on day 14 post-implantation were covered in a thick pericapsular infiltrate consisting primarily of macrophages and helper T-cells. It was found that islet graft failure in these rats corresponded with the formation of this pericapsular infiltrate (PCI). It was believed that either the islets were starved of nutrients, or the cells comprising the PCI released toxic cytokines such as IL-1 or TNF. It is believed that a similar mechanism may have been responsible for the early disappearance of the human growth hormone from the dog plasma in the dogs implanted with the APA microcapsules.

When retrieved from the peritoneal cavity of the implanted dogs on day 14 post-implantation, BaAlginate microcapsules were clear of cellular adhesions and were floating freely in the abdomen. The viability of the cells inside the capsules remained high and when recultured, the microencapsulated cells continued to secrete human growth hormone into the media, although at levels lower than that observed at the time of implantation. Later retrieval attempts on day 42, however, failed to yield any free-floating microcapsules, all of which were later found to be trapped in the omentum. Histologically, the regions of the omentum where the microcapsules were trapped showed signs of a foreign body reaction, involving fibrosis consisting primarily of macrophages and fibroblasts. A final retrieval attempt on day 60 post-implantation showed that fewer than 0.1% of the originally implanted capsule volume remained in the abdomen, and that these were all trapped in the omentum.

BPA capsules retrieved from the abdomen on day 14 post-implantation were also clear of any adhesive cells and contained cells with high viability. When recultured, the microencapsulated cells continued to secrete human growth hormone into the media, although at levels lower than those at the time of implantation. A later retrieval attempt, performed on day 42 post-implantation resulted in the recovery of ~5% of the original infusion volume, leaving more capsules remaining floating freely in the abdomen. While the viability of the cells inside these recovered BPA capsules had remained high, the cell number had decreased to 25% of the levels observed in the capsules retrieved on day 14. Additionally, though the surface of the recovered capsules appeared clear, when

recultured these capsules failed to deliver detectable levels of human growth hormone to the media. A final retrieval attempt on day 60 post-implantation showed that fewer than 2% of the initial infusion volume of microcapsules were present, and that all of them were trapped in the omentum. As was the case with the BaAlginate capsules trapped in the omentum on day 42 post-implantation, a fibrotic walling-off of the capsules in the BPA microcapsule-implanted dogs had occurred.

These data on the microcapsules retrieved from the experimental animals are in agreement with the antibody titre profiles for each experimental group. In the dogs receiving the APA capsules, the antibody titre declined to background levels after only 35 days post-implantation. This was consistent with the finding that intact microcapsules could no longer be found in these dogs by day 14 post-implantation. The early disappearance of the antigenic stimulation provided by the hGH-secreting microencapsulated cells, led to an early decline in anti-hGH antibody titre. Conversely, with some microcapsules remaining in the omentum of the BaAlginate implanted dogs by day 42 post-implantation, the antibody titre was still above background levels. Similarly, the persistence of the BPA microcapsules in the abdomen for up to 60 days post-implantation, was reflected by the decrease in anti-hGH antibodies to background levels only after about 55 days post-implantation.

By day 7 post-implantation in the APA encapsulated dogs, and much later in the BaAlginate and BPA encapsulated dogs (42 and 60 days, respectively) signs of chronic alloantigenic rejection of the microcapsules appeared in the peritoneal cavities of the implanted dogs. In this reaction, alloreactive T-cells recruited and activated macrophages, which initiated a delayed type hypersensitivity response to the microcapsules. The result was the formation of an often multi-layered fibrotic overgrowth on the surface of the microcapsules. The fibrotic overgrowth of the microcapsules was also characteristic of the capsules found trapped in the omentum. The development of fibrotic overgrowth on the pericapsular surface of microcapsules containing allogeneic tissues is common to many studies using alginate microcapsules (Fritschy et al., 1985, Mazaheri et al., 1991, Soon-Shiong et al., 1991, Horcher et al., 1994).

Human monocytes were cultured and exposed to individual alginate monomers (G-blocks, M-blocks, and M-G-blocks) of soluble commercial alginate (Soon-Shiong et al., 1991). The stimulation of macrophage-derived cytokines such as IL-1 and TNF-α, potent stimulators of fibroblast proliferation and inflammatory reactions, from these alginate monomers was examined. It was found that the M-blocks of commercial alginate stimulated the production of IL-1 and TNF-α, in an amount 2.2 times and 2.8 times respectively, the levels achieved with 1 mg/ml of lipopolysaccharide (LPS). Similarly, alginate gels composed of MG-blocks also stimulated the production of high levels of these two cytokines. In contrast, the incubation of monocytes with G-blocks resulted in the secretion of these cytokines at levels only slightly above background (Otterlei et al., 1991). It appears then, that alginates with higher concentrations of α-L-guluronic acid (>60%) show the lowest capacity for cytokine induction from monocytes (Otterlei et al., 1991), in addition to forming alginate beads with higher mechanical strength (Smidsrod and Skjak-Braek, 1990).

The role of cytokine stimulation of monocytes by monomeric components of alginate has been demonstrated by the implantation of empty microcapsules into the peritoneal cavity of experimental animals in the absence of immunosuppressants of anti-inflammatory agents (Soon-Shiong et al., 1991). After 21 days post-implantation, empty alginate microcapsules of low and high mannuronic acid composition were removed from the peritoneal cavity of Lewis rats. The low M-block capsules were largely free of cellular overgrowth while 90% of the high M-block microcapsules were covered with cellular overgrowth, consisting of both fibroblasts and macrophages. Later studies demonstrated mild fibrotic overgrowth on the surfaces of empty alginate microcapsules after three weeks *in vivo*, while some of the microcapsules enclosing allogeneic islets showed severe fibrotic overgrowth (Horcher et al., 1994).

The only significant pathology associated with the implantation of the different microcapsule types in the dogs was localized granulomatous foreign body reaction in the areas of the omentum where microcapsules had become trapped, and mild lymphadenitis in the mesenteric lymph nodes. While the formation of fibrosis could be identified in the areas surrounding the microcapsules trapped in the omentum, the other abdominal organs

were free of adhesions and other pathology associated with the microcapsules. Mild lymphadenitis is a common result of any antigenic stimulation occurring within the abdomen (K. Delaney, personal communication).

Other possible mechanisms have been proposed to account for the inflammatory and foreign-body reactions against implanted microcapsules. Cell adhesion has also been observed as a result of an inflammatory reaction to the microcapsules resulting from the charged poly-L-lysine molecules being incompletely covered by alginate (Sawhney et al., 1992). Similarly, as the diameter of the microcapsules decreases, the proportion of implanted tissue incompletely covered by the alginate capsules would increase (DeVos et al., 1993). This would result in the alloantigenic cells or tissue from inside the capsules coming into contact with the hosts' immunomodulating cells. As cells inside the microcapsules die, necrotic cellular debris could also diffuse out of the microcapsules resulting in cytokine induction in the local environment of the microcapsules (Soon-Shiong et al., 1992).

Many researchers have listed a smooth and uniform microcapsule surface as a requirement for biocompatibility (Goosen, et al., 1985). A scanning electron micrograph (SEM) of a barium-poly-L-lysine microcapsule, was taken both before and after implantation in the dog (appendix E). The surface of the pre-implantation microcapsule showed convolutions, which may have encouraged an immune response in the host (Lanza et al., 1992). If the textured surface of the microcapsules acted as an antigen presenting cell, it may have stimulated the release of hydrolytic enzymes and cytokines from activated macrophages, which may have damaged the encapsulated tissues (Abbas et al., 1994). However, the appearance of the microcapsule under SEM may have been an artifact of the sample preparation process, and may not have been reflective of the true surface appearance of an unprocessed microcapsule (Leu et al., 1993). The appearance of a BPA microcapsule retrieved from a dog on day 42 post-implantation, however, showed small holes and cracks in its surface, and provided insight into another possible mechanism by which the enclosed cells could eventually come into contact with components of the hosts' immune system. From inside the holes in the surface of the microcapsules, several cells could be seen lying adjacent to one another. If the holes and

cracks observed on the surface of this microcapsule were representative of the population of capsules inside the peritoneum of the dog on day 42 post-implantation, then this may be a contributing factor accounting for the failure of the enclosed cells, when recultured, to secrete detectable levels of human growth hormone into the culture media.

Any of the components mentioned above, either alone or in combination may play a role in the formation of fibrosis on and around the microcapsules. Further studies to elucidate the relative contribution of each mechanism will allow for the formulation of more biocompatible microcapsule implants.

Microencapsulated non-autologous cells have been successfully implanted into the abdominal cavities of mice, where they have delivered gene products of interest while remaining intact and free of pericapsular fibrotic infiltrate for as long as 100-200 days (Chang et al., 1993, Al-Hendy et al., 1995, Hortelano et al., 1996). Alginate microcapsules enclosing allogeneic pancreatic islets have survived for more than one year in larger animals such as the dog (Soon-Shiong et al., 1993). However, immunosuppressant therapy was required to prevent graft failure in the dog model. Recently, alginate-poly-L-lysine alginate-microencapsulated human islets were implanted into the peritoneal cavity of a type I diabetic patient. With concurrent immunosuppressant therapy, normoglycemia was maintained for up to 9 months post-implantation.

In this study, microcapsules with three different formulations were compared with respect to their respective abilities to deliver recombinant gene products to the circulation of a large animal model. In the absence of immunosuppressant therapy, human growth hormone (hGH) was detectable in the circulation for 14 days in dogs implanted with two types of barium-alginate microcapsules. The profile of anti-hGH antibodies appearing in the circulation by day 10 post-implantation, suggested continued secretion of the human growth hormone form the microencapsulated cells past day 14, despite the inability to detect it by hGH ELISA. The increased mechanical integrity of the barium-alginate microcapsules, due presumably to the use of higher concentrations of alginate and the maintenance of a solid alginate core, resulted in their persistence in the abdomen. Alginate-poly-L-lysine-alginate microcapsules, however, survived in the abdomen of

dogs for less than 14 days, and were less effective at delivering hGH during that time. While fibrotic overgrowth of all of the microcapsules eventually occurred, the barium alginate and barium-poly-L-lysine-alginate microcapsules remained free of this fibrosis for up to 42 and 60 days respectively. Mild hypertrophy of the mesenteric lymph nodes (MLN), observed in both the experimental and control dogs, is typical of any intra-peritoneal antigenic challenge and did not cause any clinical symptoms in these dogs. Similarly, microcapsules trapped in the omentum of all of the dogs receiving the microcapsules exhibited a foreign body, delayed type hypersensitivity reaction. The omentum of the control dogs, implanted with unencapsulated recombinant cells, also exhibited signs of mild localized foreign body reaction. This suggests that the pathology in the MLN and the omentum may have been partially due to the presence of the xenogeneic protein and cellular debris, rather than solely due to the presence of the microcapsules. The implantation of empty microcapsules would provide more insight into the validity of this theory.

There are numerous advantages to the implantation of microencapsulated cells for non-autologous cells for gene therapy. The creation of a genetically engineered allogeneic "universal" cell line, reduces the time and cost associated with isolation and genetic manipulation of each patient's own cells. The use of biocompatible, permselective microcapsules protects the enclosed tissue from coming into contact with components of the hosts' cellular and humoral immune system. Because of this, the use of immunosuppressants such as cyclosporin, which are often associated with side effects (Rang et al., 1995), are unnecessary. Improvements such as the use of alginates high in α-L-guluronic acid, better purification of alginate before microcapsule production, and the use of more stable cross-linking reagents will hopefully lead to the development of more biocompatible microcapsules. Studies such as this one helped to identify some of the contraindications associated with the use of microcapsules to deliver therapeutic products to large animal models. The identification of these issues have allowed for many of them to be addressed, and will hopefully bring non-autologous somatic cell gene therapy using microcapsules one step closer to large-scale human clinical trials.

APPENDIX A

Protocol For Microcapsule Implantation

Materials Required:

- 250 ml sterile beaker
- two 20 cc sterile syringes
- sterile PBS
- sterile 11 G French catheter
- microcapsules
- sterile test tube (for blood sample)

Before Procedure:

- pre-medicate with Ketamine acepromazine and glycopyrolate
- prepare 80-100 ml microcapsules
- obtain count of capsules/ml (packed capsule volume), cell number/capsule, average cell viability and hGH secretion rate/capsule and per cell
- bleed dog - 5 ml to provide negative (pre-immune) control to be used in ELISA's
- weigh animal using electric balance - allows monitoring of the effects of surgery on indicators of health such as food and water intake
- take animal's temperature
- prepare capsule suspension by placing capsules in sterile beaker, allowing them to settle and removing the media
- wash the capsules repeatedly with sterile 4°C PBS until all visual traces of the media have been removed (10-12 times)
- suction off as much of the solution from the top of the capsules as possible without drawing up the capsules
- transfer capsules to O.R. using covered sterile beaker on ice

Procedure:

- anaesthetize the animal using Isoflurane (1.5-2.5%)
- shave abdominal area using an animal clipper
- place animal on clean, absorbent surface under which may be placed a hot water bottle or heating blanket to maintain body temperature throughout the procedure
- scrub skin using Hibitane or other surgical soap and rinse with 70% ethanol
- follow with surgical prep solution such as Tamed iodine and wipe off with 70% ethanol
- drape around shaved area as required
- clamp drapes in place using sterile forceps
- make 2-3 cm incision through skin and linea alba puncturing the peritoneal wall

- insert sterile 11 guage French catheter without stilette into abdomen
- advance catheter caudally towards the lumbar fauca, the pelvic gutters beside the bladder
- draw capsules up into sterile 60 cc syringe and attach it to the inserted cannula via the tubing provided with the catheter
- gently extrude the contents through the cannula into the abdomen
- repeat as necessary until all of the capsules have been implanted
- withdraw cannula, while keeping a piece of sterile gauze over the wound
- suture the linea alba together using 2.0 vycril
- suture sub-cutaneous layers using 2.0 vycril sutures
- staple skin incision
- remove animal to recovery
- place animal beneath heat lamp or on heating pad in recovery cage and monitor body temperature until it returns to normal levels
- when animal is capable of sitting upright on its sternum and back legs, it can be returned to its run (approx. 30 min following the procedure)

APPENDIX B

PROTOCOL FOR MICROCAPSULE RECOVERY

Materials Required:

- sterile 16 G feeding tube
- sterile 3-way stopcock
- sterile 50 ml centrifuge tubes
- bag of sterile sterile
- sterile 60cc syringes
- sterile surgical instruments

Before procedure:

- pre-medicate with Ketamine acepromazine and glycopyrulate

Procedure:

- anaesthetize dog using Isoflurane (1.5-2.5%) and oxygen
- shave abdominal area using animal clipper
- place animal on clean, absorbent surface, under which may be placed a hot water bottle or heating blanket
- scrub skin using Hibitane or other surgical soap and rinse with 70% ethanol
- follow with surgical prep such as tamed iodine
- drape around shaved area as required
- using a sterile surgical scalpel, make 2-3 cm skin incision caudal to the umbilicus
- extend incision through the linea alba to puncture the peritoneum
- open sides of incision using sterile haemostat forceps
- insert feeding tube, attached to saline bag *via* the three wqay stopcock, and flush 250 ml of saline into the abdomen
- while providing gentle massage on the exterior of the abdomen, open stopcock to the syringe and withdraw infused fluid slowly
- gently transfer lavage into sterile 50 ml centrifuge tube and repeat as necessary
- withdraw feeding tube, remove haemostat forceps and close linea alba and sub-cutaneous layer using 2.0 vycril
- remove animal to recovery
- provide post-operative analgesic, fluids and heat as required

APPENDIX C

A view of the abdomen of a dog - ventral surface

- the fat-ladened, highly vascularized omentum extends from the greater curvature of the stomach to the transverse colon

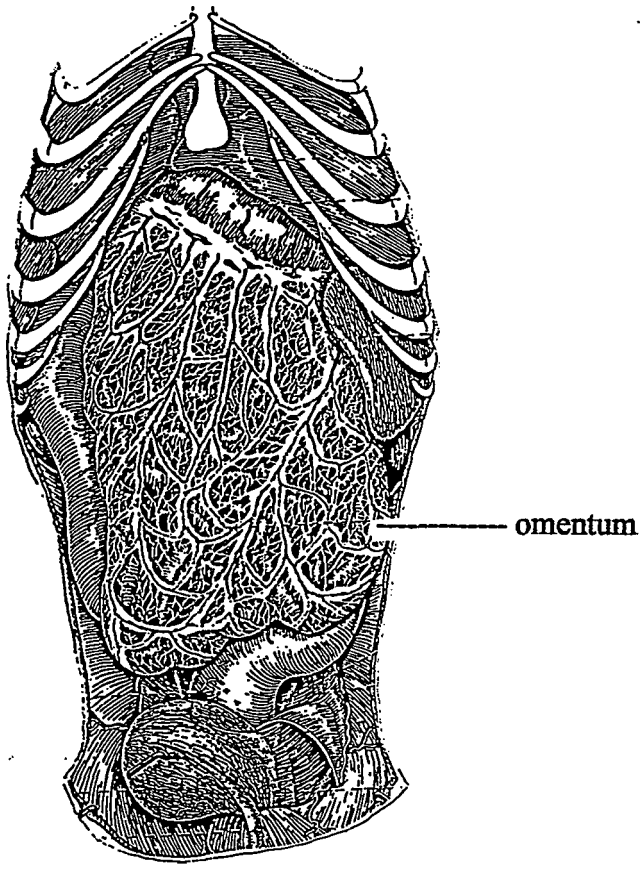

Diagram adapted from Anderson, A. The Beagle as an Experimental Dog. The Iowa State University Press, Ames, Iowa, 1987.

APPENDIX D

The Cone and Plate Apparatus

The cone and plate apparatus was developed in the laboratory of Dr.Brash at McMaster University (Karja, 1994) to study the response of platelets to various surfaces under conditions of fluid shear flow. The purpose of the apparatus is to generate a laminar fluid flow in the gap between a rotating cone cone and a well in a plate that contains the sample under study. The fluid shear rate in the plate can be varied in this system by changing either the rotational speed of the cone or the cone angle.

The cone and plate apparatus consists of four Plexiglas cones having cone angles of 4°. The cones are attached to a stainless steel drive that sits in a plate that can be adjusted vertically so that the cones may be raised and lowered into the wells. The height of all cones is adjusted simultaneously to ensure that all cones are in contact with the base of the wells at the same time. The cones are lowered into position where they can just touch the base of the wells after the wells have been filled with the samples (see figure on next page). Once the fluid has been placed in the gap between the cone and the plate, the four cones are rotated at a uniform speed.

Experimental Procedure

For this experiment, 100 µl of packed microcapsules were mixed with 1.5 ml of regular growth media. With the capsules evenly suspended in the solution, the suspension was drawn up from the eppendorf test tube using a pasteur pipette with a rubber bulb. This 1.5 ml sample was loaded into a well in the cone and plate apparatus. Suspensions of different types of microcapsules were run simultaneously and subjected to the same shearing forces by utilizing all four of the wells in the plate. Once the samples were in place, the Plexiglas cones with cone angles of 4° were lowered into the wells until the tip of the cones was lightly touching the bottom of the wells. The cones were then set to rotate at a constant speed of 333 rpm.

Prior to being subjected to the shearing forces, and then at regular timed intervals, the cones rotation was stopped, the cones were raised out of the wells and the plate containing the wells was swirled to suspend the sampling mixture. The plate was tipped on an angle to collect the sample from one side of the well. The complete capsule suspension was withdrawn form the well and photographed using a Sony CDD colour video camera. The resulting images were stored and viewed using Vidas 21 software.

The microcapsules' relative resistance to fluid shear was characterized by the percentage of the total number of capsule in the samples that remained intact as a function of time subjected to shearing forces. The entire capsule sample was photographed at each time point and an attempt was made to photograph a number of fields and to include as representative a sample as possible.

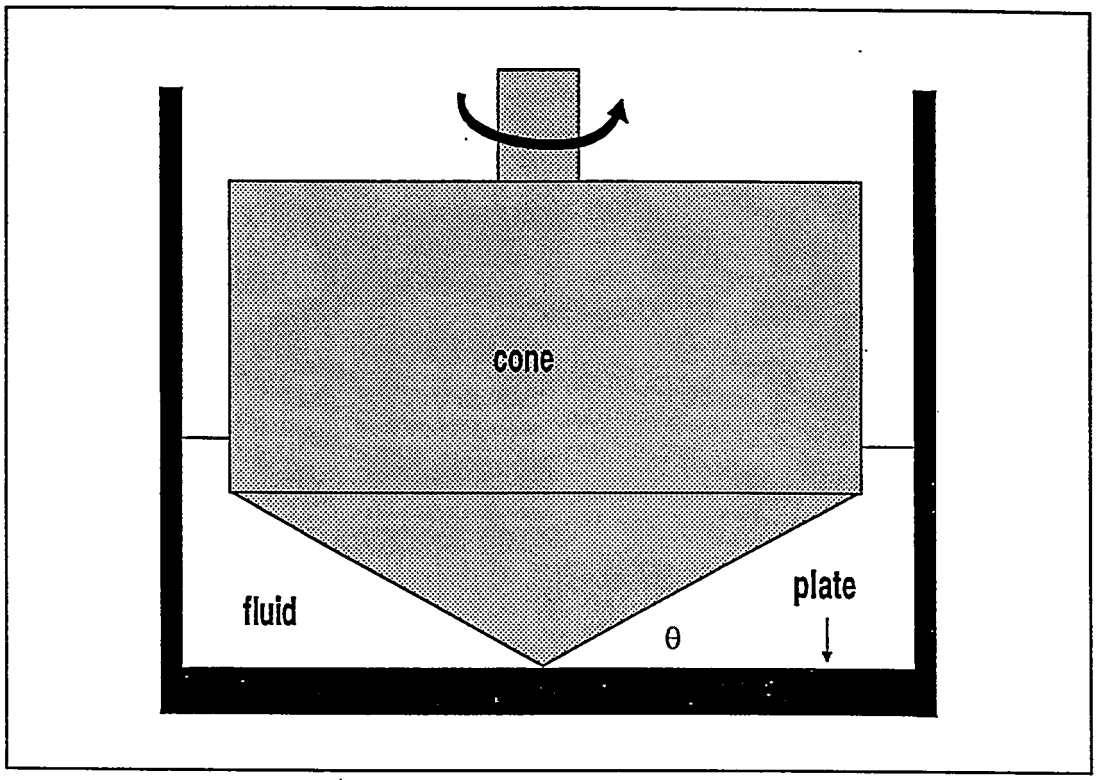

Diagram redrawn with permission from Karja, 1994.

APPENDIX E

Scanning Electron Micrographs of Microcapsules

- these scanning electron micrographs represent:

a) a barium-alginate-poly-L-lysine-alginate (BPA) microcapsule before implantation. The capsule in the electron micrograph is magnified 350x.

b) the BPA microcapsule retrieved from the peritoneum of the dog after 42 days *in vivo*. The capsule in the electron micrograph is magnified 350 x.

c) a magnification of a crack in the surface of the retrieved microcapsule indicated with an arrow in photmicrograph (b), showing the enclosed cells lying adjacent to one another inside the capsule. This section of the microcapsule is magnified 5500x.
- the reduction or loss of immunoprotection caused by this crack in the surface of the microcapsule represents one possible mechanism for eventual graft failure of the implanted alginate microcapsules

- briefly, capsules were placed upon poly-L-lysine-covered glass coverslips and fixed with gluteraldehyde. After post-fixing in OsO_4 specimen was dehydrated using graded ethanol washes and coated with gold.

Appendix E

a)

b)

c)

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