A MODEL OF CANCER IMMUNOTHERAPY
VIA IMMUNOENCAPSULATION OF GENE
MODIFIED TUMOUR CELLS

by

Saul Mandelbaum

A thesis submitted in conformity with the requirements
for the degree of M.Sc.
Institute of Medical Science
University of Toronto

© Copyright by Saul Mandelbaum (2000)
The author has granted a non-exclusive licence allowing the National Library of Canada to reproduce, loan, distribute or sell copies of this thesis in microform, paper or electronic formats.

The author retains ownership of the copyright in this thesis. Neither the thesis nor substantial extracts from it may be printed or otherwise reproduced without the author’s permission.

L’auteur a accordé une licence non exclusive permettant à la Bibliothèque nationale du Canada de reproduire, prêter, distribuer ou vendre des copies de cette thèse sous la forme de microfiche/film, de reproduction sur papier ou sur format électronique.

L’auteur conserve la propriété du droit d’auteur qui protège cette thèse. Ni la thèse ni des extraits substantiels de celle-ci ne doivent être imprimés ou autrement reproduits sans son autorisation.
Title: A Model of Cancer Immunotherapy via Immunoencapsulation of Gene Modified Tumour Cells
Degree: M.Sc.
Year of Graduation: 2000
Graduate: Saul Mandelbaum
Graduate department: Institute of Medical Science
University of Toronto

Tumour antigen was delivered simultaneously with cytokine utilizing a subcutaneously implantable microencapsulation device. It was hypothesized that animals bearing encapsulated cytokine secreting tumour cells would demonstrate a survival benefit vs control animals after tumour challenge. Tumour lines included B9/BM1 myeloma (Balb/c), MCA-38 colon carcinoma, and MMB leukemia (C57BL/6). Cytokines included interleukin-12 (IL-12), granulocyte-monocyte colony stimulating factor (GM-CSF), and soluble Flt3 ligand (sFlt3L). In vivo devices contained viable B9/BM1 tumour up to 70 days, 145 days for MCA-38, and 166 days for MMB. Device secreted IL-12 was detectable for seven weeks in vitro and 3 weeks in vivo. Challenge experiments demonstrated no survival advantage for groups bearing B9/BM1 or MCA-38 encapsulated cells. In animals bearing encapsulated MMB cells no differences amongst lymphocyte subsets, nor in CTL cytotoxicity against MMB cells, were detected. Animals implanted with encapsulated IL-12 gene modified cells before MMB tumour challenge did display a trend towards increased survival.
Thanks are due to both Dr. Keith Stewart and Dr. Steven Gallinger for support and guidance through this work.

For innumerable hours of assistance and time shared at the bench I must express my gratitude to Zhi Hua Li and Christine Dodgson.

And to my best friend, Melissa Nutik, I owe much.
# TABLE OF CONTENTS

List of Tables .................................................................................................................. vii
List of Figures .................................................................................................................. viii
Introduction ...................................................................................................................... 1
  Tumour Antigens .......................................................................................................... 1
  The Immune Response and Cancer Immunotherapy ................................................... 3
    T Cell Activation & Costimulation ................................................................................ 4
    Type 1 vs Type 2 Immune Responses ....................................................................... 5
    The Major Histocompatibility Complex (MHC) ....................................................... 6
  Antigen Presenting Cells (APCs) .................................................................................. 6
  CTLs are Required for Effective Anti-tumour Immune Responses ............................ 6
  Development of an Adaptive Immune Response is APC Dependent ....................... 6
  Cross Priming ............................................................................................................... 8
  Helper T Cells .............................................................................................................. 8
  Natural Killer (NK) & NK-T Cells ............................................................................ 9
  Mechanisms of Tumour Immune Evasion .................................................................. 10
  Cytokines and Cancer Immunotherapy ..................................................................... 13
    Interleukin-12 (IL-12) & Cancer Immunotherapy ................................................ 16
    IL-12 and CD80 ....................................................................................................... 17
    Granulocyte-Monocyte Colony Stimulating Factor (GM-CSF) ............................ 18
    Flt3 Ligand (Flt3L) ................................................................................................ 19
  Immunoe encapsulation .............................................................................................. 19
  Hypothesis and Plan .................................................................................................. 23
  Materials and Methods ............................................................................................... 24
Cell Lines and Culture ................................................................. 24
MCA-38 Retroviral Infections ..................................................... 24
Secreted Cytokine Quantitation ................................................... 24
TheraCyte™ Sterilization ............................................................. 25
TheraCyte™ Cell Loading Procedure ........................................... 25
In Vitro TheraCyte™ Experiments .............................................. 25
In Vivo TheraCyte™ Experiments ................................................ 25
Lymphocyte Preparation from Spleen .......................................... 26
Fluorescence Activated Cell Sorting ............................................ 27
CTL Cytotoxicity Assays .............................................................. 27
Statistical Analysis ...................................................................... 27
Results ....................................................................................... 28

Immuoencapsulation Based Tumour Immunotherapy in a Myeloma Model .... 28

B9/BM1 Myeloma Cells Implanted in TheraCyte™ Devices Survive in vitro for up to 4 Weeks ................................................................. 28
IL-12 is Secreted from B9/BM1 Loaded TheraCyte™ Devices in vitro for 5 Weeks .............................................................................. 28

In vivo Survival of TheraCyte™ Implanted B9/BM1 Cells .................. 29

In vivo IL-12 Production in TheraCyte™ Implanted Mice .................. 29

B9/BM1 TheraCyte™ Tumour Challenge Experiments ..................... 29

Immunoencapsulation Based Tumour Immunotherapy in a Colon Carcinoma Model ................................................................. 30

Retroviral Transductions & Cytokine Production ............................. 31

MCA-38 Tumorigenesis ................................................................. 31

In vivo Survival of TheraCyte™ Implanted MCA-38 Cells ................. 32
MCA-38 TheraCyte™ Tumour Challenge Experiments.................................32

In vivo IL-12 Production in MCA TheraCyte™ Implanted Mice..................33

Imunoencapsulation Based Tumour Immunotherapy in an Acute Myelogenous
Leukemia Model......................................................................................33

In vivo Survival of TheraCyte™ Implanted MMB Cells............................34

MMB Cell Lines & Cytokine Production....................................................34

MMB Tumorigenesis..................................................................................34

MMB TheraCyte™ Tumour Challenge Experiments....................................34

FACS Analysis of Splenocytes from MMB TheraCyte™ Implanted Mice....35

In vitro Murine Cytotoxicity Assays..........................................................36

In vivo Cytokine Production in MMB TheraCyte™ Implanted Mice...........36

Discussion...............................................................................................37

Future Work............................................................................................46

Conclusions............................................................................................48

Figures....................................................................................................49-63

Tables.....................................................................................................64-66

References.............................................................................................67-91
LIST OF TABLES

1. Cell Numbers Placed In TheraCyte™ Devices.................................................................25
2. U266 Based Gene Modified Tumour Lines and Secretion Levels.................................28
3. Viability of MCA-38 Cells in Devices in vivo.................................................................64
4. Viability of MMB3.19 Cells in Devices in vivo.................................................................65
5. IL-12 Concentration in Serum from Mice from Tumour Challenge Experiments..........66
6. FACS Analysis of Splenocytes from Vaccinated Mice.......................................................66
7. TheraCyte™ Therapy of Pre-existing MCA-38 Tumours by Baxter Healthcare Corp....44
LIST OF FIGURES

1. IL-12 Production from B9/BM1 Cells in TheraCyte™ Devices in vitro ......................49
2. TheraCyte™ Device and Murine Subcutaneous Implantation ........................................50
3. Photograph of histopathology of TheraCyte™ device containing B9-12.B7 Cells at one and five weeks .................................................................51
4. Serum IL-12 Concentration of Balb/c Mice with TheraCyte™ Devices Implanted with B9-12 cells ...........................................................................52
5. B9/BM1 Tumour Vaccination Experiments (n=3) Utilizing Single 4.5µl TheraCyte™ Devices: Day 28 Challenge ...............................................................53
6. B9/BM1 Tumour Therapy Experiment Utilizing Single 4.5µl TheraCyte™ Devices: Same Day Challenge .................................................................54
7. B9/BM1 Tumour Vaccination Experiment Utilizing Two 4.5µl TheraCyte™ Devices: Day 14 Challenge ..............................................................55
8. B9/BM1 Tumour Vaccination Experiment Utilizing Single 20µl TheraCyte™ Devices: Day 28 Challenge .................................................................56
9. Photograph of histopathology of TheraCyte™ device containing MCA-38 Cells ........57
10. MCA-38 Tumour Vaccination Experiment Utilizing Single 20µl TheraCyte™ Devices .................................................................................58
11. MCA-38 Tumour Therapy Experiment Utilizing Two 4.5µl TheraCyte™ Devices .................................................................................59
12. Photograph of histopathology of TheraCyte™ device containing MMB 3.19 Cells ......60
14. MMB3.19 Tumour Vaccination Experiment Utilizing Single 20µl TheraCyte™ Devices: Day 19 Challenge .................................................................62
15. Cytotoxicity of B6 Splenocytes from TheraCyte™ Implanted Mice vs. MMB and B9 Cells in vitro .................................................................63
Cancer immunotherapy can be defined as any treatment that seeks to promote host immune system mediated tumour destruction or control. It was the goal of this thesis to develop a novel method of cancer immunotherapy by altering deliberately the molecular interaction(s) between the host immune system and cancer cells.

Many intriguing associations between the immune system and cancer have been noted previously, for example, immune deficient individuals such as transplant recipients on immunosuppressive medications, patients treated with antineoplastic chemotherapy (Penn 1972, Frei 1993, Sheil 1993), AIDS patients (Zeigler 1987) and those with congenital immunodeficiencies (Dent 1966) are known to be susceptible to particular malignancies. Conversely, the immune system has also been credited with anti-tumour responses: spontaneous regression of malignant melanomas and renal cell carcinomas are thought to be immune mediated (Bystryn 1987). Patients with leukemia are more likely to be cured by allogeneic rather than autologous bone marrow transplantation, an observation attributed to an immunologically mediated graft-versus-leukemia effect (Truitt 1995). By extension, adoptive immunotherapy with donor leukocyte infusions has resulted in complete remission in some patients with relapsed chronic myeloid leukemia, demonstrating convincingly that it is possible to harness the immune system for therapeutic clinical benefit against cancer (Porter 1999). Consequently, recent investigative efforts have sought to further understand and exploit the host-tumour interaction to generate effective anti-tumour immune responses.

TUMOUR ANTIGENS

Tumour cells are now known to express antigens, known as tumour associated antigens (TAAs), that can be recognized by cells of the immune system, and can result in immune mediated tumour destruction. This central tenet of cancer immunotherapy has its origins in studies that demonstrated T cell mediated rejection of transplantable murine tumours after immunization procedures (Foley 1953, Prehn 1965). These findings were bolstered by the observation that syngeneic murine tumours caused by a tumorigenic virus were eliminated by cytotoxic T cells (CTLs) with specificities for epitopes from the tumorigenic virus (Rosenberg 1986), indicating that the tumours shared TAAs. The observed specificity of anti-tumour CTLs was subsequently used to help define TAAs and their causative
mutations (Lurquin 1989, Sibille 1990, Boon 1978, Van Gel 1983). In addition, infiltrating lymphocytes are prominent features in many human tumours, particularly melanomas and renal cell carcinomas (Strohal 1994). Cell lines established from these infiltrating lymphocytes are capable of lysing different melanoma cell lines that share the HLA-A2\(^+\) haplotype, indicating that TAAs recognized by autologous CTLs are shared by tumours of the same histological type (Kawakami 1992, Slingluff 1993).

Currently, five categories of TAAs have been defined using tumour cDNA expression libraries and human specific CTLs. Antigens identified to date include: normal differentiation antigens, tumour-specific fetal antigens, overexpressed oncogenes and tumour suppressor genes, viral antigens and antigens unique to individual patients. Differentiation antigens are those expressed by both malignant and untransformed cells specific to the tissue from which the malignancy has arisen. Examples include the melanoma antigens MART-1/Melan A, gp100 and tyrosinase, which were first identified using CTLs obtained by culture with autologous tumour, which lysed both allogeneic melanomas and normal allogeneic melanocytes (Anichini 1985 & 1993, Darrow 1989, Kawakami 1992). Tumour-specific fetal antigens, MAGE-1 and MAGE-3 (Melanoma AntiGEN-x) (Gaugler 1994) for example, are encoded by genes not normally expressed in adult tissues other than testis and placenta (De Smet 1997). Expression of such antigens is cell transformation dependent, and is related to the demethylation of promotor DNA, known to occur in tumour cells and prominent in metastases (Gama-Sosa 1983, Liteplo 1987).

Examples of oncogene encoded tumour antigens include ras and p185\(^{HER-2/neo}\). The ras oncogene is the most frequently expressed activated oncogene in human malignancies (Barbacid 1990). Both human and murine CD4\(^+\) class II restricted memory cells and CD8\(^+\) class I restricted CTLs have been generated against mutated ras proteins (Jung 1991, Gedde-Dahl 1992, Peace 1991). The p185\(^{HER-2/neo}\) gene encodes a transmembrane tyrosine kinase that is overexpressed in many adult carcinomas. Human breast cancer infiltrating CD8\(^+\) lymphocytes have been shown to recognize and lyse ovarian carcinoma cells displaying a HER-2/neu encoded HLA-A2 restricted peptide (Linehan 1995).

Viral antigens can provide tumour specific epitopes that promote vigorous immune responses, as such antigens generally encode proteins not expressed endogenously.
Immunization of C3H/HeN mice by syngeneic HPV-16 E7 gene transfected fibroblast-like cells conferred CTL based protection against transplanted cells from a HPV-16 E7-positive syngeneic tumour (Chen 1991). The simian virus 40 large T antigen also provides CTL targets on virally transformed cells (Tevethia 1985).

The tumour specific idiotypes of immunoglobulin producing B-cell malignancies are an example of a group of patient specific antigens. F(ab')2 and Fab idiotypic fragments from patients with lymphoma or myeloma have been used to generate in vitro and in vivo T cell responses (Bianchi 1997, Dabadghao 1998).

Non-peptide TAAs also exist, as demonstrated by CD1a+ dendritic cells (DCs) found in and around a number of tumours (Goldman 1998, Hillenbrand 1999). These facilitate the presentation of lipid and glycolipid antigens to a subset of restricted T cells (Porcelli 1998).

Thus, it is evident that tumour antigens exist, and that they can form the basis of specific, and perhaps ultimately, therapeutic, immune responses. However, although TAAs do exist, it is clear that their existence alone, as demonstrated by tumour growth in immunocompetent individuals, is normally insufficient to engender protective immunity.

THE IMMUNE RESPONSE & CANCER IMMUNOTHERAPY

The understanding of the mechanisms of CTL generation and anti-tumour immune responses in general have been altered dramatically of late. We will therefore briefly review the relevant components of the immune system, as the design of effective cancer immunotherapies is dependent on these principles.

The immune system is divided functionally into the innate and adaptive, based on receptor type. Receptors of the innate immune system are germline encoded and have evolved to recognize infection and/or danger from the uninfectious and/or non-dangerous. In contrast, receptors of the adaptive immune response vary at random and are not germline encoded, and thus can, in principle, engage any molecule, including self. Harmless entities are ignored by the adaptive system since activation of adaptive immune responses depends largely upon the activation of the innate immune system (Medzhitov 1998).
T Cell Activation & Costimulation

Naïve T cells require two concurrent signals for activation. The two signal hypothesis was proposed by Bretcher and Cohn in response to the observation that mature B cells with self specific Ig receptors were self tolerant (Bretscher 1970), and is now generally accepted as a central tenet of naïve T cell activation (Guinan 1994).

The antigen specific signal delivered through the T cell receptor (TCR) via MHC is defined as signal one, and the costimulatory signal is defined as signal two.

Signal 1 is dependent on the half life of the TCR-MHC-peptide interaction (Lyons 1996), and this interaction is stabilized by the formation of a specialized contact, termed the immunological synapse (Grakoui 1999, Dustin 1996). The immunological synapse is defined by a specific pattern of receptor segregation formed in three distinct stages with a central cluster of TCRs surrounded by a ring of integrin family adhesion molecules (Monks 1998). With sustained aggregation of a threshold of MHC-peptide complexes with sufficient affinity for the TCRs, T cell activation takes place via recruitment of intracellular signaling molecules such as p56lac by CD4, for example. In addition, TCR-MHC-peptide interactions are dependent on T cell and APC actin cytoskeleton mediated reorganization of cell surface proteins. Lipid microdomains ("lipid rafts") of sphingolipids and cholesterol within the glycerophospholipid-rich plasma membrane (Simons 1997), are enriched in signal transduction molecules and function as sites of TCR aggregation and signaling (Moran 1998).

Signal two may be delivered by a variety of ligand-receptor pairs, including: B7/CD28 (Kuchroo 1998), 4-1BB ligand (4-1BBL)/4-1BB (Takahashi 1999), and OX40 receptor/ligand (Weinberg 1998). The two signal paradigm of T cell activation also applies to signals delivered through CD1 displaying non-peptide antigens (Exley 1998). If the two signals are not delivered simultaneously, TCR engagement results in apoptosis or an antigen specific state of T cell unresponsiveness termed anergy (Jenkins 1987a,b). Further stimulation of an anergic T cell, even in the presence of costimulation, does not result in IL-2 production nor proliferation, although the T cells can still respond to IL-2 (Jenkins 1992, Schwartz 1990).

The B7/CD28 costimulatory system consists of two ligands, B7-1 (CD80) and B7-2 (CD86), and two receptors, CD28 and CD152 (cytotoxic T lymphocyte antigen-4). The
specificity of the costimulatory signal rests in the tissue and temporal distribution of the costimulatory ligands. The B7 molecules are expressed primarily by antigen presenting cells, including B lymphocytes, monocytes, macrophages and dendritic cells (DC) (Greenfield 1998). Activation of the APC increases the expression of both ligands (Freeman 1993, Azuma 1993).

CD28 is expressed on 95% of resting human CD4+ T cells and 50% of resting CD8+ T cells in peripheral blood (June 1994). The expression of CD28 is upregulated after T cell activation, and while it has affinity for both CD80 and CD86 these molecules may bind different sites on the molecule for differing effects (Truneh 1996). Costimulation through CD28 via CD80 results in a number of T cell effects including: stabilization of IL-2 mRNA, an increase in expression of all IL-2 receptor chains (Fraser 1992), prolongation of the duration of CD40L expression (Johnson-Leger 1998), and stabilization of CTLA-4 mRNA (Lindsten 1993). Multiple T cell derived cytokines, including interferon-gamma (IFNγ), granulocyte-monocyte colony stimulating factor (GM-CSF), and tumour necrosis factor-alpha (TNFα) (Fraser 1992), are also produced upon CD28 signaling. The effects of CD28 costimulation on T cell proliferation are marked, as it is required to sustain the late proliferative response and enhance long term T cell survival (Greenfield 1998, Wells 1998).

Type 1 vs Type 2 Immune Responses

The type 1 and type 2 patterns of cytokine secretion correspond to the activated effector phenotypes generated during an immune response. Naïve CD4+ T cells principally produce IL-2 when first stimulated and then differentiate into committed phenotypes (Th1 or Th2) secreting other cytokines (Abbas 1996). The type 1 profile includes IL-12, IFNγ and lymphotoxin, with Th1 cells regulating cell mediated inflammation and immunity against intracellular pathogens. The type 2 cytokine profile consists of IL-4, IL-5 and IL-10, which encourage antibody production, eosinophil proliferation and allergic responses (Muraille 1998, Mosmann 1996). The cytokines of each phenotype downregulate the other, as IFNγ selectively inhibits Th2 cell proliferation (Wynn 1995), and IL-10 inhibits Th1 cell cytokine secretion (Yamamura 1991). Developmental commitment to the Th2 lineage abolishes IL-12 signaling by downregulation of the IL-12β receptor (Szabo 1995).
The Major Histocompatibility Complex (MHC)

CD8+ and CD4+ T cells recognize peptide antigens via the T cell receptor (TCR) as peptides complexed with an MHC class I or class II molecule, respectively (Yague 1985, Julius 1993). Peptides presented on MHC I were classically found to be derived mainly from a cytosolic, or "endogenous", endosomal pathway of protein digestion suggesting strictly intracellular protein sources (reviewed by Germain 1993, York 1996). This is in contrast to the "exogenous" pathway of MHC II presentation of peptides derived from proteins of an extracellular nature (Morrison 1986). However, recent findings have challenged this hypothesis (see below).

Antigen Presenting Cells (APCs)

The defining characteristic of an APC is the ability to support the generation of a primary adaptive immune response. While so-called professional APCs include macrophages and B cells, dendritic cells (DC) are the prototypical APCs with marked ability to ingest and present antigen (Ag), thus promoting specific T-lymphocyte responses in organized lymphatic tissue (Knight 1985, McLellan 1996). Myeloid derived interstitial DCs form a network of sentinel cells in virtually all organs of the body except the brain, testes and parts of the eye (Tuft 1984). Such cells develop from circulating precursors (Hart 1981), and function to sample the environment continuously by picking up local debris. APC produced IL-12 subsequently plays a crucial role in the development and clonal expansion of Th1 cells (Maruo 1996, Murphy 1994, Kennedy 1994).

CTLs are Required for Effective Anti-tumour Immune Responses

Effective anti-tumour immune responses both in animal models and in humans are often mediated by the cellular immune system with CTL involvement (Zitvogel 1996a,b, Farrar 1999, Pham-Nguyen 1999). Type 1 responses generating CTLs are critical for anti-tumour immunity (Coughlin 1995, Toes 1997, Pardoll 1998, Yang 1999), and in general Th2 responses have been associated with a lack of anti-tumour immunity (Rodolfo 1998, Kobayashi 1998). However, a more detailed understanding of CTL generation is leading to the development of novel strategies of cancer immunotherapy.

Development of an Adaptive Immune Response is APC Dependent

The "endogenous" model of MHC I restricted peptide generation and presentation was initially attractive in that it allowed for CTLs to be tolerant towards self antigens and to kill
only infected or tumour cells (Moore 1988, Yewdell 1988). However, this theory was ultimately untenable in light of the facts that T cells require two signals for activation and that naive T cells rarely traffic to peripheral tissues (Butcher 1996). In other words, the model was inconsistent in that CTLs require costimulation to become activated to kill a target, but the only encounter that any given CTL would have by the “endogenous” model was with its target, which could not give a costimulatory signal. There was no means for a costimulation dependent CTL recognizing an intracellular pathogen, or tumour, to become primed to kill if it recognized its antigen only on an infrequently encountered peripheral cell that expressed no costimulatory molecules.

It has become clear of late that exogenous antigen can indeed access the MHC I processing pathway for CTL activation, and this phenomenon of APC uptake and presentation of exogenous antigen is termed “cross priming” (Carbone 1998). DCs have now been shown to incorporate, process and present exogenous antigen in association with both class I and class II MHC in local lymph nodes (Mitchell 1998, Shen 1997).

As the cross-priming model predicts that APCs will, to some extent, encounter and present normal peripheral antigen, it begs the question of how the decision is made between tolerance and aggression. Indeed, tolerance may be induced by Ag carried in the cross-priming pathway as evidenced in an OVA expressing mouse model (Heath 1998, Kurts 1997) and in an islet expressed haemaglutinin model (Lo 1992). The decision between tolerance and eradication of Ag bearing cells may be made on the basis of antigen dose. For example, a higher dose of islet cell antigen was found to prime naïve CTLs in local lymph node via a cross-presentation mechanism, while a lower dose did not (Kurts 1998). In addition, only relatively large amounts of apoptotic material were able to promote DC maturation to an allostimulatory phenotype (Rovere 1998b). Consistent with this model, agents such as papillomavirus, with low level Ag expression and exclusive peripheral tissue tropism, fail to prime effective CTL responses, likely due to insufficient access to the cross-priming pathway (Tindle 1994). Similarly, a peripheral tumour with a model antigen does not prime CTLs on its own (Speiser 1997). Conversely, molecules that increase antigen uptake would be predicted to prime CTLs efficiently. Examples of such molecules include heat shock proteins, which chaperone peptides to APC endosomes.
raising peptide specific CTL responses (Suto 1995, Arnold-Schild 1999), and chemical chaperones (Ghumman 1998).

Cross Priming

The cross priming model of CTL generation has profound implications for tumour immunotherapy. The essential point is that appropriately targeting sufficient antigen to the cross-priming pathway can generate effective anti-tumour responses. This has been demonstrated directly in studies that have generated effective cancer immunotherapy in animal models by delivering dendritic cells loaded ex vivo with tumour lysate or peptides. For example, all animals were cured of day 8 established metastatic MCA205 or TS/A tumours by 4 doses of biweekly administered tumour lysate pulsed bone marrow derived DCs (Zitvogel 1996b). There are numerous other examples of DC based immunotherapy in animal models (Flamand 1994, Paglia 1996, Poragdor 1996). Clinical trials of DC based immunotherapy are currently underway and initial results have included complete and partial responses in melanoma and B cell lymphoma (Hsu 1996, Lotze 1997, Nestle 1998).

While antigen dose is critical in the generation of tumour specific CTLs, delivery of antigen as a single dose is not sufficient to maintain an anti-tumour immune reaction to tumour eradication. Even though CTL responses were present after even a single injection of DCs or virus, multiple treatments were required to cure mice of established tumours (Oschsenbein 1999). Speiser et al. noted that the simple presence of tumour antigen did not lead to eradication after the initiation of a tumour specific CTL response (Speiser 1997). It is therefore likely that immunotherapy protocols will call for multiple rounds of stimulation or prolonged antigen exposure at adequate doses in order to eradicate completely tumour via CTL generation.

Helper T cells

The CD4+ T cell is an essential component of the cross-priming model of T cell activation. Co-localization of CD8+ and CD4+ T cells on host APC allows for the paracrine delivery of helper derived IL-2 (Bennett 1997, Kirberg 1993), or alternatively, the APC may be altered by the T helper cell, as CD4+ cells can condition DCs to stimulate CTL activation by modulating CD40, even bypassing the requirement for IL-2 (Ridge 1998). These principles also apply to anti-tumour immune responses. Tumour eradication
mediated by adenovirally delivered IL-12, or IL12 and B7, was abrogated by CD4+ T cell depletion (Coughlin 1995, Pham-Nguyen 1999). Similar CD4+ dependence was demonstrated in both a vaccinia virus/melanocyte antigen model (Overwijk 1999), in a MUC1 antigen model (Agrawal 1998), and in DC based therapy (Zitvogel 1996b). Thus, both CTL and CD4+ T cells are required for effective anti-tumour immunity.

**Natural Killer (NK) and NK-T Cells**

NK cells are TCR and surface immunoglobulin (slg) negative, large granular lymphocytes with functions straddling innate and adaptive immune responses (Trinchieri 1989). A higher cytotoxic activation state, termed lymphokine activated killer (LAK) cell, can be generated by exposing NK cells to IL-12 and particularly IL-2 (Grimm 1982, Frederick 1997). NK cells are intimately involved in the generation of Th1 responses. NK cells migrate early to sites of inflammation and draining lymph nodes, and subsequent NK cytokine production, particularly IFNγ, both recruits leukocytes to the site and drives the immune response along the Th1 pathway (Peritt 1998, Trinchieri 1998, Scharton 1993). NK cells are necessary for CTL differentiation (Kos 1996) and can direct B cell Ig production toward IgG2A (Yuan 1994, Gray 1995).

NK cells, and particularly LAK cells, display cytotoxicity against tumours in vitro and in vivo. NK lines can kill β2-microglobulin (β2M) deficient tumour (Porgador 1997), and IL-2 and IL-12 LAK cells have demonstrated activity against leukemia and neuroblastoma cells (Uharek 1996, Rossi 1994). NK cells are likely involved in the protection against metastases (Pham-Nguyen 1999), and LAK cells may be generated from peripheral blood lymphocytes and are being reinfused back into patients for a form of passive immunotherapy (Herrera 1997).

NKT cells consist of at least two subsets, one TCR+ and CD1d restricted and the other TCR- (Chiu 1999). The TCR expressed by NKT cells is composed of a single invariant α chain and a small set of Vβ regions in both mice and humans (Lantz 1994). The triggering of cytotoxicity of these cells is more akin to NK cells than T cells, as recognition and signaling through the CD1d molecule was required only for cell activation and proliferation (Kawano 1998, Spada 1998).

NKT cells have demonstrated anti-tumour cytotoxicity. These cells were an absolute requirement, in contrast to either NK cells or CD8+ T cells, for the IL-12 based rejection
of a number of tumours in a C57BL/6 model (Cui 1997). While at least one study has disputed such a large role for NKT cells in antitumour immunity (Pham-Nguyen 1999), the results overall have suggested that NKT cells may represent a proportion of the antitumour effects stimulated by IL-12. NKT cells also mediated MHC unrestricted tumour lysis and produced large amounts of IFNγ and IL-4 upon activation (Kawano 1998).

In summary, to develop effective cell mediated anti-tumour immune responses, a number of criteria will likely need be met, namely that treatments must engender a predominantly type 1 immune response, the treatment must ultimately target antigen to host APCs, and the stimulus must be either long lasting or repeated in multiple rounds. It is likely therefore, that successful strategies will recruit cells from both innate and adaptive arms of the immune system, and many immunotherapeutic strategies are being designed based on these principles (reviewed by Colaco 1999).

MECHANISMS OF TUMOUR IMMUNE EVASION

While TAAs are present on many human tumours, they do not in general lead to protective immune responses. A number of mechanisms have been put forward to explain the fact that clinically evident tumours are not in general eliminated by the immune system: downregulation of MHCI, molecular alterations of tumour vasculature, anergy, clonal deletion, clonal diversion, immunosuppression and ignorance.

As CD8+ T cell peptide recognition is dependent on peptide presentation by MHCI, it follows that CTL recognition of TAAs is dependent on tumour MHCI expression. However, the expression of MHCI on human tumours is extremely variable and subject to downregulation (Garrido 1993, Stern 1996). Between 39 and 88% of tumours from MHCI positive epithelia are HLA deficient, with heterogeneity within a single tumour not uncommon (Garrido 1993). Five classes of altered tumour HLA class I phenotypes have been defined: total HLA loss, HLA haplotype loss, HLA locus loss, HLA allelic loss and complex altered phenotypes (Garrido 1997). The Daudi cell line provides an example of total HLA loss. A point mutation in the initiation codon of the β2m gene of this Burkitt's lymphoma line destabilizes all MHCI complexes for lack of β2m (Rosa 1983). There are few examples of HLA haplotype loss, for example MZ2-MEL (Boon 1994), whereas HLA locus loss is more common. This latter phenomenon may be transcriptional, as selective
HLA-B downregulation is correlated with increased c-myc transcription in some melanomas (Versteeg 1989). Allelic loss is also common and may result from point mutations or partial deletions of class I genes (Browning 1993). Fresh human tumours reflect this variability in MHCI expression. For example, in colon carcinomas ~15% of tumours examined lacked HLA expression (Moller 1992). Tumours of the cervix, ovary, stomach amongst many others have also exhibited defective class I expression (Cromme 1994, Kabawat 1983, Natali 1989, Moller 1992, Elliott 1989, Hammerling 1987).

Molecular alterations in tumour vasculature can affect tumour host immune interactions. For instance, decreased L-selectin and α4β7 expression discourages lymphocyte-endothelial cell binding, and correlates with decreased lymphocytic infiltration in murine tumours (Onrust 1996). Similarly, low tumour expression of binding molecules such as ICAM-1 and -2 also preclude immune activity, and may be counteracted by angiogenesis inhibitors (Griffioen 1998).

Anergy is the likely outcome of the interaction between naïve T cells and tumour if the tumour does express MHCI, since the vast majority of tumours are not APCs and do not express any costimulatory molecules (Sotomayor 1996). By directly following naïve tumour antigen specific CD4+ cells injected into tumour bearing mice, it was noted that tumour induced antigen specific tolerance is dramatic and occurs early in the course of the disease (Staveley-O’Carrol 1998). This is perhaps not surprising as tumours, at least early, appear immunologically more like normal than diseased host tissue. The normal tolerization of the host immune system to its own tissue would thus supervene over anti-tumour responses. The transduction of tumour cells with B7-1 rendering them more immunogenic helped confirm this paradigm (Chen 1992, Townsend 1993). However, expression of B7-1 or B7-2 alone does not lead to systemic regression of native tumour in many models and seems to be dependent on the inherent immunogenicity of the tumour (Chen 1994).

Fas-Fas ligand (FasL) interactions can result in T cell apoptosis under certain circumstances. Some tumours, such as melanoma and hepatoma, have been found to be FasL positive and may utilize this pathway to evade host immune responses (Hahne 1996, Strand 1996). However, the regenerative and recombinative capabilities of T cell
development and TCR generation make it likely that a pool of potentially anti-tumour reactive lymphocytes will exist even in late stages of disease.

Clonal diversion refers to a shift of a tumour immune response from a Th1 response to a Th2 pattern of cytokine secretion. While it is clear that each of these patterns of response tend to downregulate the other (Muraille 1998), the evidence supporting this as a mechanism of tumour immune evasion in vivo is contradictory (Ghosh 1995, Handel-Fernandez 1997).

Global immunosuppression is present in humans at late stages of disease (Takasugi 1977), and may reflect alterations in T cell, macrophage and/or dendritic cell function (Cardoso 1996, Bhatia 1995, Gabrilovitch 1996). T cells from patients with advanced tumours have been shown to have signaling defects such as decreased expression of the ε and ζ chains of the TCR (Reichert 1998). This is most marked within tumours as some tumour infiltrating lymphocytes lack the ζ chain, and hydrogen peroxide from macrophages isolated from lymph nodes bearing metastatic melanoma cause ζ chain downregulation (Kono 1996). Suppressor T cells may also play a role as the passive transfer of L3T4+ T-cells from normal donor mice to tumour bearing mice treated with cyclophosphamide abrogated the drug’s protective effect (Awaad 1989). Locally induced immunosuppression may be induced by tumour produced soluble inhibitory factors such as prostaglandins (Leung 1989), TGF-β (Arteaga 1993), or IL-10 (Lopez 1996).

Ignorance may be a mechanism governing the maintenance of peripheral tolerance and tolerance to tumour. Since tolerance to a peripheral antigen can be ablated with the delivery of sufficiently antigenic stimuli (Ohashi 1991), the corollary is that tolerance could be maintained without such stimuli. Indeed, there is direct evidence for ignorance as a mechanism mediating tumour immune evasion, particularly to solid tumours. Mice were subcutaneously challenged with identical numbers of TAA bearing sarcoma cells either as a single cell suspension or as small tumour fragments. The fragments grew while the cells in the suspension were rejected. It was demonstrated that the tumour was ignored in this model because tumour antigens did not reach organized lymphoid tissue early after the challenge (Ochsenbein 1999). If tumour antigen had access to organized lymphoid tissue CTL effectors were generated both early and late in the course of the disease. These experiments parallel the clinical situation where tumours will not generally become
apparent until it is too late for native immune responses to deal with the tumour, and demonstrated that anergy is not a central mechanism of tumour immune evasion.

**CYTOKINES & CANCER IMMUNOTHERAPY**

A number of strategies for cancer immunotherapy have led to the eradication of established tumour in murine models, and the experience with IL-12 is particularly illustrative. In addition, the cytokines GM-CSF and Flt3 ligand (Flt3L) have also had potent anti-tumour effects.

**Interleukin-12 (IL-12) & Cancer Immunotherapy**

IL-12 is a crucial immunoregulatory cytokine encoded as two separate amino acid chains of 35 and 40kDa, respectively, that must be united as a heterodimer for biological activity. Physiologically, IL-12 is produced mainly by APCs, particularly DCs (Macatonia 1995, Heufler 1996), by one of three mechanisms. During antigen presentation activated T cells promote DC IL-12 production by virtue of the interaction of CD40L on the T cell with CD40 on the DC (Germann 1993, Shu 1995). Pathogens such as S. aureus, bacterial LPS and endotoxin promote a CD40 independent DC IL-12 production (Maruo 1997, D’Andrea 1992), as do the low molecular weight fragments of hyaluronic that accumulate during inflammation (Hodge-Dufour 1997). IL-12 influences activated T cells to promote efficient IFNγ production from these cells (Chan 1991). IFNγ in turn enhances macrophage activation in the innate response (Gazzinelli 1993), and supports a positive feedback loop as it greatly potentiates the ability of DCs to produce IL-12 (Kubin 1994a, Cassatella 1995). This synergy promotes activated T and NK cell proliferation (Kubin 1994b, Murphy 1994), and enhances cytotoxicity against virally infected cells, antibody coated cells and tumour cells (Gately 1992, Chehimi 1993). Thus IL-12 is required during the early phase of the immune response for Th1 priming, and its influence is dependent on its concentration relative to IL-10 and/or IL-4 in the area of inflammation (Tripp 1994, Vieira 1994, Muraille 1998).

IL-12 has had dramatic effects on established tumours in a number of murine studies. Systemically delivered rmIL-12 resulted in regression of day 7 to day 45 established murine tumours and long term immunity (Brunda 1993, Nastaia 1994, Zou 1995). While the dependence on NKs and CD4+ T cells was variable, common findings included that
the protection was evident only if the treatment was begun after the tumour was established (after day 3), that it was dependent on INFγ and CD8$^+$ T cells, and that cured mice retained long lasting tumour specific immunity (Mu 1995, Tannenbaum 1996, Vagliani 1996).

These regressions caused by systemic IL-12 have subsequently been attributed mainly to innate immunity and antiangiogenic mechanisms. For example, in two studies IL-12 based regression was dependent on IFNγ and the subsequent upregulation of inducible NO synthase (iNOS) (Yu 1996, Tsung 1997). In the latter study the iNOS producing cells were determined to be tumour infiltrating macrophages based on Mac1$^+$ expression with marked degradation of tumour vasculature. In these models IFNγ induced the intratumoral expression of IP-10, a CXC chemokine with antiangiogenic properties that is chemoattractive for activated T lymphocytes (Tannenbaum 1996, Cavallo 1999). In a direct comparison, systemic IL-12 was superior to intratumoral IL-12, and the regression was PMN and CD8 dependent (Cavallo 1999). As above, NKT cells are also a major in vivo target of IL-12 with MHC unrestricted tumour cytotoxicity (Cui 1997). Interestingly, IL-4 producing T cells were prominent in regressing tumours (Tsung 1997, Cavallo 1999), highlighting the contribution of mechanisms other than a Th1 adaptive response for tumour regression.

IL-12 delivered systemically to patients has had mixed results. A phase II dose escalation study, based on good results from a phase I study (Atkins 1997), resulted in the death of 2 of 12 patients hospitalized after only two doses at 500ng/kg of rhIL-12, as a result of a change in the schedule of administration (Leonard 1997). Deleterious effects associated with the systemic delivery of IL-12 have included fatigue, anemia, leukopenia, lymphopenia, thrombocytopenia and splenomegaly, amongst others (Sarmiento 1994, Tare 1995, Leonard 1997), while the marked tumour regression seen in mice was not reproducible in humans. Although further studies detailing positive CTL and NK effects of safely delivered systemic IL-12 to cancer patients have been recently published (Robertson 1999), the encountered toxicities have prompted the development of alternative strategies of delivery.

The delivery of IL-12 in combination with tumour cells has had success in generating Th1 responses and tumour eradication. IL-12 delivered by fibroblasts admixed with
tumour cells in repeated doses delayed onset of some tumours and could cure established disease at distant sites in other models (Tahara 1994, Zitvogel 1995), with the amount of secreted IL-12 correlating positively with regression. Similar results were found using tumour cells transduced retrovirally to express IL-12, and in keeping with the data from systemically delivered IL-12, protection was dependent on the cooperation between NK, CD4+ and CD8+ T cells (Tahara 1995). Again, the amount of IL-12 secreted at the tumour site was critical to regression (Colombo 1996). IL-12 expressing TSA BALB/c mammary adenocarcinoma cells, compared to the same cells transduced to express a variety of other cytokines, were best able to immunize some mice against wild-type challenge and cure a proportion of animals with distant established disease, while the efficacy of systemically delivered rmIL-12 was higher (Cavallo 1997). In this study only a single clone of cells secreting a relatively low amount of IL-12 was utilized. It has also been demonstrated that rejection of wild type tumour following therapy with an IL-12 expressing transduced P815 mastocytoma line was associated with, and dependent on, upregulation of host CTLA-4 ligand (Fallarino 1997). Since IL-12 was delivered in these studies in conjunction with tumour cells, and since tumour specific immunity developed in these models, it may be concluded that IL-12 is able to potentiate the development of a protective, or curative, immune response to coadministered tumour antigen, if the antigens are delivered in cellular form. Notably, multiple treatments were required for an effect, the immune reaction was biased towards the type 1 phenotype, and the antigens forming the basis of the CD4+ and CD8+ T cell reactivity were unknown in these models.

Taken together, these findings demonstrate that IL-12 is a cytokine with marked anti-tumour properties stemming from effects on both the innate and adaptive immune systems. IL-12 alters the host/tumour relationship through multiple pathways, including the enhancement of CD4+ T cell differentiation and the facilitation of CTL proliferation, the activation of NK and NKT cells directly, the inhibition of angiogenesis by IFNγ and/or IP-10 via NK cells, T cells and macrophages, and the suppression of tumour cell proliferation by macrophage derived iNOS. Given the toxicities of systemic IL-12 and the demonstration that long term immunity could be produced by delivery of IL-12 with tumour cells, many approaches have subsequently been explored for more efficient and localized IL-12 delivery.
Adenovirus mediated intratumoral IL-12 delivery has proven to be a promising strategy based on the local delivery of cytokine. Intratumoral injection of AdIL-12 caused complete regression and long term tumour specific immunity in 1/3 of mice bearing mammary adenocarcinoma, and it was demonstrated that the IL-12 production was highly localized to the tumour, with large secondary increases in local IFNγ production (Bramson 1996). Treatment of MCA-26 colon carcinoma murine hepatic metastases with an AdIL-12 vector resulted in a similar cure rate (Caruso 1996), and 100% of mice with a bladder carcinoma so treated showed regression and long term tumour specific immunity, dependent on CD4⁺ and CD8⁺ T cells (Chen 1997). In another model, the growth of day 3 established Renca hepatic metastases was prevented almost completely by intravenous administration of an AdIL-12 construct. Curiously, in this case PMN, macrophage and antiangiogenic responses alone were responsible for regression (Siders 1998). The absence of involvement of any adaptive response may have been due to the early time point of treatment, as innate mechanisms may have eradicated the small tumours before the development of an adaptive response. A high frequency of tumour specific CD8⁺ T cells has been demonstrated in regressing tumour and in all lymphoid compartments in mice bearing locally AdIL-12 treated P815 mastocytoma tumours (Fernandez 1999).

Taken together, these studies demonstrate that the local administration of IL-12 via an adenovirus can mediate marked anti-tumour activity based on the effects of IL-12, and that these effects can be achieved without signs of toxicity. Other immunostimulatory molecules have been used in conjunction with IL-12 to promote synergistic anti-tumour immune responses and the most promising of these synergies is that between IL-12 and CD80.

**IL-12 & CD80**

The delivery of the combination of IL-12 and CD80 as cancer immunotherapy is perhaps the current gold standard. Synergy between IL-12 and CD80 was first demonstrated in vitro. IL-12 and CD80 together activated naïve T cells and enhanced their subsequent production of IFNγ more effectively than either alone (Kubin 1994, Murphy 1994). IL-12 in conjunction with anti-CTLA-4 monoclonal antibody (mAb) was sufficient to convert a tolerigenic stimulus into an immunogenic one, confirming the synergy between IL-12 and CD80 (Van Parijs 1997).
Anti-tumour immunity has been enhanced by the delivery of IL-12 and CD80 together. In one early study, CD80 expressing transduced SCK mammary carcinoma cells formed tumours in 3/4 of animals while wild type tumours were fatal in all mice given systemic IL-12. However, identical administration of IL-12 to mice inoculated with the CD80 transduced cells cured 3/4 of animals, and this phenomenon was dependent on both CD4+ and CD8+ T cells (Coughlin 1995). Delivery of a mixture of CD80 and IL-12 transduced TS/A adenocarcinoma functioned as a vaccine against wild-type tumour at a distant site, where either alone was not efficacious. This synergistic effect was dependent on the level of local IL-12 production (Zitvogel 1996a). Treatment of Lewis lung carcinoma with an IL-12/CD80 doubly transduced variant significantly reduced the frequency of metastases compared to treatment with singly transduced variants (Kato 1997).

IL-12 and CD80 have been co-delivered most effectively by an adenovirus in a murine model. A single injection of a low concentration of an IL-12 and CD80 encoding adenovirus into day 21 established polyoma middle T transgenic (PyMidT) MT1A2 mammary adenocarcinoma tumours mediated complete regression and long term tumour specific immunity in 9/10 animals without any visible toxicity (Putzer 1997). Interestingly, the delivery of this virus was more efficacious than coadministration of two separate viruses, one encoding IL-12 and one encoding CD80, confirming in vivo the synergy seen in vitro with the combination of IL-12 and CD80. Recently, the construction of a tricistronic adenovector encoding the human IL-12 subunits and human CD80 has been completed, and will soon be used for human clinical trials (Stewart 1999, manuscript submitted).

Although the molecular and cellular mechanisms mediating regression in the preceding system have yet to be examined, it is informative to note the effect of forced CD80 expression by tumours. Tumour cells transduced to express CD80 alone do not act as APCs on their own, but do trigger some CTL cytotoxicity that does not necessarily lead to long term immunity (Wu 1995, Huang 1996). Although clonal expansion of T cells occurs at the tumour site, host APCs are likely necessary for systemic antitumour immunity when priming with CD80+ or CD80- tumour, consistent with the cross-priming hypothesis (Yang 1997, Maric 1998). It is unknown if the expression of IL-12 and CD80 will confer APC functionality to the tumour cell, but synergy is certainly apparent and likely to be due to
the recruitment of the cellular machinery of the innate response leading to an increased availability of antigen for the cross-priming pathway.

**Granulocyte-Monocyte Colony Stimulating Factor (GM-CSF)**

GM-CSF was first identified based on its ability to stimulate the clonal proliferation of myeloid precursors *in vitro* (Nemunaitis 1993). GM-CSF plays a vital role in hematopoiesis by inducing the growth of several different cell lineages, and is a principal mediator of proliferation, maturation, and migration of dendritic cells (Sallusto 1994, Young 1995, Caux 1992). GM-CSF increases DC expression of MHCII, B7 and adhesion molecules such as intercellular adhesion molecule (ICAM), and also enhances production of cytokines such as IL-1, TNF, and IL-6 (Jones 1994, Sallusto 1994). GM-CSF primes T cells for IL-2-induced proliferation (Al-Aoukaty 1994), and augments lymphokine-activated killer (LAK) cell generation in conjunction with IL-2 (Schiller 1996, Stewart-Akers 1993).

GM-CSF has been utilized to generate significant anti-tumour immunity in both murine models and in human clinical trials. In a comparison of 10 different cytokines, irradiated B16 melanoma cells retrovirally transduced to express GM-CSF stimulated the potent, long lasting, tumour specific immunity in C57BL/6 mice, superior to that resulting from cells producing other cytokines. Protection in this model was CD4+ and CD8+ dependent (Dranoff 1993). Similarly, Lewis lung carcinoma cells adenovirally modified to secrete GM-CSF *in vitro* successfully treated a proportion of mice with established tumour (Lee 1997). The GM-CSF dependent mechanism of regression is likely DC dependent as large numbers of DCs are visible at the site of inoculation of GM-CSF transductants (Lee 1997, Armstrong 1996, Gabrilovich 1996a,b). A single injection of 1x10^5 irradiated GM-CSF secreting leukemia cells cured mice of 100% of day 7, 90% of day 14, and 20% of day 21 established leukemias. Furthermore, 6 of 9 mice so treated at week two displayed long term specific immunity after rechallenge at 4 months (Dunussi-Joannopoulos 1998).

A number of clinical trials have demonstrated anti-tumour effects in patients with advanced disease. GM-CSF administered subcutaneously to patients with metastatic renal cell carcinoma (Wos 1996, Rini 1998) and intralesionally to patients with metastatic melanoma (Si 1996) mediated a small number of partial responses without toxicities. GM-CSF delivered as an adjuvant to s/c injected irradiated autologous melanoma cells caused
complete regression in 2 of 20 stage IV melanoma patients, and significant partial responses in 2 (Leong 1996). A similar phase I protocol has also demonstrated extensive tumour destruction in 11 of 16 treated metastatic melanoma patients (Soiffer 1998).

**Flt3 Ligand (Flt3L)**

Flt3L is another cytokine with anti-tumour immunological effects. It is one of a network of cytokines that regulates clonogenic survival, growth and maturation of early hematopoietic progenitors, and is most active in synergy with other cytokines (Drexler 1996). Murine injections of Flt3L caused massive increases in the numbers of cells expressing DC characteristics in spleen, lymph node, peripheral blood (Maraskovsky 1996) and liver (Shurin 1997, Steptoe 1997). Such cells are functionally active as APCs in that they are able to support the generation of primary adaptive immune responses (Shurin 1997). Ten days of s/c Flt3L injections beginning one day after tumour inoculation retarded significantly the growth of B16 and C18-1 melanomas, and EL-4 lymphoma in immunocompetent mice in association with DC and CD8+ T cell tumour infiltrates (Esche 1998). Similarly, established murine fibrosarcomas were treated successfully by Flt3L, with CD8+ T cells involved intimately with tumour regression (Lynch 1997). NK cells are also increased in number following Flt3L administration (Shaw 1998) and were responsible for the inhibition of murine colon adenocarcinoma liver metastases (Peron 1998).

**IMMUNOENCAPSULATION**

Immunocapsulation may be defined as the segregation of viable allogeneic or xenogeneic tissue within a host by enclosure within a semipermeable membrane providing protection from host immune destruction while ensuring adequate nutrition and oxygenation. Viable tissue so implanted could theoretically supply to the host soluble hormones or factors of which the host is deficient. Diseases under study for such therapy include insulin dependent diabetes (Lanza 1992, Soon-Shiong 1993), hemophilia (Josephs 1999), anemia (Koo 1993), and pituitary disorders (Hymer 1981). Immunocapsulation as a cancer immunotherapy arose from the observation that soluble antigens could access the cross priming pathway to engender an immune response and that encapsulated xenogeneic tissue stimulates a strong immune response (Brauker 1996). Two conditions
must be met for success of any therapy based on immunoencapsulation: first, the membrane must support implanted cell viability and function. Second, the membrane must prevent immune rejection. Cell viability and function is dependent on the supply of both nutrients and oxygen. Low molecular weight solutes such as glucose and macromolecules such as albumin must be accessible to the encapsulated cells. Oxygen supply depends on the site of implantation and local \( pO_2 \), the number of host blood vessels in the area, the oxygen permeability of the tissue surrounding the biomembrane and the encapsulated tissue, the rate of \( O_2 \) consumption of the encapsulated tissue, the geometry of the device, and the tissue density and spatial arrangement of the encapsulated cells.

The host foreign body response to implanted materials is a limiting factor in cell encapsulation. This response is characterized by an initial infiltrate of macrophages and/or foreign body giant cells, a secondary avascular region of multiple fibroblast layers of 30-100\( \mu m \) thick, and vascularized tissue overlying the fibroblast layers. The encapsulated tissue is thus walled-off, inhibiting oxygen and nutrient transport to the cells (Lacy 1991).

Preventing immune rejection requires that cells of the immune system have no direct contact with encapsulated tissue and that large molecules of the humoral response, particularly IgM and complement components, are also unable to reach the enclosed cells. Soluble factors such as IL-1 and macrophage released oxygen and nitrogen metabolites would be able to reach the enclosed tissue, but may be hindered by the distance of diffusion and subsequent inactivation (Colton 1996).

The criteria for successful immunoencapsulation seem to have been met by Baxter Healthcare Corporation in a novel device termed the TheraCyte™. The device is composed of a dual membrane of polytetraethylene (PTFE), with the inner cell isolating PTFE membrane of a small 0.45\( \mu m \) pore size not more than 15\( \mu m \) thick, bonded to an outer PTFE membrane of 5\( \mu m \) pore size. The outer membrane supports host cell penetration and neovascularization directly adjacent to the inner PTFE membrane in a manner that is dependent not only on pore size, but also on membrane architecture. A suffocating foreign body response is averted by the same membrane architectural characteristics that support the neovascularization. (Brauker 1995).

This system has protected allogeneic tissue viably from rejection in immunocompetent hosts for long periods of time. Allogeneic tissue implanted within TheraCyte™ devices in
rats was viable for more than one year (Brauker 1996). Secreted factors produced by encapsulated cells have been detected. Human plasma factor IX was detectable for greater than 100 days in the serum of athymic rats implanted with transduced human fibroblasts within TheraCyte™ devices (Brauker 1998), and human growth hormone was similarly detectable in nude rats for six months (Josephs 1999).

The TheraCyte™ has also been utilized in a model of cancer immunotherapy. Syngeneic murine colon carcinoma cells (1x10⁶) placed in devices and implanted subcutaneously in C57BL/6 mice survived for up to nine months, although cells that had been previously implanted in a mouse survived better than cultured cells in vitro. All (8 of 8) mice implanted with two small tumour loaded devices did not form tumours when challenged intramuscularly three weeks after implantation. Furthermore, 4/5 mice rechallenged 8 weeks after implantation were resistant to tumour challenge. Thus immune protection against tumour was engendered without cell to cell contact (Geller 1997a). Further work in the same system noted that the implanted cells were unable to protect from tumour challenge given 3 days prior to device implantation and that a slow release form of IL-2 given with the device at this time point resulted in protection of approximately 60% of animals (Geller 1997b). Thus a natural extension of this model which may enhance protection against established tumour, would be the genetic modification of the encapsulated cells with diffusable immunostimulatory molecules.

With regards to the mechanism it was hypothesized that macromolecular tumour debris shed across the device membrane accessed the immune system as antigen, cross priming T cells via dendritic cells, as there was consistently a proportion of necrotic cells contained within the device lumen. It was therefore the intent of this thesis to examine the additive benefit using cytokine expressing tumour.

There are a number of potential advantages to this system over current models of cancer vaccines: (i) in contrast to cancer vaccines, in which the transfected tumour cell is manipulated into functioning as an APC, this system would be expected to utilize native APCs, (ii) encapsulation minimizes the risk of the establishment of re-introduced viable tumour, (iii) anergy to shed antigen may be averted without cell to cell contact between tumour cells and immune cells, (iv) the antigenic stimulus and cytokine delivery from the
immunoencapsulation device is continuous and of long duration, and (v) the cells and device may be removed at the end of the treatment period.

Importantly, immunoencapsulation also provides advantages over strategies in which particular tumour antigens are targeted, since the identity of the antigen(s) need not be known, thus increasing the number of tumours that could be treated, and decreasing the likelihood of escape mutants. Potential disadvantages envisioned include poor survival of primary encapsulated tumour cells \textit{in vivo}, poor efficacy of the device due to the low concentration of antigens secreted (particularly in the human setting), the loss of cell-cell contact, and the possibility of generating autoimmunity.
HYPOTHESES & PLAN

It was hypothesized that extending the duration of tumor associated antigen presentation in the context of appropriate immunostimulatory cytokines would enhance the vaccine potential of gene modified tumor cells. To achieve this goal it was reasoned that immunoencapsulation would provide an environment in which viable tumor cells could provide a continuous antigen source to the host immune system at a level sufficient to result in immunoprotection. Further, it was anticipated that the previously reported synergistic effects of adding CD80 to cytokines in tumour vaccine models would be abrogated by loss of cell-cell contact. To evaluate this model we sought first to establish the survival characteristics of tumours both in vitro and in vivo inside TheraCyte™ immunoencapsulation devices, and to demonstrate cytokine secretion from the cells across the device membrane. Immunoencapsulation based protection and therapy experiments were then conducted to evaluate the hypothesis in vivo. Finally, we planned to evaluate the immune response conferring protection from tumor challenge using immunoencapsulated gene modified tumor cells.
MATERIALS AND METHODS

Cell Lines and Culture

The B9/BM1 cell line and all its derivatives were grown in Iscove's modified Dulbecco's medium (IMDM) with 10% fetal bovine serum (FBS), 1% penicillin-streptomycin-amphotericin (PSF), and 2% interleukin-6 (Sandoz). The MCA-38 cell line, a gift of Dr. S. Cohen (Roswell Park Memorial Cancer Institute, Buffalo, NY), was grown in RPMI medium with 10% FBS and 1% PSF (designated RPMI++). The MMB3.19 (MMB) cell line and its derivatives, gifts of Dr. Andre Schuh (University of Toronto, Toronto ON), were grown in IMDM with 10% FBS and 1% PSF. Trypsinization was required for the MCA-38 and MMB lines, and was performed with 0.25% trypsin in phosphate buffered saline (PBS) for 2 min at 37°C. Unless otherwise stated, all media and additives were purchased from GIBCO BRL (Burlington, ON). Plasticware was obtained from Corning (Corning, NY), and all cells were cultured at 37°C, in 5% CO₂.

MCA-38 Retroviral Infections

MCA-38 cells were incubated for 8-16h at 37°C, in 5%CO₂ in the presence of 8μg/ml polybrene with 5ml of retroviral supernatant from GP+E cells producing MSCV-murine IL-12, MSCV-soluble Flt3L, or MSCV-GM-CSF. The resulting MCA-38/IL-12 (MCA-12) and MCA-38/sFlt3L (MCA-Flt3) cells were selected in 50 μg/ml puromycin (Sigma, St. Louis, MO) for 1week, and then 100μg/ml puromycin for 2 additional weeks. One group of MCA-Flt3 cells were removed from puromycin at 2 weeks. MCA-38/GM-CSF (MCA-GM) cells were was selected in 0.4mg/ml zeocin (Invitrogen, Carlsbad, CA) for 3 days, and thereafter in 0.6 ng/ml for 3 weeks.

Secreted Cytokine Quantitation

ELISAs (R&D Systems, Minnesota, MN) of cell free supernatants of 1x10⁶ cells grown in 4 or 6 ml of media for 24h were performed to quantitate IL-12 and GM-CSF. Quantitation of sFlt3L cell free supernatants was performed by bioassay utilizing the Ba/F3 cell line (interleukin-3/sFlt3L dependent), with recombinant human Flt3L (10ng/ml) (R&D Systems) as the standard. Quantitation of GM-CSF cell free supernatants was performed by bioassay utilizing the OTT-1 cell line (GM-CSF dependent), with recombinant human GM-CSF (10ng/ml) (R&D Systems) as the standard.
TheraCyte™ Sterilization

TheraCyte™ devices of two sizes, 4.5μl and 20μl, were received packaged within an 8cm outer plastic capsule (Figure 2). The devices were rinsed first with 95% ethanol (Fisher Scientific, Fair Lawn, NJ), through the cleaning port, and then rinsed with and submerged in 70% ethanol for 2-4h. Devices were then rinsed twice with PBS. Fresh PBS was placed in each device, all air bubbles were expressed, and the cleaning port was grasped with a hemostat and heat sealed.

TheraCyte™ Cell Loading Procedure

A specialized plastic collar was placed over the loading port and Hamilton syringes (Hamilton Co., Reno, NV) with blunted needles were passed through the collar into the device for cell loading. A silicone sealant (Dow-Corning, Midland, MI) was then injected into the port to the level of the device lumen via a 20G needle. The loading port was cut at the border of the outer plastic capsule. Cell concentrations utilized for device loading are summarized in Table 1.

Table 1. Cell Numbers Placed In TheraCyte™ Devices.

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>4.5μl Device</th>
<th>20μl Device</th>
</tr>
</thead>
<tbody>
<tr>
<td>B9/BM1</td>
<td>0.5 x 10⁶</td>
<td>2.0 x 10⁶</td>
</tr>
<tr>
<td>MCA-38</td>
<td>1.0 x 10⁶</td>
<td>2.0 x 10⁶</td>
</tr>
<tr>
<td>MMB</td>
<td>N/A</td>
<td>1.0 x 10⁶</td>
</tr>
</tbody>
</table>

In Vitro TheraCyte™ Experiments

Individual 4.5μl devices, as loaded above with IL-12 expressing B9/BM1 cells (B9-12) or IL-12 and B7 expressing B9/BM1 cells (B9-12.B7), were placed into 3ml of medium in 24 well tissue culture plates for 12 weeks. Media was changed weekly and stored at -20°C for IL-12 quantitation by ELISA.

In Vivo TheraCyte™ Experiments

Balb/c and C57BL/6 mice (Charles River, Quebec) were housed in pathogen free facilities in clear individually aerated cages at the Toronto Hospital, Toronto, ON. All procedures detailed here were approved by the Toronto Hospital Animal Care Committee. For abdominal subcutaneous (s/c) device implantation, mice were anesthetized with
intraperitoneal (i/p) xylazine (10mg/kg) (Bayer, Etobicoke, ON) and ketamine (100mg/kg) (Rogar/STB, London ON). Mice were shaved and prepped with betadine (Purdue-Frederick, Pickering, ON) and 70% ethanol. Mice were implanted with one or two 4.5μl devices, or a single 20μl device. For 20μl device implantation, a vertical incision to the left of the midline was made with s/c dissection carried contralaterally. For the 4.5μl devices the incision was made in the midline. The device was rinsed once in PBS and placed in a vertical orientation under the skin flap. Wounds were closed with 11mm Michel clips (Harvard Apparatus, St. Laurent, Quebec). Animals were given s/c buprenorphine postoperatively (0.05mg/kg) (Reckitt&Colman Pharmaceuticals, Richmond, VA), and placed under a heat lamp until consciousness was regained.

Control groups were injected either s/c or i/p as appropriate with cell types and numbers identical to those placed in the devices in the particular experiment. The B9-12.B7 positive controls were administered as 5 injections separated by 1 week each, begun 4 weeks prior to the implantation date, and the remainder of the control injections were single vaccinations given the day of device implantation.

B9/BMI and MCA-38 tumour challenge was performed s/c in the groin(s) or dorsally, in 100μl volumes with cell numbers as indicated. Tumour size was measured with calipers in two dimensions and animals were sacrificed if tumour size was equal to 15mm in one dimension. MMB challenge was 1x10^7 cells i/p in 200μl of PBS, and mice were monitored for ascites and sacrificed before lethargy was apparent. Upon sacrifice, the device(s) with adjacent skin and peritoneum were preserved in 10% formalin (Fisher). Serum was separated from blood by centrifugation at 1500 rpm, and then frozen at -20°C.

Sectioning and staining (hematoxylin and eosin) of devices was carried out by Baxter Healthcare Corporation or the pathology department at the Hospital for Sick Children, (Toronto, ON).

**Lymphocyte Preparation from Spleen**

Murine spleens were collected into PBS under sterile conditions and injected via a 25G needle with 1cc of 100 U/ml type V collagenase (Sigma), diluted in Hanks Balance Salt Solution (HBSS) containing Ca^{++} and Mg^{++}. Spleens were shredded and pipetted vigorously, and remaining clumps were incubated for 30 min in 400 U/ml collagenase, and pressed through a 200μm nylon screen (Becton-Dickenson, Lincoln Park, NJ). Cells were
then washed in PBS and resuspended in red blood cell lysis buffer for 5 minutes. The pellet was resuspended in RPMI++, and viable lymphocytes were counted in a hemacytometer by trypan blue exclusion.

**Fluorescence Activated Cell Sorting**

Cells (5x10^5) were suspended in 50μl of PBS and stained with 2μl of one or a combination of the following monoclonal antibodies (mAb): anti-CD3 FITC, anti-CD45RPE, anti-CD8PE, anti-CD11cPE, anti-CD4 biotin (all from Pharmingen, Mississauga, ON), anti-MHCII biotin (Serotec, Hornby, ON) and streptavidin-cyochrome (Serotec). Cells were incubated on ice for 20-30 min, washed twice with sheath fluid and analyzed on a FACS scanner (Becton-Dickenson) using CellQuest™ software.

**CTL Cytotoxicity Assays**

All remaining splenocytes were incubated for 5 days with either irradiated MMB cells or irradiated B9/BM1 cells at a ratio of 20:1 in RPMI++ at a concentration not exceeding 6x10^6 cells/ml. Nonadherent cells were removed, washed in RPMI++ and counted using a hemacytometer, assessing viability by trypan blue exclusion. Cells were then resuspended to generate effector:target ratios via serial dilution in triplicate wells in 96 well V-bottom microtitre plates (Nunc, Rochester, NY). MMB and B9/BM1 cells were incubated separately with 100μCi of ⁵¹Cr (Amersham, Baie d’Urfe, Quebec) for 1.5h, washed three times in RPMI++ medium and resuspended to a concentration of 4x10^5 cells/ml in RPMI++ medium. 100μl of the resulting target cell suspension was added to each well, the plates were spun at 1200 rpm for 4 min, and were then incubated for 4h at 37°C, in 5% CO₂. At the end of the incubation period the plates were spun at 1200 rpm for 4 min and 20μl of 2% Triton-X (Fisher) was added to the maximal release wells. 100μl of supernatant from each well were placed in 3ml of scintillant and radioactivity was measured using a gamma counter (Beckman-Coulter, Fullerton, CA). Percent specific lysis was calculated as follows: ((Experimental - spontaneous release)/(maximal release)) multiplied by 100.

**Statistical Analysis**

Comparisons between mouse treatment groups were made using the χ² test.
RESULTS

Immunoeiicapsulation Based Tumour Immunotherapy in a Myeloma Model

Our initial experiments employed a murine model of multiple myeloma. The B9/BM1 cell line is a genetically engineered IL-1 producing derivative of the IL-6 dependent B cell hybridoma B9, and it preferentially metastasizes to bone marrow after intravenous (iv) injection. It may also become established in the vertebral column where it causes hind limb paralysis as well as lytic bone lesions by one to three months. If injected s/c tumour nodules will generally be palpable and measured easily at two to three weeks (Hawley 1991). Genetic modification of B9/BM1 lines had previously been accomplished in our laboratory using a murine stem cell (MSCV) based retroviral vector (Hawley 1992) with the GP+E murine fibroblast producer line used for generation of retroviral particles. Lines available for use and levels of transgene expression are summarized in Table 2.

Table 2. B9/BM1 Based Gene Modified Tumour Lines and Expression Levels

<table>
<thead>
<tr>
<th>Molecules Expressed</th>
<th>Cytokine Secretion ng/10⁶ cells/24h</th>
<th>CD80 Expression (%)</th>
<th>Name</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-12</td>
<td>1</td>
<td>N/A¹</td>
<td>B9-12</td>
</tr>
<tr>
<td>IL-12 and CD80</td>
<td>1</td>
<td>&gt;97%</td>
<td>B9-12.B7</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>70</td>
<td>N/A</td>
<td>B9-GM</td>
</tr>
<tr>
<td>Soluble Flt3L</td>
<td>200</td>
<td>N/A</td>
<td>B9-Flt3L</td>
</tr>
</tbody>
</table>

¹. Not Applicable

*B9/BM1 Myeloma Cells Implanted in TheraCyte™ Devices Survive in vitro for up to 4 Weeks*

To examine the viability of encapsulated B9/BM1 myeloma cell lines 4.5μl TheraCyte™ devices containing B9-12.B7 cells were cultured in vitro and were then opened under sterile conditions at one and four weeks. Viable cells grew in culture from opened devices from 2 of 3 devices at one week and from 1 of 3 devices at four weeks.

*IL-12 is Secreted from B9/BM1 Loaded TheraCyte™ Devices in vitro for 5 Weeks*

To ensure that cytokine was secreted across the membrane, and to measure duration of expression, 4.5μl devices loaded with either B9-12.B7 or B9-12 cells were placed in
medium in vitro. The IL-12 concentration of medium harvested weekly was assayed by ELISA. IL-12 levels secreted by B9-12 cells were higher at all times than those secreted by B9-12.B7 cells. IL-12 levels peaked at week 2, declined slightly and remained steady thereafter until after week 5. Cytokine levels then rapidly declined, and were undetectable by week 8 (Figure 1). These data suggest that viable cells persist in vitro for approximately 4-5 weeks post encapsulation.

**In vivo Survival of TheraCyte™ Implanted B9/BM1 Cells**

4.5μl TheraCyte™ devices were loaded with B9 cells and implanted s/c in mice. Initial pathological evaluation of B9-12.B7 and parental B9/BM1 (B9) loaded devices was carried out one week and one month after implantation. At one week, all devices contained viable cells associated with cellular debris. However, at one month, only a few nests of viable cells remained in the lumen, with a high ratio of debris to viable cells. Microscopic analysis of 13 devices retrieved after 14 to 70 days in vivo revealed that viable cells were present in 6 of 13 devices, again with a high ratio of debris to viable cells. Beyond one week, the duration of s/c implantation of the device did not correlate with the viable tissue remaining, nor was there any difference in survival of the cytokine expressing transductants as compared to parental cells (Figures 2, 3).

**In vivo IL-12 Production in TheraCyte™ Implanted Mice**

Serum from balb/c mice implanted subcutaneously with a single 4.5μl or 20μl device loaded with B9-12 cells was assayed for IL-12 weekly for 3 weeks (Figure 2). The IL-12 serum level was 9.7ng/ml at week 1 in a mouse implanted with a 20μl device, but declined by week three to levels equivalent to those in mice implanted with the 4.5μl device approximately 1ng/ml. Control mice contained levels of IL-12 not exceeding 60pg/ml. Mice implanted with B9-12 cells in 20μl devices exhibited signs of potential IL-12 toxicity and took longer to recover from surgery than other animals implanted with the same sized devices with any other contents. These mice were less mobile, and displayed unkempt and ruffled fur, and often did not eat nor drink as quickly after the surgery as other animals.

**B9/BM1 TheraCyte™ Tumour Challenge Experiments**

In three separate experiments balb/c mice were implanted with a single 4.5μl device loaded with B9 cell lines. Animals were challenged s/c 28 days later with 1x10⁵ parental B9 cells bilaterally in the groin. Groups included B9-12.B7 in device, B9-12 in device, B9
in device, and medium alone in device (medium). In the latter two experiments a positive control was added. These positive control mice were vaccinated s/c weekly for five weeks with $2 \times 10^5$ B9-12.B7 cells, beginning at the time other experimental groups of mice were being implanted with devices. No survival benefit against tumour challenge was conferred by implanting devices loaded with B9-12.B7, B9-12, or B9 over medium alone (Figure 5). The B9-12.B7 s/c vaccinated positive control mice survived significantly longer than did all the other groups. These data demonstrate that the protective effect achievable with IL-12/CD80 gene modified B9-12.B7 cells used as s/c vaccines is lost when encapsulated cells are used as the vaccine. Mice implanted with a single small device were also challenged with parental tumour on the day of surgery (Figure 6). No differences in survival were detected among the various groups.

To determine whether failure of encapsulated cells to protect challenged mice may be related to cell number, we next implanted two 4.5μl devices (total $1 \times 10^6$ cells) containing B9-12 cells or medium alone. In this experiment Flt3L expressing cells were also used alone or in combination with B9-12 cells (each in one device in the same mouse). Irradiated $2 \times 10^5$ B9-Flt3L cells (30cGy) were administered s/c in five weekly doses as a positive control, as data in our lab indicated that this regimen prevented the formation of B9/BM1 tumours in roughly half of treated animals. The mice were challenged two weeks after implantation. Statistically significant differences in survival were not observed (Figure 7).

The effect of an increase in the cell dose was studied further by loading B9-12.B7, B9-12 or B9 cells into 20μl devices ($2 \times 10^6$ cells). After challenge 28 days after implantation, all mice implanted with B9-12.B7 encapsulated cells had to be sacrificed at day 48 due to tumour growth, and as a group their overall survival did not differ significantly from that of the controls (Figure 8). Taken together, these data together demonstrate that at the maximal possible encapsulated tumour vaccine dose, mice were not protected from wild type tumour challenge. Indeed, the protective effects of s/c IL-12 and CD80 gene modified cells were abrogated.

**Immunoencapsulation Based Tumour Immunotherapy in a Colon Carcinoma Model**

Due to the failure of encapsulated B9 myeloma cells to confer protection against B9 challenge, the MCA-38 cell line was obtained for further studies, as this line was
previously utilized in work published by the manufacturer of the TheraCyte™ device. In these previously published studies, encapsulated MCA-38 cells consistently protected against subsequent tumour challenge (Geller 1997a,b). The MCA-38 line is a C57BL/6 methylcholoranthrene derived colon carcinoma adherent cell line that will metastasize to liver if injected intracecally, and which forms distinct nodules 10-12 days after s/c injection (Gallinger 1990).

**Retroviral Transductions & Cytokine Production**

The MCA-38 cell line was transduced retrovirally with IL-12, GM-CSF and sFlt3 ligand vectors also carrying the puromycin or zeocin resistance selectable markers. After 3 weeks of selection in puromycin, the IL-12 transductant (MCA-12) produced 16ng/10⁶cells/24h of IL-12. A GM-CSF MCA-38 transductant produced 1ng/10⁶cells/24h by OTT-1 bioassay and by ELISA. Further subcloning by limiting dilution produced a line secreting 14ng/10⁶cells/24h GM-CSF (MCA-GM) by ELISA. Two sFlt3 ligand lines were produced by selection in puromycin for 2 or 3 weeks respectively and using a Ba/F3 bioassay (see materials and methods), the lines produced 20ng/10⁶cells/24h and 300ng/10⁶cells/24h respectively (MCA-Flt3L). The higher secreting MCA-Flt3L and MCA-GM were used in the following studies.

**MCA-38 Tumorigenesis**

A cell dose of 4x10⁶ MCA-38 cells s/c was found to generate tumours consistently in vivo by day 15. The tumorigenicity of the MCA-38 retrovirally transduced lines was analyzed using 4x10⁶ MCA-12, MCA-GM or MCA-Flt3L cells. MCA-Flt3L cells caused tumour in 1 of 3 mice, while neither MCA-12 nor MCA-GM were tumorigenic in a series of 3 mice each. All survivors were immune to parental MCA-38 s/c challenge on day 49.

The potential of the retrovirally transduced lines to protect mice from same day wild type challenge was assayed by injecting 4x10⁶ parental MCA-38 and 4x10⁶ of one of MCA-12, MCA-GM or MCA-Flt3L cells s/c on opposite groins simultaneously. In animals treated with MCA-GM or MCA-12 cells, 2 of 3 mice in each group grew parental tumour, while 0 of 3 mice with MCA-Flt3L developed tumours. All survivors were immune to s/c rechallenge with MCA-38 cells at day 47. These data demonstrate that cytokine expressing gene modified MCA-38 tumour cells administered s/c can protect mice from wild type tumour challenge.
In vivo Survival of TheraCyte™ Implanted MCA-38 Cells

A total of 18 devices loaded with MCA-38 cells were implanted in mice and retrieved 14 to 195 days later (Table 3). Microscopic examination revealed that while viable cells were seen at 14, 40 and 50 days, these live cells were scattered in clumps within the lumen together with moderate amounts of cellular debris. Overall, nests of viable cells, were present in 5 of 14 devices containing parental MCA-38 cells. Small numbers of dispersed but viable individual cells were seen in 2 of the remaining 9 devices. In 3 of the 5 containing viable nests of cells, the living cells were concentrated around the perimeter of the lumen, as previously described by the manufacturer (Geller 1997a) (Figure 9). The microscopic appearance of the devices containing transduced cells was identical to that of the devices containing parental cells. All devices contained some fibrinous debris. The length of time the devices had been implanted in vivo did not correlate with the amount of viable tissue, as the three devices demonstrating the best survival were explanted after 43, 50 and 148 days. Thus cell viability was highly variable.

MCA-38 TheraCyte™ Tumour Challenge Experiments

To test the capacity of encapsulated MCA cells to protect against tumour formation in a vaccine setting, mice were implanted with a single 20μl device containing MCA-GM, or MCA-parental cells, or medium alone, and were then challenged dorsally s/c on day 27 with 4x10⁶ MCA-parental cells. Negative controls included naïve mice and surgery alone animals. Two positive control groups were included, a single s/c inoculation of 2x10⁶ irradiated MCA-parental cells, or a single s/c inoculation of 2x10⁶ irradiated MCA-GM cells administered at the time of surgery. Due to post-operative wound complications, only 2 of the device controls, containing medium alone could be evaluated. Figure 10 shows the control groups compared to the encapsulated MCA-parental, and cytokine modified MCA-GM groups. The MCA-parental encapsulated cells did not provide any greater protection from challenge (1 survivor of 3) compared to non-encapsulated irradiated cells (1 survivor of 4), although both groups exhibited slightly prolonged survival as compared to naïve mice and surgery alone mice challenged with parental cells s/c. Irradiated MCA-GM cells injected s/c protected completely all 5 mice from challenge while 2/5 mice treated with encapsulated MCA-GM cells survived long term. All mice in the surgery only and naïve groups were sacrificed by day 32 and 36, respectively. Thus, a pattern similar to
that achieved in the B9 model emerged, in that encapsulating gene modified cells abrogated the protective effects of their unencapsulated counterparts.

In a concurrent experiment, mice were challenged with tumour 2 days prior to implantation of devices to assess the potential of encapsulated cells as a tumour therapy. Two 4.5μl devices per mouse were used in this experiment as 20μl device availability was limited. Groups included: MCA-parental, MCA-GM and MCA-12 in devices, irradiated MCA-parental, MCA-GM or MCA-12 cells (all given s/c), medium alone, surgery alone and naïve. All mice in the naïve and empty device groups were sacrificed by day 34 post tumour challenge, whereas a single mouse with surgery alone rejected its tumour. Figure 11 compares controls with MCA-parental (s/c and encapsulated) and MCA-12 cells (s/c and encapsulated), and MCA-GM (s/c and encapsulated) groups. No statistically significant differences in survival were evident between any two groups, except between naïve and irradiated MCA-GM cells (the negative and positive controls) (p value 0.015).

In vivo IL-12 Production in MCA TheraCyte™ Implanted Mice

At the conclusion of challenge experiments, (range day 20 to 27 post implantation) mice were assayed for serum IL-12. The average serum IL-12 concentration of 3 MCA-12 mice at the time of sacrifice was 38.3pg/ml (Table 5). Other serum IL-12 concentrations were as follows: unmanipulated control 41.1pg/ml, empty device 39.8pg/ml, irradiated MCA-12 34.1pg/ml, and encapsulated MCA-parental 41.8pg/ml. Since samples were not collected serially it is not known if higher IL-12 concentrations were present at earlier time points.

Immunoencapsulation Based Tumour Immunotherapy in an Acute Myelogenous Leukemia Model

Since neither B9 nor MCA-38 encapsulated cells protected against tumour challenge, and since cell viability was poor, we were not confident that the loss of protection from tumour challenge observed after encapsulation was meaningful. Therefore we explored cell lines which may survive for longer periods after encapsulation. MMB is a c-myc retrovirus transformed myeloid leukemia line of C57BL/6 origin that expresses monocyte/macrophage markers including Mac-1, Mac-2, as well as MHCI and MCHII (Korngold 1994).
**In vivo Survival of TheraCyte™ Implanted MMB Cells**

Early microscopic analysis of MMB loaded devices was promising. Overall, a total of 22 MMB loaded 20μl TheraCytes™ were sectioned and stained (Figure 12), and the duration of implantation ranged from 7 to 166 days. All devices were found to be thick with viable cells around the perimeter of the lumen (~4-8μm), with the central area of the lumen packed with necrotic debris (~10-20μm) (Table 4). In all devices, except those explanted at day 7, copious numbers of viable cells were evident around the perimeter of the lumen, and large areas filled with cellular debris were evident. At day 7 post implantation, the debris was less prominent, but rather a thin core of mostly viable cells (~3-4μm) was present. Devices containing MMB-12 and MMB-Flt3L transductants appeared identical pathologically to the MMB cells. Thus in contrast to the B9 and MCA-38 lines, the encapsulated MMB cells survive very well for long periods of time in vivo.

**MMB Cell Lines & Cytokine Production**

The parental MMB line, an IL-12 secreting version (MMB-12) and a transductant secreting sFlt3L (MMB-Flt3L) were obtained from the lab of Dr. A. Schuh. The rates of IL-12 and Flt3L secretion, as determined in our laboratory, were 13.8 and 25ng/10^6 cells/24h, respectively. Greater than 94% of both MMB and MMB-12 cells were positive constitutively for CD80, and approximately 3.4% of cells from both lines were positive for MHCII.

**MMB Tumorigenesis**

Intraperitoneal (i/p) injection of 1×10^7 MMB cells results in ascites and lethargy in 100% of mice in 2 to 4 weeks. Data from the laboratory of Dr. Schuh indicated that MMB-12 cells would not grow in vivo.

**MMB TheraCyte™ Tumour Challenge Experiments**

To test the capacity of encapsulated MMB cells to enhance protection against tumour formation in a vaccine setting, mice were implanted with 20μl devices loaded with MMB, or MMB-12 cells or with medium alone, and were then challenged with 1×10^7 MMB cells i/p on day 19 post surgery. Negative control groups included naïve mice and surgery alone animals. Two further groups were included as controls to exploit the protective effects of MMB-12 non-encapsulated cells. These latter groups underwent surgery on the same day that they were injected i/p with 2×10^6 MMB-12 or irradiated (30cGy) parental MMB cells.
Results demonstrated a rapid development of tumour in the naïve and surgery only mice while encapsulated parental MMB cells, or i/p administered irradiated cells, caused a small delay in tumour onset as compared to controls, although there were no long term survivors (Figure 13). Of the IL-12 expressing groups, encapsulated cells also caused a slight delay in tumour onset with a single long term survivor. Interestingly, once again the non-encapsulated MMB-12 cells group proved most efficacious with a greatly extended survival and 2/5 long term survivors.

The experiment was repeated utilizing 20μl sized devices once again and mice were challenged on day 25. All naïve and surgery only animals were sacrificed by day 16 post challenge, and 50% of those with an empty implant were sacrificed at this time, while the remainder survived until day 48. Both the encapsulated parental MMB and irradiated parental MMB groups demonstrated a delay in tumour onset relative to controls (Figure 14). Although encapsulated MMB-12 cell treated mice also demonstrated a delay in tumour onset, they were considerably inferior as compared to MMB-12 i/p animals, which were all subsequently noted to be resistant to challenge. The MMB-Flt3L group all had to be sacrificed early compared to the negative controls, consistent with earlier findings, suggesting that Flt3L may accelerate the growth of MMB cells in vivo. The results from these latter two experiments were pooled for statistical analysis of survival and significant differences were noted between MMB-12 administered i/p and all other groups (p values not greater than 0.02). No differences seen between the empty device group and encapsulated MMB nor MMB-12 cell groups (p values 0.33 and 0.54). The survival of the MMB-12 i/p group, as well as the encapsulated MMB and MMB-12 groups, varied significantly from that of the naïve and surgery only groups, while the MMB-Flt3L group did not (p value 0.23 and 0.31). Thus MMB-12 encapsulated cells conferred less protection than a single i/p injection of these cells.

**FACS Analysis of Splenocytes from MMB TheraCyte™ Implanted Mice**

To examine the immunologic basis for the survival difference between i/p vaccinated mice versus mice implanted with encapsulated MMB-12 cells, splenocytes were harvested at weeks 1 and 3 and stained with various mAb (Table 6). No gross differences in splenic cell immunophenotype were observed between the groups that could account for the differences in survival.
**In vitro Murine Cytotoxicity Assays**

Splenocytes were harvested from mice implanted 1 week prior with a 20μl TheraCyte™ device loaded with parental MMB cells, or MMB-12 cells, or medium alone. Following stimulation of splenocytes for 5 days with irradiated MMB parental cells, control splenocytes showed no differences in cytotoxicity against parental MMB cells compared to splenocytes from MMB-12 i/p injected controls (Figure 15). Similarly, at three weeks no differences in cytotoxicity against MMB cells was observed between the two groups, and at both 1 and 3 week time points the absolute level of cytotoxicity was low in all animals. As a positive allogeneic control, splenocytes were also incubated *in vitro* with irradiated B9/BM1 cells, and significant cytotoxicity was generated against B9/BM1 cells. In summary, no differences in splenic derived CTL cytotoxicity against parental MMB cells could be demonstrated to explain the survival differences conferred by i/p injection of MMB-12 cells.

**In vivo Cytokine Production in MMB TheraCyte™ Implanted Mice**

Average IL-12 concentrations in mice with MMB-12 encapsulated cells was 38.1 and 43.9 pg/ml respectively (Table 5). Mice bearing encapsulated MMB cells had serum IL-12 concentrations of 31.6 and 41.8 pg/ml, respectively in two sequential experiments, while mice with empty devices had serum IL-12 values of 37.2 and 50.1 pg/ml. An unmanipulated C57BL/6 mouse had a serum IL-12 value of 41.1 pg/ml.

In summary, no differences in circulating IL-12 levels, splenic CTL or splenic immune cell phenotype were observed. Thus, the reasons for the enhanced protection against the parental MMB cells in the MMB-12 i/p vaccinated mice, and the small effect of the encapsulated MMB-12 cells, are not currently clear.

No Flt3L was detected in the serum of control mice nor in the serum of 3 mice implanted with MMB-Flt3L encapsulated cells sacrificed at days 46, 47 and 48.
DISCUSSION

In this study we postulated that tumour antigen would be delivered continuously together with cytokine through a semi-permeable immunoencapsulation device. We also hypothesized that extending the duration of tumor associated antigen presentation in the context of appropriate immunostimulatory cytokines would enhance the vaccine potential of gene modified tumor cells.

In all three murine tumour models utilized in this thesis encapsulated cells failed to provide protection against wild type tumour challenge, compared to protective effects generated by unencapsulated gene modified tumour cells. In the B9 model, the protective effects of the B9-12.B7 cells delivered s/c were abrogated by tumour encapsulation at all doses. Similarly, there was no statistically significant prolongation of survival seen in the MCA model. Finally, while mice with encapsulated MMB or MMB-12 cells survived significantly longer than naïve or surgery only controls, they did not vary significantly from the empty device controls.

There are a number of potential explanations for the failure of the TheraCyte™ devices to protect mice from wild type tumour challenge compared to controls. These possibilities include (i) failure of antigen to cross the membrane, (ii) failure of antigen to access the APC cross priming pathway due to macrophage uptake, (iii) failure of antigen to access the APC cross priming pathway due to alternate methods of antigen uptake by APCs (akin to loss of cell to cell contact), (iv) inadequate cell and/or antigenic dose, (v) rate of antigen release, (vi) development of tolerance, (vii) molecular alterations of encapsulated tumour cells, and (viii) inadequate cytokine levels.

No direct evidence that antigen does indeed cross the device membrane was put forward in this work and thus, theoretically, it is possible that antigen never accessed APCs. Although possible, this is unlikely given that IL-12 has been shown to cross the membrane.

Antigen released from the device may have failed to access the cross-presentation pathway due to uptake by macrophages. Macrophages, like DCs, are attracted to sites of inflammation and actively take up antigen by phagocytosis and pinocytosis. The result of macrophage antigen uptake, however, is immune downregulation (Savill 1997, Albert 1998a). While DCs cross-presented ingested antigen on MHCI leading to CTL priming, macrophages failed to prime CTLs after antigen uptake, and also abrogated the priming
response in a competitive assay (Ronchetti 1999, Albert 1998a,b). After ingestion of apoptotic cells, macrophages can inhibit proinflammatory cytokine production through mechanisms involving TGF-β and PGE2 (Fadok 1998), or via the induction of apoptosis of bystander leukocytes (Brown 1999). Thus, if antigen shed from the device were taken up primarily by macrophages, as opposed to DCs, a CTL response may have been prevented or downregulated.

The mechanism of APC antigen uptake may vary between the encapsulated and nonencapsulated cells, and this may have led to divergent immunological outcomes. CTL mediated cytotoxicity results in apoptosis of target cells (Griffiths 1995, Henkart 1997). Apoptotic cells, but not necrotic cells (Albert 1998a), are taken up by DCs for antigen presentation to T cells (Albert 1998b, Rovere 1998c). Immature DCs mediate phagocytic uptake of apoptotic material by DCs via αβ integrins and CD36, and these antigens rapidly access the cross priming pathway (Albert 1998b). These former mechanisms require cell to cell contact, but antigen transfer to APC may also include shedding of antigenic vesicles (Zhou 1992) and the release of immunogenic, apoptotic blebs (Casciola-Rosen 1996). Since the diameter of apoptotic blebs by electron microscopy is in the order of 0.18μm (Segundo 1999), it is unclear if soluble antigen is the only material able to cross the 0.45μm TheraCyte™ membrane. Uptake of soluble antigens may be mediated differently from that of antigens encountered as cellular constituents (Steinman 1991, Reis-e-Sousa 1993, Sallusto 1995, Lanzavecchia 1996), and has been associated with the induction of antigen specific tolerance in some models (Liblau 1996, Galbiati 1998). Thus, a number of pathways for antigen collection operate in APCs. These mechanisms may target differentially derived antigens to different processing compartments, such as the cell membrane, early endosomes, lysosomal-like vesicles, or the cytosol, potentially leading to differential peptide repertoires for MHC binding and presentation (Jondal 1996).

Assuming antigen does traverse the TheraCyte™ membrane and is taken up by APCs, differences in antigen concentration initially, and over time, may exist between the encapsulated and nonencapsulated groups. While it was unclear what degree of cell survival within an encapsulation device would be required for the generation of an antitumour immune response, the inconsistent survival of both the B9 and MCA-38 cells in devices in vivo likely corresponds to a high variability in antigen exposure. The consistent
survival of the MMB cells in the device probably reflects a more reproducible amount of antigen release as compared to the other models, however, in no way can it be assumed that the cell turnover in the devices in vivo resulted in a similar amount of antigen release as the positive controls. Thus, the total amount of antigen delivered may not be identical, and this is significant because antigen load can determine the outcome of cross presentation (Kurts 1998, Rovere 1998a).

The rate of antigen release into surrounding tissue from encapsulated and nonencapsulated groups could vary significantly. A surge of antigen release from a newly implanted cell-loaded device would be anticipated, as it would be from any of the nontumorigenic positive controls. However, given that some of the encapsulated cells would be expected to survive, and that the membrane would slow antigen diffusion, the initial antigen load might be considerably smaller than that from nonencapsulated cells. The large buildup of debris in the MMB implanted devices also highlights the possibility that antigen release in this instance may have been quite slow for long periods of time. In general, studies demonstrating protection with cell lysate vaccines have utilized repeated vaccinations rather than a constant stimulation, likely simply due to technical reasons (Zitvogel 1996, Mitchell 1998).

Soluble antigen release can tolerize an animal to tumour (Liblau 1996, Galbiati 1998). It is possible therefore, that ongoing low level antigen release in our model may have served to tolerize the animals to the tumour in a mechanism suggested by the deletion of autoreactive CD8+ T cells via cross-presentation (Kurts 1997). Similarly, tolerance may be an outcome of cross-presentation if the precursor frequency is low, as was seen in the rat insulin promoter/hemagglutinin (HA) expressing mice (Lo 1992). In these animals, expression of a foreign antigen, influenza HA, on pancreatic beta cells led to tolerance, even in the face of an HA-expressing viral challenge. Only adoptive transfer of lymphocytes from transgene negative mice in addition to viral challenge led to inflammation and autoimmunity. These results argue against constant antigen stimulation and in favor of a repetitive cycle of stimulation with antigen levels above a threshold for CTL generation as demonstrated by Oschenbein et al (Oschenbein 1999).

Even if antigen was delivered and presented appropriately, it cannot be assumed that the cells implanted in the TheraCyteTM and the cells used for the challenge were identical after
4 weeks in vivo. Two populations of multiplying cells from a common origin will necessarily undergo expressional and genetic changes that will not necessarily be equivalent in rate nor direction over time. In the late 1970s, leukemic cells were placed in diffusion chambers in vivo to study the cellular effects of chemotherapeutic agents. After periods of time in vivo various morphological features and surface molecular phenotypes of some cell types were found to be altered, and a tendency towards differentiation was seen in a number of instances (Jager 1982, Thiel 1981, Hoelzer 1977). Therefore, it is possible that at some point after implantation the cells in the TheraCyte™ could have become so dissimilar from the challenging tumour cells that the animal was being exposed to a set of antigens quite distinct from that expressed by the challenging tumour.

IL-12 secretion from encapsulated tumour did not enhance the vaccine efficacy of parental cells in the TheraCyte™ device, and failed to elicit protection in any of the three models. IL-12 has been delivered in models of tumour immunotherapy in one of three general ways: locally at the tumour site, systemically, or in conjunction with a cross-priming stimulus. An aim of the current study was to utilize IL-12 as an adjuvant to shed antigen as a cross priming stimulus, but this system must also be considered as a means to deliver systemic cytokine.

The differences between the TheraCyte™ system and other models of systemic IL-12 delivery for cancer immunotherapy can likely be accounted for in the relative doses of IL-12. Cavallo et al. gave mice 100ng/day in two courses of five doses each to cure balb/c mice of established mammary adenocarcinomas (Cavallo 1997, 1999). Mu et al. utilized a dose of 500ng in five doses over two weeks against an ovarian tumour, while Brunda et al. gave C57BL/6 mice 15 doses of 1μg/day over 3 weeks to treat melanoma and found the effect substantially reduced at a dose of 0.1μg, and reduced to zero at a dose of 0.001μg (Mu 1995, Brunda 1993). In these studies it is unknown if serum levels of IL-12 correlate with tumour regression although it is clear that these effects are dose dependent. The above regimens differed drastically from the amount of IL-12 released from the device. Assuming all 2x10⁶ MCA-38 cells survived in the 20μl device, 32ng/day of IL-12 could be delivered maximally, which is approximately 1/3 of the lowest dose seen to be effective. In addition, the preceding studies delivered IL-12 beginning on the day of challenge or later, capitalizing on the effects of IL-12 against established tumour, in contrast to our
system in which therapy was begun 2 to 4 weeks prior to challenge in most instances. Thus in light of the cellular survival documented in the devices, the amount of IL-12 actually delivered was likely quite low at the time of the tumour challenge. Thus, it is would not be expected for IL-12 to act systemically against tumour challenge in our models.

IL-12 was found in the serum of balb/c mice implanted with encapsulated B9-12 cells at weeks 1 to 3 whereas no IL-12 was found in mice bearing MMB-12 loaded Theracyte™ devices. This is a curious observation as the B9 cells secrete IL-12 at a rate a log lower than do the MMB-12 cells. This discrepancy may be partially explained by the patterns of cellular survival. The survival of these cells in devices in vivo demonstrated inverse patterns, while many B9 cells were present at week 1 and few thereafter, few live MMB cells were visible at week 1 with many thereafter.

In the Theracyte™ system IL-12 failed to act as an adjuvant to a cross-priming stimulus in our models. IL-12 has previously been delivered in various models as an adjuvant to tumour vaccines or therapies in vivo in three general ways, with whole tumour cells (gene modified and/or irradiated), in combination with dendritic cells cultured ex vivo with exposure to antigen, and rarely together with soluble antigen. Many studies have documented tumour specific immunity after vaccination with IL-12 delivered with tumour (Tahara 1995, Zitvogel 1995, Colombo 1996, Fallarino 1997, Rodolfo 1998, Sumimoto 1998), and IL-12 can improve the efficacy of peptide pulsed DC based vaccinations to generate an antigen specific CTL response (Gabrilovich 1996a,b). Significantly, doses of IL-12 well below those effective systemically, delivered concomitantly with peptide pulsed DCs, has evoked strong anti-tumour CTL responses (Fallarino 1999), demonstrating that locally delivered IL-12 in combination with an antigenic stimulus can induce an effective type 1 response. Thus, IL-12 is an excellent adjuvant in the development of tumour specific CTL responses in the context of whole cells or primed APCs. Soluble protein alone delivered continuously via a mini-oxmotic pump inhibited the development of a type 1 response and promoted a type 2 response, tolerizing balb/c mice to tumour. IL-12 given with the soluble protein at 1.2ng/h for 12 days promoted a vigorous Type 1 response although protection in vivo was not assessed (Galbiati 1998). This dose was likely in the same order of magnitude as that delivered by the MMB-12 cells but was likely higher than
that in the B9 and MCA systems due to the lower amount of IL-12 produced and poor cell survival, respectively.

Thus, the use of IL-12 as an adjuvant to promote tumour specific CTL responses is sound and it may have failed due to inadequate dose. However, the variability in the stimulating antigen(s) is likely also a crucial factor. The amount of antigen accessing the cross-priming pathway could have been below a threshold at which IL-12 could have acted effectively as an adjuvant. Therefore, it is also possible that IL-12 did not fulfill this objective in this work due to a low antigenic stimulus.

The ability of the cytokine secreted from encapsulated cells to access compartments outside of the device was confirmed by the B9 IL-12 secretion seen in vitro and the serum IL-12 levels found in vivo. The tapering of IL-12 secretion in the B9 model probably reflected ongoing cellular loss rather than downregulation of IL-12 production, as it paralleled the histological findings documenting scanty cellular survival of B9 cells in devices in vivo. As IL-12 is a cytokine with antiangiogenic properties it was anticipated that high concentrations of IL-12 around the devices in vivo could inhibit angiogenesis into the device upon which the survival of encapsulated cells were ultimately dependent. Blood vessel formation around the encapsulation devices containing IL-12 secreting cells upon explantation in all three models was not grossly different from that around other encapsulated cells, and MMB-12 cells were supported to the same degree as were the other MMB lines. Thus, although not formally tested, it is unlikely that IL-12 affected angiogenesis in the proximity of the TheraCyte™, and the lack of protection in the challenge experiments could not be attributed to poor cell survival mediated by the antiangiogenic properties of IL-12.

To compare the encapsulated cells with the positive controls, CD80 expression must also be considered. CD80 expression alone promotes CTL cytotoxicity against a variety of CD80 positive targets (Chen 1994, Wu 1995, Zheng 1997). A regimen of vaccinations with CD80 expressing cells has generated long term immunity in some models, but failed to achieve this goal in others (Chen 1994, Wu 1995, Yang 1997). The variable results in these studies has been ascribed to the native immunogenicity of the tumour. As seen elsewhere (Coughlin 1995, Zitvogel 1996a), synergy would be expected between IL-12 and CD80 in this setting, by attracting cells of the innate immune system and triggering a
cascade of cytokines and effector molecules. MMB-12 cells are rejected in immunocompetent mice while MMB cells grow progressively, notwithstanding that both are strongly CD80 positive. It is possible that synergy between IL-12 and CD80 results in CTL cytotoxicity against the MMB-12 cells resulting in antigen delivery into the cross priming pathway and systemic immunity. Encapsulated groups could not benefit from CD80 expression as cell to cell contact between targets and effectors is prohibited by the TheraCyte™ membrane.

Antigen specific anti-tumour immune responses have been promoted in patients via GM-CSF delivered together with irradiated cells (Soiffer 1998, Leong 1999), and GM-CSF also promotes allostimulatory DC presentation of soluble antigen in vitro. (Jones 1994, Sallusto 1994). The levels of GM-CSF delivered in these models was consistently higher than that which could have been delivered by the TheraCyte™ encapsulated B9 or MCA-38 cells. As in the case of IL-12, it is possible that the tumour antigen dose could have been below a threshold under which GM-CSF would have been able to act as a vaccine adjuvant.

Unexpected results were obtained in the MMB model utilizing Flt3L, as the MMB-Flt3 group died significantly earlier than the empty control group (p value 0.04). Since Flt3L has marked proliferative effects on DCs (Maraskovsky 1996, Shurin 1997, Steptoe 1997), stimulates NK mediated anti-metastatic effects (Peron 1998), and has prevented the development of peripheral tolerance to a soluble antigen (Pulendran 1998), it was anticipated that it would be particularly suited to the TheraCyte™ system. However, it is appreciated that many leukemic cells express Flt3 and exhibit a proliferative response to Flt3L exposure in vitro (Drexler 1996), although Flt3L administration has inhibited growth of the C57BL/6 syngeneic EL-4 lymphoma in vivo (Esche 1998). In the latter study, 10μg/mouse/day was administered after tumour challenge, and this is many times that which could have been maximally secreted by the encapsulated MMB-Flt3L cells in the current studies. Although no Flt3L was detected in the serum of mice bearing MMB-Flt3L loaded devices, it is unknown if serum Flt3L levels correlate with its effects, particularly since level were measured after forty days in vivo. Interestingly, Flt3L has been implicated in the enhancement of the induction of oral tolerance to very low doses of soluble antigen (Viney 1998). This again points to questions regarding the nature of the antigens released
from the TheraCyte\textsuperscript{TM}, as it is possible that these antigens tolerized the mice in a mechanism similar to those delivered orally.

We attempted to use the same tumour model as the manufacturers (Geller1997a,b), C57BL/6 syngeneic MCA-38 cells, because immunoencapsulation as a tumour vaccine may not have been testable in the B9/BM1 model due to the inability of the cells to survive in the devices \textit{in vivo}. We acquired the MCA-38 line not from the company, but from an alternative source. Although our cells grew consistently in mice challenged with $4 \times 10^6$ cells, this was a larger dose than those cell doses used by Geller et al. The survival of our MCA-38 line in devices \textit{in vivo} was also not as good as expected given the results of Geller et al (Geller 1997a). It is unknown how divergent molecularly these lines are, and whether these potential differences affected cellular survival in the devices \textit{in vivo}.

Our decision to use encapsulated IL-12 expressing cells was based on the reported success of the TheraCyte\textsuperscript{TM} in conjunction with a slow release form of IL-2 (Geller 1997b). Animals with preexisting tumour were treated (Table 5) and in these experiments a free injection of $1 \times 10^6$ irradiated MCA-38 cells in the area of the device was added. Devices were implanted three days after intramuscular tumour cell challenge consisting of $1 \times 10^3$ cells.

Table 7. TheraCyte\textsuperscript{TM} Therapy of Pre-existing MCA-38 Tumours by Baxter Healthcare Corp.

<table>
<thead>
<tr>
<th>Group #</th>
<th>Device Contents</th>
<th>Free Irradiated Cells</th>
<th># Animals Tumour Free at 60 Days</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>No Cells</td>
<td>No</td>
<td>0 / 3</td>
</tr>
<tr>
<td>2</td>
<td>No Cells</td>
<td>Yes</td>
<td>0 / 3</td>
</tr>
<tr>
<td>3</td>
<td>Live MCA Cells</td>
<td>No</td>
<td>0 / 4</td>
</tr>
<tr>
<td>4</td>
<td>Live MCA Cells</td>
<td>Yes</td>
<td>1 / 4</td>
</tr>
<tr>
<td>5</td>
<td>Irradiated MCA Cells</td>
<td>No</td>
<td>1 / 5</td>
</tr>
<tr>
<td>6</td>
<td>Irradiated MCA Cells</td>
<td>Yes</td>
<td>3 / 5</td>
</tr>
<tr>
<td>7</td>
<td>Irradiated MCA Cells &amp; Slow Release IL-2</td>
<td>No</td>
<td>3 / 5</td>
</tr>
</tbody>
</table>

Based on these data, it was concluded that direct cell to cell contact was not required for protection, notwithstanding the use of irradiated cells inside the device. This again leads to the question what form the antigens leaving the device are taking. It is clear that TheraCyte\textsuperscript{TM} encapsulated MCA-38 cells did not protect all mice from tumour challenge in our hands, regardless of the state of the cells \textit{in vivo}. This was the case in this work both in
the setting of device implantation prior to tumour challenge (vaccination), as well as in the setting of tumour challenge prior to device implantation (therapy). In the setting of tumour therapy, the best results of Geller et al. (Geller 1997b), occurred using irradiated cells, thus, the argument could be made that these positive results stemmed from a particularly large initial antigen load rather than an ongoing low level antigenic stimulation.

While the promise of immunoencapsulation to deliver a continuous antigenic signal sufficient to generate an anti-tumour type 1 immune response was not realized in this work, our results do not necessarily invalidate this strategy. Given the current state of knowledge with respect to tumour antigens, samples of a spontaneously arising neoplasm are likely to play an important role in cancer immunotherapy as a source of tumour antigen. Since only small amounts of tumour may be available at any one time, immunoencapsulation as a strategy for cancer immunotherapy thus remains conceptually attractive. With further work efficacy may be evident in particular situations and it may find a place amongst the myriad of proposed cancer immunotherapy strategies.
FUTURE WORK

With regards to the TheraCyte™ device, work could be directed towards animal tumour challenge experiments looking for working formulae, or work could focus on the molecular and cellular interactions of this device and extruded antigen. It is the opinion of this researcher that further detailed study of the fundamental interactions between neoplastic cells and the immune system will particularly enhance our ability to design effective cancer immunotherapy strategies. The TheraCyte™ device provides a unique opportunity to study the molecular aspects of antigen uptake both in normal and neoplastic systems. A better understanding of antigen uptake will better allow us to target tumour antigen to APCs, thus increasing the likelihood of generating effective anti-tumour immune responses.

The constitution of the material and rate of its release from the device is of central interest, and could be investigated by a determination of the molecular nature of secreted contents. Do the materials secreted contain protein, lipid, and/or nucleic acids, and in what proportions? How does the secretion rate change over time? Are the materials contained in vesicles, or soluble? In conjunction with this work it would be crucial to assess the role of APCs in accumulating tumour antigen released from the device. Using a cell line engineered to express a particular protein, in vitro work could investigate a number of APC functions, whether APCs can take up secreted material, which subcellular APC mechanisms assist in its processing, and whether the APC can present the proteins to T cells to generate an effective response. Comparison should be made to antigen derived from unencapsulated tumour cells. To demonstrate antigen uptake/processing in vivo, APCs could be harvested from a mouse implanted with encapsulated cells that express a protein with an epitope known to bind to the MHC of the mouse, hen egg lysozyme for example, and a T-cell clone could be used in a functional assay.

If work was to be carried past this level, an exploration of in vitro and in vivo murine tumour cell line survival in the devices would also be important, as this was likely the single most important factor affecting the outcome of these studies. An assessment of the survival of human tumour lines as well as fresh human tumour in vitro and in nude mice could also be considered. One could also assess the effect of other cytokines on the ability of the device to engender protection, particularly IL-2, given the results of Geller et al., as
well as IL-3, in light of its effects on antigen uptake and processing (Yeh 1998, Pulaski 1996). Further delivery of cytokine could be accomplished by direct repeated injection of cytokine into the area of the device to ensure adequate cytokine levels and maintain a molecular milieu favorable for the generation of an effective anti-tumour type 1 response.
CONCLUSIONS

In this study we have demonstrated that TheraCyte™ devices loaded with gene modified, or wild type, tumour cells functioned neither as a cancer therapy nor as a cancer vaccine in the B9/BM1 and MCA-38 murine cancer models. Based on the duration of IL-12 secretion across the device membrane and the differences seen in the sectioned devices, it is evident that tumour cells can survive in the devices both *in vitro* and *in vivo* for extended, albeit highly variable periods of time. This variability is dependent on the particular characteristics of the tumour, and likely reflected a variability in tumour antigen release. Thus, it is possible that the poor survival of the tumour cells in the devices impacted significantly on the ability of encapsulated cells to protect the mice from wild type tumour challenge.

It may also be concluded that the TheraCyte™ device was not efficacious as a cancer vaccine in the MMB tumour model since mice bearing encapsulated cells did not survive statistically significantly longer than mice with devices containing medium alone. However, as there was a significant prolongation of survival of mice bearing encapsulated cells compared to the negative controls it is possible that there was some positive effect of encapsulated cells (although numbers are small).

Immunoencapsulation of gene modified tumour cells is extremely attractive as a strategy for cancer immunotherapy due to reasons elaborated above. However, overall the studies performed here were not successful in demonstrating this potential of immunoencapsulation as an effective cancer therapy. Although manipulation of a number of the factors in these models, particularly in the MMB model, has the potential to lead to a positive result, proof that this approach can consistently overcome host tolerance to tumour is necessary and will not be easily forthcoming.
Figure 1. IL-12 Production from B9/BM1 Cells in TheraCyte™ Devices *in vitro*. $5 \times 10^5$ B9-12 or B9-12.B7 cells were loaded into 4.5ml TheraCyte™ devices *in vitro* and placed in medium in separate wells of a 24 well plate. Medium was changed weekly, and IL-12 levels were determined by ELISA weekly for 12 weeks.
Figure 2. TheraCyte™ Device and Murine Subcutaneous Implantation. TheraCyte™ devices (top) are displayed within the outer plastic sterilization capsule. The 4.5μl size is above and the 20μl size is below. The sterilization port (left, at 0.5cm), and cell loading port (right, at 9.0 cm) are displayed. Implantation was accomplished via a skin flap created on the abdominal surface by subcutaneous dissection across the midline (bottom).
Figure 3. Pathology of implanted TheraCyte™ devices containing B9-12.B7 cells at 7 days (top) and 35 days (bottom). Live cells were present in vivo in devices loaded with B9-12.B7 cells harvested at one week (left). Few live cells were found in the lumen of devices harvested past one week. Note the capillary ingrowth into the outer membrane (bottom).
Figure 4. Serum IL-12 Concentration of Balb/c Mice bearing Theracyte™ (TC) Devices Implanted with B9-12 cells. Balb/c mice were implanted with a single 4.5ul or 20ul device loaded with B9-12 cells and sacrificed each week. Controls are Balb/c mice without an implant.
Figure 5. B9/BM1 Tumour Vaccination Experiments (n=3) Utilizing Single 4.5ul TheraCyte™ Devices: Day 28 Challenge. Balb/c mice were implanted s/c with a single 4.5ul TheraCyte™ device containing medium alone (Medium), or 5x10^5 B9 parental (B9-Parental), B9-12 (B9-12) or B9-12.B7 (B9-12.B7) cells. A positive control group was vaccinated with five weekly s/c injections of 5x10^5 B9-12.B7 cells (SCIL12/B7) beginning on the day of surgery. Animals were challenged with 2x10^5 parental B9 cells s/c bilaterally in the groins 28 days after device implantation. Mice were assessed biweekly and sacrificed when tumour diameter reached 15mm. Numbers of mice per group are indicated in parentheses.
Figure 6. B9/BM1 Tumour Therapy Experiment Utilizing Single 4.5ul TheraCyte™ Devices: Same Day Challenge.

Balb/c mice were implanted s/c with a single 4.5ul TheraCyte™ device containing medium alone (Medium), or $5 \times 10^5$ B9 parental (B9-Parental), B9-12 (B9-12) or B9-12.B7 (B9-12.B7) cells. Animals were challenged with $2 \times 10^5$ B9-parental cells s/c bilaterally in the groins on the day of surgery (Day 0). Numbers of mice per group are represented in parentheses. Mice were assessed biweekly and sacrificed when tumour diameter reached 15mm.
Figure 7. B9/BL1 Tumour Vaccination Experiment Utilizing Two 4.5ul TheraCyte® Devices: Day 14 Challenge. Balb/c mice were implanted s/c with two 4.5ul TheraCyte® devices containing medium alone (Medium), $5 \times 10^5$ B9-12 (B9-12) or B9-Flt3L (B9-Flt3L) cells, or with the two latter lines in separate devices (B9-12/B9-Flt3L). A positive control group was vaccinated with five weekly s/c injections of $5 \times 10^5$ irradiated B9-Flt3L cells (Irr B9-Flt3L s/c) beginning on the day of surgery. Animals were challenged with $2 \times 10^5$ parental B9 cells s/c bilaterally in the groins 14 days after device implantation. Numbers of mice per group are indicated in parentheses. Mice were assessed biweekly and sacrificed when tumour diameter reached 15mm.
Figure 8. B9/BM1 Tumour Vaccination Experiment Utilizing Single 20ul TheraCyte™ Devices: Day 28 Challenge. Balb/c mice were implanted s/c with a single 20ul TheraCyte™ device containing medium alone (Medium), or $5 \times 10^5$ B9 parental (B9-Parental), B9-12 (B9-12) or B9-12.B7 (B9-12.B7) cells. A positive control group was vaccinated with five weekly s/c injections of $5 \times 10^5$ B9-12.B7 cells (B9-12.B7 s/c) beginning on the day of surgery. Animals were challenged with $2 \times 10^5$ parental B9 cells s/c bilaterally in the groins 28 days after device implantation. Numbers of mice per group are indicated in parentheses. Mice were assessed biweekly and sacrificed when tumour diameter reached 15mm.
Figure 9. Pathology of implanted TheraCyte™ devices containing parental MCA-38 cells. Survival of MCA cells in vivo in the devices was highly variable. A device removed at day 25 (top) contains debris with no live cells. A device removed at day 148 (bottom) contains a rim of viable cells around the periphery of the lumen.
Figure 10. MCA-38 Tumour Vaccination Experiment Utilizing Single 20ul Theracyte™ Devices. C57BL/6 mice were implanted s/c with a single 20ul Theracyte™ device containing medium alone (Medium), or 2x10^5 MCA-38 parental (MCA-parental) (left), or 2x10^6 MCA-GM cells (MCA-GM) cells (right). Control groups included: surgery alone (Surgery Only), unmanipulated mice (Naive), and a single s/c injection of 2x10^6 irradiated MCA-38 parental cells (Irr MCA-parental) (left), or 2x10^6 irradiated MCA-GM parental cells (Irr MCA-GM) (right) on the day of surgery. Number of mice per group is indicated in parentheses. Animals were challenged s/c with 4x10^6 parental MCA-38 cells 27 days after device implantation, assessed biweekly, and sacrificed when tumour diameter reached 15mm.
Figure 11. MCA-38 Tumour Therapy Experiment Utilizing Two 4.5ul TheraCyte™ Devices. C57BL/6 mice were challenged s/c dorsally with 4x10^6 MCA-p parental cells two days prior to device implantation. Animals were implanted s/c with two 4.5ul TheraCyte™ devices containing medium alone (Medium), 1x10^6 MCA-38 parental cells (MCA-parental) (left), 1x10^6 MCA-12 cells (MCA-12) (middle), or 1x10^6 MCA-GM cells (MCA-GM) (right) each. Positive controls included bilateral single s/c injections of 1x10^6 irradiated MCA-38 parental cells (Irr MCA-p) (left), 1x10^6 irradiated MCA-12 cells (Irr MCA-12) (middle), or 1x10^6 irradiated MCA-GM cells (Irr MCA-GM) (right) on the day of surgery. Control groups included: surgery alone (Surgery Only) and unmanipulated mice (Naive). Numbers of mice per group are indicated in parentheses. Animals were assessed biweekly and sacrificed when tumour diameter reached 15mm.
Figure 12. Pathology of implanted TheraCyte™ devices containing MMB3.19 cells. A device harvested at 99 days in vivo contains parental MMB3.19 cells with a rim of viable cells by the lower membrane and debris in the center of the lumen (top). A device harvested at 49 days contains MMB-flt3 cells (bottom). All devices with MMB3.19 cells had a similar appearance.
Figure 13. MMB Tumour Vaccination Experiment Utilizing Single 20ul TheraCyte™ Devices: Day 25 Challenge. C57BL/6 mice were implanted s/c with a single 20ul TheraCyte™ device containing media alone (Medium), 1x10^6 MMB parental cells (MMB-parental) (left), or 1x10^6 MMB-12 cells (MMB-12) (right). Positive controls included a single i/p injection of 1x10^6 irradiated MMB parental cells (Irr MMB-parental) (left), or 1x10^6 MMB-12 cells i/p (MMB-12 i/p) (right) on the day of surgery. Control groups included: surgery alone (Surgery Only), and unmanipulated mice (Naive). Animals were challenged i/p with 1x10^7 parental MMB cells 25 days after device implantation. Number of mice per group are indicated in parentheses. Animals were assessed biweekly and sacrificed when ascites developed.
Figure 14. MMB Tumour Vaccination Experiment Utilizing Single 20ul TheraCyte™ Devices: Day 19 Challenge. C57BL/6 mice were implanted s/c with a single 20ul TheraCyte™ device containing media alone (Medium), 1x10^6 MMB parental cells (MMB-parental) (left), 1x10^6 MMB-12 cells (MMB-12) (middle), or 1x10^6 MMB-Fit3L cells (MMB-Fit3L) cells (right). Positive control groups included: a single i/p injection of 1x10^6 irradiated (30cGy) MMB parental cells (Irr MMB-parental) (left) or a single i/p injection of 1x10^6 MMB-12 cells (MMB-12 i/p) (middle) on the day of surgery. Control groups included surgery alone (Surgery Only), unmanipulated mice (Naive) mice. Animals were challenged i/p with 1x10^7 parental MMB cells 19 days after device implantation. Number of mice per group are indicated in parentheses. Animals were assessed biweekly and sacrificed when ascites developed.
Figure 15(A-D). Cytotoxicity of B6 Splenocytes from Theracyte™ Implanted Mice vs. MMB and B9 Cells in vitro. C57BL/6 mice were vaccinated with 1x10^5 MMB-12 cells i/p (circle), or were implanted with Theracyte™ devices. Devices contained medium (star), or 1x10^6 parental MMB cells (square), or 1x10^6 MMB-12 cells (triangle). An unmanipulated control was included (line). Single cell suspensions prepared from spleens harvested at one or three weeks were cocultured with MMB and B9 cells, respectively (both irradiated with 30cGy each). On day 5 of coculture 4x10^3 ^51Cr labeled MMB or B9 cells were incubated for 4h with the MMB or B9 cocultured splenocytes, respectively, at the indicated effector to target ratios in triplicate. 2% Triton-X was added to maximal release wells at the end of the incubation, and after centrifugation 100μl of supernatant of each well was placed in 3ml of scintillant. Agitated samples were gamma counted. Percent specific lysis was calculated as: ((Experimental - spontaneous release)/(maximal release))*100. A - Week 1 splenocyte cytotoxicity against MMB cells, B - Week 1 splenocyte cytotoxicity against B9 cells, C - Week 3 splenocyte cytotoxicity against MMB cells, D - Week 3 splenocyte cytotoxicity against B9 cells.
Table 3. Viability of MCA-38 Cells In Devices *In Vivo*

<table>
<thead>
<tr>
<th>Cells</th>
<th>Days <em>in vivo</em></th>
<th>% Viable Area</th>
<th>Viable Nests</th>
<th>Viable Indv.</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 MCA-parental</td>
<td>15</td>
<td>&lt;1</td>
<td>no</td>
<td>yes</td>
</tr>
<tr>
<td>2 MCA-parental</td>
<td>40</td>
<td>3</td>
<td>yes</td>
<td>yes</td>
</tr>
<tr>
<td>3 MCA-parental</td>
<td>50</td>
<td>40</td>
<td>yes</td>
<td>yes</td>
</tr>
<tr>
<td>4 MCA-parental</td>
<td>148</td>
<td>3</td>
<td>yes</td>
<td>yes</td>
</tr>
<tr>
<td>5 MCA-parental</td>
<td>148</td>
<td>35</td>
<td>yes</td>
<td>yes</td>
</tr>
<tr>
<td>6 MCA-parental</td>
<td>195</td>
<td>0</td>
<td>no</td>
<td>no</td>
</tr>
<tr>
<td>7 MCA-parental</td>
<td>195</td>
<td>0</td>
<td>no</td>
<td>no</td>
</tr>
<tr>
<td>8 MCA-parental</td>
<td>195</td>
<td>0</td>
<td>no</td>
<td>no</td>
</tr>
<tr>
<td>9 MCA-parental</td>
<td>54</td>
<td>&lt;1</td>
<td>no</td>
<td>yes</td>
</tr>
<tr>
<td>10 MCA-parental</td>
<td>55</td>
<td>0</td>
<td>no</td>
<td>no</td>
</tr>
<tr>
<td>11 MCA-parental</td>
<td>25</td>
<td>0</td>
<td>no</td>
<td>no</td>
</tr>
<tr>
<td>12 MCA-parental</td>
<td>25</td>
<td>0</td>
<td>no</td>
<td>no</td>
</tr>
<tr>
<td>13 MCA-parental</td>
<td>25</td>
<td>&lt;1</td>
<td>no</td>
<td>yes</td>
</tr>
<tr>
<td>15 MCA-parental</td>
<td>27</td>
<td>0</td>
<td>no</td>
<td>no</td>
</tr>
<tr>
<td>16 MCA-12</td>
<td>43</td>
<td>&lt;5</td>
<td>yes</td>
<td>yes</td>
</tr>
<tr>
<td>17 MCA-GM</td>
<td>43</td>
<td>&lt;5</td>
<td>yes</td>
<td>yes</td>
</tr>
<tr>
<td>18 MCA-GM</td>
<td>21</td>
<td>&lt;1</td>
<td>no</td>
<td>yes</td>
</tr>
</tbody>
</table>

Summary (Range) 15-195 0-40 61/18 (33%) 11/18 (61%)

Table 3. Viability of MCA-38 Cells in Devices *in vivo*. Days *in vivo* represent the number of days the mouse lived with the device implanted. % viable area is a visual estimation of the percentage of area of the lumen of the device that contained viable cells by light microscopy at time of sacrifice. A nest of cells was arbitrarily defined as a group of 15 or more viable cells in contiguous contact. Viable Indv. represents the presence of viable individual cells anywhere within the lumen.
Table 4. Viability of MMB Cells In Devices In Vivo

<table>
<thead>
<tr>
<th>Sample</th>
<th>Cells</th>
<th>Days in vivo</th>
<th>% Viable Area</th>
<th>Viable Nests</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>MMB</td>
<td>166</td>
<td>30</td>
<td>yes</td>
</tr>
<tr>
<td>2</td>
<td>MMB-Flt3L</td>
<td>46</td>
<td>40</td>
<td>yes</td>
</tr>
<tr>
<td>3</td>
<td>MMB-Flt3L</td>
<td>47</td>
<td>30</td>
<td>yes</td>
</tr>
<tr>
<td>4</td>
<td>MMB-Flt3L</td>
<td>48</td>
<td>35</td>
<td>yes</td>
</tr>
<tr>
<td>5</td>
<td>MMB-Flt3L</td>
<td>39</td>
<td>25-30</td>
<td>yes</td>
</tr>
<tr>
<td>6</td>
<td>MMB-Flt3L</td>
<td>39</td>
<td>25-30</td>
<td>yes</td>
</tr>
<tr>
<td>7</td>
<td>MMB</td>
<td>50</td>
<td>25-30</td>
<td>yes</td>
</tr>
<tr>
<td>8</td>
<td>MMB</td>
<td>99</td>
<td>25-30</td>
<td>yes</td>
</tr>
<tr>
<td>9</td>
<td>MMB</td>
<td>113</td>
<td>25-30</td>
<td>yes</td>
</tr>
<tr>
<td>10</td>
<td>MMB-12</td>
<td>67</td>
<td>25-30</td>
<td>yes</td>
</tr>
<tr>
<td>11</td>
<td>MMB-12</td>
<td>70</td>
<td>25-30</td>
<td>yes</td>
</tr>
<tr>
<td>12</td>
<td>MMB-12</td>
<td>70</td>
<td>25-30</td>
<td>yes</td>
</tr>
<tr>
<td>13</td>
<td>MMB-Flt3L</td>
<td>7</td>
<td>60</td>
<td>yes</td>
</tr>
<tr>
<td>14</td>
<td>MMB-12</td>
<td>7</td>
<td>60</td>
<td>yes</td>
</tr>
<tr>
<td>15</td>
<td>MMB</td>
<td>7</td>
<td>60</td>
<td>yes</td>
</tr>
<tr>
<td>16</td>
<td>MMB</td>
<td>63</td>
<td>25-30</td>
<td>yes</td>
</tr>
<tr>
<td>17</td>
<td>MMB-12</td>
<td>56</td>
<td>25-30</td>
<td>yes</td>
</tr>
<tr>
<td>18</td>
<td>MMB-Flt3L</td>
<td>37</td>
<td>25-30</td>
<td>yes</td>
</tr>
<tr>
<td>19</td>
<td>MMB-Flt3L</td>
<td>37</td>
<td>25-30</td>
<td>yes</td>
</tr>
<tr>
<td>20</td>
<td>MMB</td>
<td>52</td>
<td>25-30</td>
<td>yes</td>
</tr>
<tr>
<td>21</td>
<td>MMB-12</td>
<td>54</td>
<td>25-30</td>
<td>yes</td>
</tr>
<tr>
<td>22</td>
<td>MMB-12</td>
<td>55</td>
<td>25-30</td>
<td>yes</td>
</tr>
<tr>
<td>Summary (Range)</td>
<td>7-166</td>
<td>25-60</td>
<td>100%</td>
<td></td>
</tr>
</tbody>
</table>

Table 4. Viability of MMB Cells in Devices in vivo. Days in vivo represent the number of days the mouse lived with the device implanted. % viable area is an estimation of the percentage of area of the lumen of the device that contained viable cells by light microscopy at time of sacrifice. A nest of cells was arbitrarily defined as a group of 15 or more viable cells in contiguous contact.
Table 5. IL-12 Concentration in Serum from C57BL/6 Mice from Tumour Challenge Experiments.

<table>
<thead>
<tr>
<th>Model</th>
<th>Expt.</th>
<th>Group</th>
<th>Days in vivo</th>
<th>IL-12 pg/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>MCA-38</td>
<td>Figure 10</td>
<td>MCA-12</td>
<td>27</td>
<td>34.7</td>
</tr>
<tr>
<td></td>
<td></td>
<td>MCA-12</td>
<td>20</td>
<td>38.5</td>
</tr>
<tr>
<td></td>
<td></td>
<td>MCA-12</td>
<td>24</td>
<td>41.8</td>
</tr>
<tr>
<td></td>
<td></td>
<td>MCA-par</td>
<td>20</td>
<td>41.8</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Medium</td>
<td>20</td>
<td>39.8</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Irr MCA-par</td>
<td>20</td>
<td>34.1</td>
</tr>
<tr>
<td>MMB</td>
<td>Figure 13</td>
<td>MMB-12</td>
<td>68</td>
<td>38.5</td>
</tr>
<tr>
<td></td>
<td></td>
<td>MMB-12</td>
<td>29</td>
<td>38.5</td>
</tr>
<tr>
<td></td>
<td></td>
<td>MMB-12</td>
<td>56</td>
<td>37.2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>MMB-par</td>
<td>63</td>
<td>31.6</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Medium</td>
<td>38</td>
<td>37.2</td>
</tr>
<tr>
<td>MMB</td>
<td>Figure 14</td>
<td>MMB-12</td>
<td>55</td>
<td>37.2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>MMB-12</td>
<td>54</td>
<td>45.9</td>
</tr>
<tr>
<td></td>
<td></td>
<td>MMB-12</td>
<td>55</td>
<td>48.7</td>
</tr>
<tr>
<td></td>
<td></td>
<td>MMB-par</td>
<td>52</td>
<td>41.8</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Medium</td>
<td>34</td>
<td>50.1</td>
</tr>
<tr>
<td>Control</td>
<td>Figure 14</td>
<td>n/a</td>
<td>n/a</td>
<td>41.1</td>
</tr>
</tbody>
</table>

Table 5. IL-12 Concentration in Serum from C57BL/6 Mice from Tumour Challenge Experiments. Serum was obtained from mice implanted with TheraCyte™ devices containing medium (Medium), IL-12 secreting cells, or parental cells from the various experiments upon sacrifice. Irr MCA-par are the irradiated controls in experiment 10. The control was sacrificed at 50 days of age. Days in vivo represents the day of sacrifice after the date of device implantation in that experiment. (n/a represents not applicable)

Table 6. FACS Analysis of Splenocytes from Vaccinated Mice

<table>
<thead>
<tr>
<th>Marker</th>
<th>Naive Week 1</th>
<th>Naive Week 3</th>
<th>Medium Week 1</th>
<th>Medium Week 3</th>
<th>MMB-parental Week 1</th>
<th>MMB-parental Week 3</th>
<th>MMB-12 Week 1</th>
<th>MMB-12 Week 3</th>
<th>MMB-12 i/p Week 1</th>
<th>MMB-12 i/p Week 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD45RO</td>
<td>46.7</td>
<td>57.1</td>
<td>52.7</td>
<td>66.3</td>
<td>n/a</td>
<td>53.9</td>
<td>56.1</td>
<td>49</td>
<td>45.4</td>
<td>n/a</td>
</tr>
<tr>
<td>CD3</td>
<td>52.4</td>
<td>54.5</td>
<td>53.9</td>
<td>47.3</td>
<td>n/a</td>
<td>58</td>
<td>46.7</td>
<td>50.7</td>
<td>45.9</td>
<td>n/a</td>
</tr>
<tr>
<td>CD4</td>
<td>58.8</td>
<td>61.7</td>
<td>59</td>
<td>60</td>
<td>n/a</td>
<td>58.8</td>
<td>54.4</td>
<td>61.9</td>
<td>56.2</td>
<td>n/a</td>
</tr>
<tr>
<td>CD8</td>
<td>38.8</td>
<td>36.1</td>
<td>38.4</td>
<td>36.8</td>
<td>n/a</td>
<td>38.7</td>
<td>42.9</td>
<td>35.4</td>
<td>40.5</td>
<td>n/a</td>
</tr>
<tr>
<td>MHCII &amp; CD11c</td>
<td>20.2</td>
<td>n/a</td>
<td>24.7</td>
<td>16.2</td>
<td>21.9</td>
<td>16.7</td>
<td>22.71</td>
<td>16.4</td>
<td>14.9</td>
<td>n/a</td>
</tr>
</tbody>
</table>

Table 6. FACS Analysis of Splenocytes from TheraCyte™ Bearing Mice. Mice were implanted with a single 20μl TheraCyte™ device containing medium (Medium), MMB-parental cells (MMB-parental) or MMB-12 cells (MMB-12). Controls included unmanipulated mice (Naive), as well as mice given a single i/p injection of MMB-12 cells (MMB-12 i/p). Splenocytes were harvested from the respective groups at 1 and 3 weeks and stained with mAb as labeled (as described in Materials and Methods). CD4 and CD8 values represent percentages of CD3+ cells. (n/a represents not applicable)
REFERENCES


Boon, T., A. Van Pel. 1978. Teratocarcinoma cell variants rejected by syngeneic mice: protection of mice immunized with these variants against other variants and against the original malignant cell line. Proc Natl Acad Sci U S A. 75: 1519-23.


