STUDIES OF THE ANTINEOPLASTIC ACTIVITY OF VEROTOXIN

IN VITRO AND IN VIVO

by

Sara Arab

A thesis submitted in conformity with the requirements for the degree of

Doctor of Philosophy in the Graduate Department of Clinical Biochemistry,

University of Toronto,

Toronto, Canada

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.

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.

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 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.

0-612-27869-7

Canadä

ABSTRACT

Studies of the neoplastic activity of verotoxin in vitro and in vivo

Sara Arab, Ph.D. Thesis, 1997, Graduate Department of Clinical Biochemistry, University of Toronto, Toronto, Canada.

In this thesis, the use of verotoxin1 as a new approach to the management of certain human neoplasms and its mechanism of action in causing cell death is discussed.

VT1 cytotoxicity was demonstrated on human cervical cancer , astrocytoma and ovarian tumor and multidrug resistant cell lines. Moreover, primary ovarian tumors, metastases and multidrug resistant tumor cells in particular, were found to contain elevated levels of Gb_3, the receptor for VT, as compared with normal ovarian epithelia. Overlays of frozen tumor sections show the toxin binds selectively to tumour tissue and the lumen of invading blood vessels. Extensive binding of toxin was observed in angiogenic astrocytoma tumor sections which may represent an anti-angiogenic property of VT1.

The second part of these studies concerned the mechanism of action of VT cytotoxicity in two cell systems: the induced VT1 hypersensitivity of human astrocytoma cell lines cultured in the presence of sodium butyrate, and the increased sensitivity of multiple drug resistant mutants as compared to parental ovarian carcinoma cells. In both cases, an alteration in the intracellular routing of the receptor-bound internalized toxin from the Golgi-lysosomal pathway to that of retrograde transport to the nuclear envelope occurred resulting in a significant increase in cell sensitivity to VT1. This change in intracellular routing may be due to sorting of Gb_3

isoforms, since retrograde nuclear targeting was found to correlate with the synthesis of Gb3 containing shorter chain fatty acid species. To our knowledge this is the first demonstration of a large protein targeting to the nucleus without a nuclear targeting signal. In less sensitive cells, toxin was targeted to components of the Golgi. These results indicate that intracellular routing can determine sensitivity to VT and that Gb3 binding may provide an intracellular route from the cell surface to the nuclear membrane for Gb3 binding proteins. Verotoxin defines a new signal transduction pathway for the rapid induction of Bcl-2 independent apoptosis in several human tumor cell lines. This pathway is most marked in multiple drug resistance and in activated endothelial cells which vascularize the tumor.

The third part of these investigations was to study the anticancer effect of VT1 in mice xenografted with human astrocytoma tumors. Tumors injected with VT1 showed 100% regression without recurrence. Using the TUNEL assay, apoptosis was observed in the tumor. These experiments demonstrate the feasibility of using VT1 therapy to selectively induce apoptosis in human astrocytoma tumors.

In the name of God, the Beneficent, the Merciful.

Acknowledgments

First I would like to thank my wonderful supervisor Dr. Lingwood for giving me the opportunity to work in his laboratory. Dear Dr. Lingwood, your support, availability, and interest throughout this work and preparation of this thesis is greatly appreciated. It has been a pleasure being your student and working with you. You are a great teacher and a wonderful human being.

I would like to thank Dr. Sarma for his "words of wisdom" and encouragement throughout these past years. Dr. Sarma you are an exceptional human being and a great teacher.

Many thanks to Dr. J. Rutka for his encouragement throughout this work and writing of this thesis. Dr. Rutka it was a pleasure working with you.

I would also like to thank other members of my advisory committee - Dr. R. Hill, Dr. J. Boggs, and Dr. L. Becker for their guidance and ideas for the writing of this thesis and Dr. C. Erlichman for accepting to be the external examiner.

Many thanks to Ms. Beth Boyd not only for her technical help but also for willing to lend a sympathetic ear in times of need. Thanks to Ms. Anita Nutikka for constantly reminding me to "stop and smell the roses" when I

had wrapped myself in too many dead lines of mine. Thanks to Mrs Shirley Thompson for her assistance.

In many ways, I share this thesis and the occasion of graduation with my family, all my friends and those that have helped me learn variety of laboratory techniques and scientific methods.

Many thanks to ministry of health and medical education of Iran for initiating this study by their scholarship.

Finally, I would like to thank my parents, my brother and sisters and my family for their continued support and unconditional love throughout this seemingly never-ending education of mine.

This thesis is dedicated to my family

my husband Naser Roushan

my daughter Ala Roushan

my son Amir Mohammed

Roushan

Table of Contents

Page

Results:

List of Figures

List of Tables

List of Abbreviations

A/M	acetone/methanol
BSA	bovine serum albumin
C/M/W	chloroform/methanol/water
DABCO	1,4-Diazabicyclo(2,2,2)- Octane
FBS	fetal bovine serum
Gb_3	globotriaosyceramide
GL	glycolipid
GSL	glycosphingolipid
HPLC	high performance liquid chromatography
MEM	minimum essential medium
p	Gb_4, antigen of P blood group
P1	Gala1-4GalB1-4GlcNAcB1-3GalB1-4GlcB1-Cer
PBS	phosphate buffered saline
P^k	Gb_3, antigen of P blood group
PPMP	1-phenyl-2- decanoylamine-3-morpholino-1-propanol
RME	Receptor mediated endocytosis
rRNA	Ribosomal ribonucleic acid
RPMI-1640	Roswell Park Memorial Institute-medium H 1640
TBS	tris buffered saline
TLC	thin layer chromatography
VT	verotoxin
VT1	verotoxin 1
VT2	verotoxin 2
VT2c	verotoxin 2c
VT2e	verotoxin 2e

CHAPTER 1: Introduction and Background

In this thesis the molecular mechanism of verotoxin cytotoxicity in astrocytoma and ovarian tumors *in vitro* and *in vivo* is discussed. This work resulted in patents in the area of verotoxin therapy for certain types of cancer using verotoxins as an antimitogenic and antiangiogenic drug, diagnosis of MDR tumors, and identification of sensitive candidates for this therapy.

In the introduction (chapter 1) of this thesis the relevant background information about verotoxins, glycosphingolipids (metabolism, functions; trafficking), retrograde transport, tumor biology and different MDR (multidrug resistant) mechanisms, ovarian and brain tumors, and apoptosis is presented.

1.1. Verotoxins

Verotoxins (VT), also known as Shiga-like toxins or SLTs, comprise a family of toxins that are produced by verotoxin producing *Escherichia coli* (VTEC). The term VTEC refers to all *Escherichia coli* strains that produce VT and includes many strains of enteropathogenic *E.coli* (EPEC) such as serotype H-30 (O'Brien et al., 1983), and some enterohemorrhagic *E. coli* (EHEC) strains such as serotype 0157:H7 (Scotland et al., 1985; Karmali, 1989). Originally verotoxin was detected by Konowalchuk in 1977 (Konowalchuk et al., 1977), as an agent with a distinct cytotoxic effect on African Green Monkey (Vero) cells, hence the name "verotoxin".

1.1.1 Verotoxin variants and their properties

At least four members of this toxin family have been reported; VT1, VT2c, VT2, and VT2e (O'Brien et al., 1982; Scotland, Smith et al., 1985; Strockbine et al., 1986; Linggood et al., 1987; Marques et al., 1987). These toxins show some similar biological activities and amino acid sequences but differ in antigenicity (Strockbine, et al., 1986; O'Brien et al., 1987). Some strains of VTEC (eg. *E. coli* 0157: H7) produce

two distinct toxins (VT1 and VT2), both of which show cytotoxicity for Vero cells, but only VT1 can be neutralized by antiserum against Shiga Toxin (ST) produced by Shigella dysenteria (O'Brien et al., 1983). VT1 and ST are identical with the exception of one amino acid in the A subunit (Jackson et al., 1987; Strockbine et al., 1988). The cytotoxicity of VT2c (produced by *E.coli* strain E32511), the second member of the VT family, is not neutralized by anti-Shiga Toxin or anti-VT1 antibody (Head et al., 1988). The third toxin,VT2 (SLTII), isolated from strain 933 (Strockbine, et al., 1986) is completely neutralized by anti VT2c, whereas anti-VT2 only partially neutralizes VT2c (Head, et al., 1988). The fourth member of this family, termed VT2e, is associated with edema disease in swine (Linggood and Thompson, 1987). VT2e is also neutralized by anti VT2c but not anti-VT1 (Marques, et al., 1987; Gannon et al., 1990).

1.1.2 Molecular structure, and biological activities of VTs

At the molecular level verotoxins are subunit toxins. VT, like several other bacterial toxins such as cholera, pertussis, and diphtheria, has been shown to possess an A (active) subunit and multiple B (binding) subunits (Middlebrook et al., 1984; Calderwood et al., 1987; Head, et al., 1988; Ramotar et al., 1990). Verotoxins are the most potent biological toxins thus far described (Head et al., 1991). The activity of VT1 has been reported at concentrations as low as 10^{-17}M (Robinson et al., 1994). Sensitivity to verotoxin has been shown to vary during the growth phases both for epithelial (Pudymaitis et al., 1992) and endothelial cells (Obrig et al., 1988) in culture. In both cases, growing cells are significantly more sensitive than stationary phase cells.

Although verotoxins are potent inhibitors of protein synthesis, holotoxin mediated cell killing is often via apoptosis (Section 1.11) rather than necrosis (Mangeney et al., 1993; Inward et al., 1995; Arab et al., in press). The classical

scheme for the mechanism of action of verotoxins in cell killing by inhibition of protein synthesis is depicted in Fig. 1.1.

Although there are minor variations among the different types of VT, the molecular weight (MW) of the holotoxin is about 70 kD with one A subunit of MW ~ 32 kDa and five monomeric B subunits, each of MW ~ 7.6 kDa (Ramotar, et al., 1990). The B subunits form a pentamer which mediates the binding of holotoxin to specific surface receptors on susceptible cells (Donohue-Rolfe et al., 1986). Internalization of cell-bound VT1 occurs by receptor-mediated endocytosis into clathrin coated pits (Figure 1.1) (Sandvig et al., 1991). Once internalized, VT1 has been shown to target the rough endoplasmic reticulum (Sandvig et al., 1992). Inside the cell the A subunit of VT1 is proteolytically cleaved to A1 and A2 fragments. The A2 fragment maintains association of A subunit with the B pentamer (Fraser, et al. 1994). The A1 fragment has N-glycanase activity, resulting in the catalytic inactivation of ribosomes (Igarashi et al., 1987). Specifically, the A1 chain removes an adenine base at position 4324 in 28S rRNA. This cleavage results in an inhibition of elongation-factor-1-dependent binding of aminoacyl transfer RNA to the A site of the 60S ribosomal subunit thereby preventing peptide chain elongation (Obrig et al., 1987).

The crystal structure of the B-subunit of VT1 shows that it is very similar in structure to the E. coli heat-labile enterotoxin in overall conformation, despite the absence of detectable sequence similarity between these two proteins (Sixma et al., 1993). The binding component contains five identical B-subunits each consisting of two three-stranded anti-parallel β–sheets at the outer surface of the pentamer and an α- helix which lines a central pore. β–sheets from pairs of adjacent B subunits interact to form a cleft, often associated with carbohydrate binding proteins, which is postulated to be the glycolipid- binding site (Figure 1.2) (Stein et al., 1992).
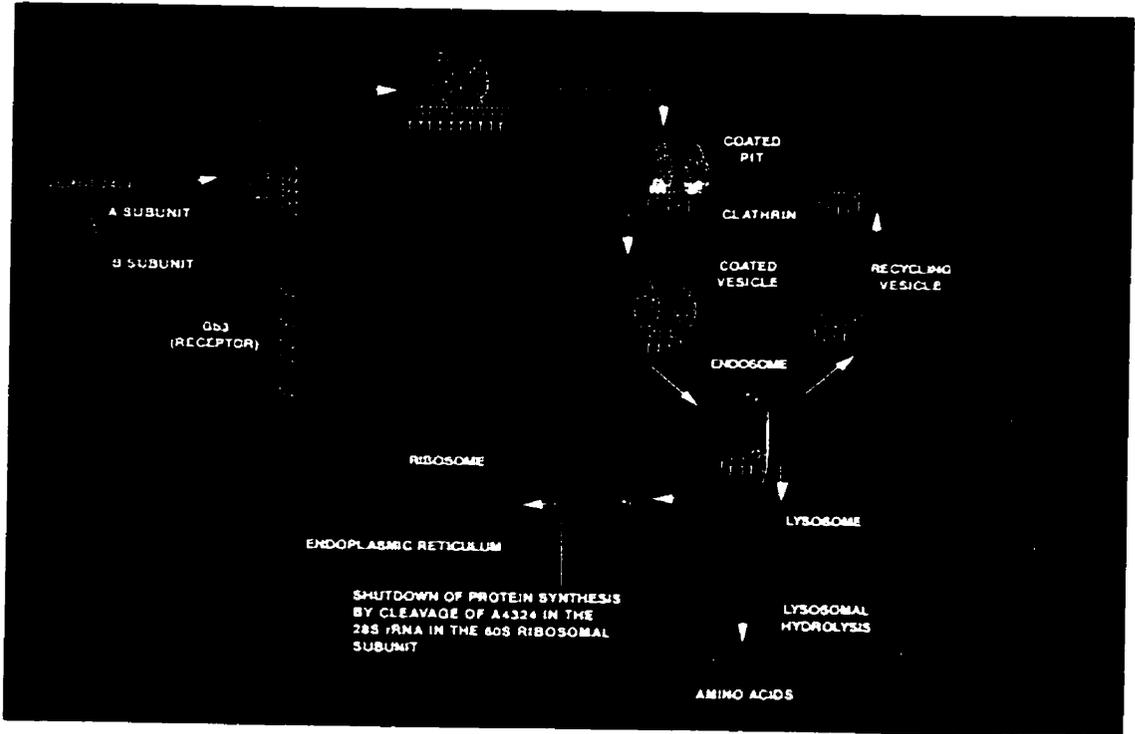
Figure 1.1   Schematic representation of verotoxin internalization and action in inhibition of protein synthesis.

Figure 1.2 **Crystal structure of the B-subunit of VT1.** β-strands are represented by arrows and α-helices by cylinders. Viewed along (top) and perpendicular (bottom) to the 5-fold axis. (Stein, et al, 1992).

Genetically, the structural genes for VT1 and VT2 are phage-encoded and the two toxins share a 58% nucleotide homology (Jackson, et al., 1987). Furthermore, they share a similar subunit stoichiometry, bind to the same glycolipid receptor (Lingwood et al., 1987; DeGrandis et al., 1989) and inhibit protein synthesis by an identical mechanism (Igarashi, et al., 1987). VT1 and VT2c show approximately 60% homology in the B (receptor-binding) subunit (Newland et al., 1987), while VT2 and VT2c are 97% identical (Head, et al., 1988; Ito et al., 1990). VT2c and VT2e are 87% identical in the B-subunit (Marques, et al., 1987).

Although VT1 and VT2c share many properties, they have interesting differences in biological activity (Head, et al., 1988). They both bind the same glycosphingolipid (GSL) receptor, globotriaosyl ceramide (Gb3), yet VT2c shows a thousand fold lower specific cytotoxic activity on Vero cells and is 100 times less lethal to rabbits than VT1 (Head, et al., 1988; 1991). VT1 and VT2c also differ in their physical properties such as thermal stability. VT2c is more heat labile than VT1. At 60°C the activity of VT2c is reduced to 50% after 30 min. and to 25% after 60 min., whereas VT1 remains unaffected (Head, et al., 1988).

Studies of hybrid toxins have been carried out to determine the roles of the A and B subunits in the differential biological activities of VT1 and VT2c (Head, et al., 1991). By separation of subunits and reannealing to form chimeric toxins, the reduced cytotoxicity of VT2c relative to VT1 was shown to be a function of the B subunit, likely mediated by the reduced binding affinity of the VT2c B subunit for Gb3. The A subunits of VT1 and VT2c were found to be equally potent inhibitors of cell-free protein synthesis (Head, et al., 1991). VT1 and VT2c have identical glycolipid binding specificities, however hybrid toxin studies of these two toxins showed that the B subunits of both VT1 and VT2c modulate the cytotoxic potential of the A subunit. Thus, both holotoxin VT2c and hybrid VT1A:VT2cB had approximately one thousand fold less cytotoxic activity than VT1 holotoxin and

VT2cA:VT1B. VT1 and VT2 with 60% homology in their B subunit share the same biological activities.

1.1.3 Role of Verotoxin in Pathogenesis of Human Disease

Clinically, infection with verotoxin producing E. coli (VTEC) is associated with two microangiopathies, that of hemorrhagic colitis, HC (Riley, 1985) and hemolytic uremic syndrome, HUS (Karmali et al., 1985; Griffin et al., 1991). In both HC and HUS, the primary site of damage is the vasculature (of the large bowel in HC and of the capillaries within the glomerulus of the kidney in HUS). The endothelial cells are damaged, resulting in the production of a fibrin clot and local occlusion of the vasculature, which in turn results in infarct and rupture of the small blood vessels (Richardson et al., 1988). HUS is characterized by microangiopathic hemolytic anemia, thrombocytopenia, and acute renal failure. Glomerular necrosis and intestinal edema are the result of capillary rupture (Richardson, et al., 1988). Histological examination of HUS patients shows swelling of the glomerular endothelial cells and fibrin deposition and inflammatory cell influx into the lumen of the glomeruli. HUS is primarily a disease of the very young and the elderly following VTEC infection (Lieberman et al., 1966; Rowe et al., 1991); Tarr, 1990], although rare cases have been reported in middle age people (Neill et al., 1985).

1.1.4 Verotoxin Specificity of glycolipid receptor binding

Reconstitution of receptor negative cells has shown that Gb3, a glycosphingolipid, is the functional receptor for verotoxin (Waddell et al., 1990; Jacewicz et al., 1994). It was shown that this glycolipid receptor is present in the pediatric renal glomerulus but absent in the equivalent adult tissue (Lingwood, 1994). This finding may explain the risk factor for the development of HUS following infection with VTEC in infants (Taylor, 1995). Figure 1.3A demonstrates a lateral view of the space

filling model of VT1B (top) and three favoured conformers of Gb3 (bottom), based on molecular mechanics calculations (Nyholm, et al., 1993). The verotoxin B-subunit is positioned with its receptor-binding side (cleft between adjacent monomers) directed downwards. Subunits 3 and 4 are shown in red and green respectively, whereas subunits 1, 2 and 5 are show in gray. The structures shown for Gb3 are the thermodynamically favoured conformers (Stromberg et al, 1991). Figure 1. 3B illustrates the modeling of the binding of VT1B pentamer to Gb3.

Human renal endothelial cells in infants are exquisitely sensitive to VT due to their very high Gb3 expression level (Obrig et al., 1993). The Gb3 expression level of cultured endothelial cells can be modulated by various cytokines, eg., IL1ß, TNF-α (Louise et al., 1991; van der Kar et al., 1992; Kaye et al., 1993), and it is possible that stimulation of quiescent cells in intact tissue to grow in culture (e.g. renal endothelial cells prepared from human adult glomeruli) results in maximal stimulation of Gb3 synthesis (Lingwood, 1994). Thus these quiescent cells do not contain Gb3 *in vivo* , but synthesis may be stimulated. This may relate to the finding that TNF-α was elevated in macrophages after VT2 treatment and macrophage-defective mice were found to be resistant to VT toxicity (Barrett et al., 1990). Such results would imply "pathological amplification", whereby VTEC enhances TNF-α production which increases endothelial Gb3, increasing the sensitivity to VT and exacerbating endothelial damage. It could be speculated therefore that HUS is the result of two insults. An inappropriate response to bacterial infection, eg., due to systemic LPS (Louise et al., 1992) resulting in elevated levels of cytokines which turn on Gb3 synthesis in the adult glomerular endothelial cells which then become sensitive to the second insult, systemic verotoxin. Thus, in the elderly population (and the adult population in general) it is likely that systemic verotoxemia in the absence of a concomitant infection, will not result in any pathological effect.
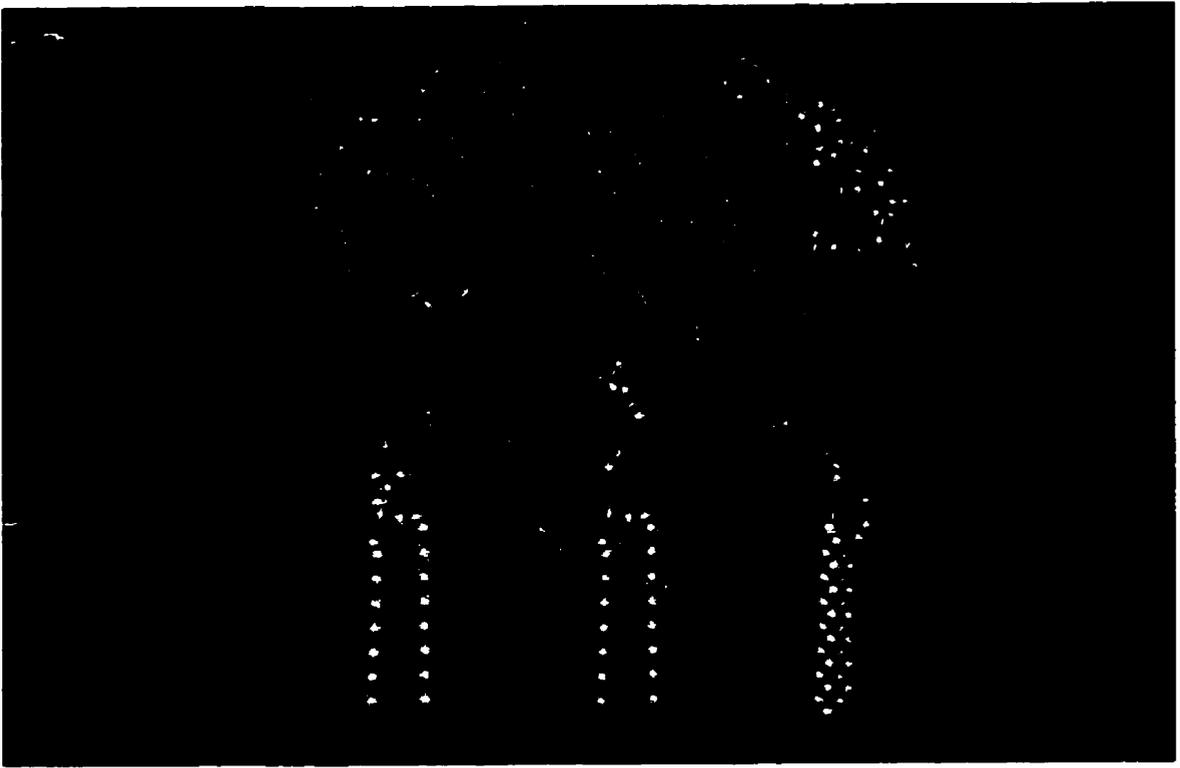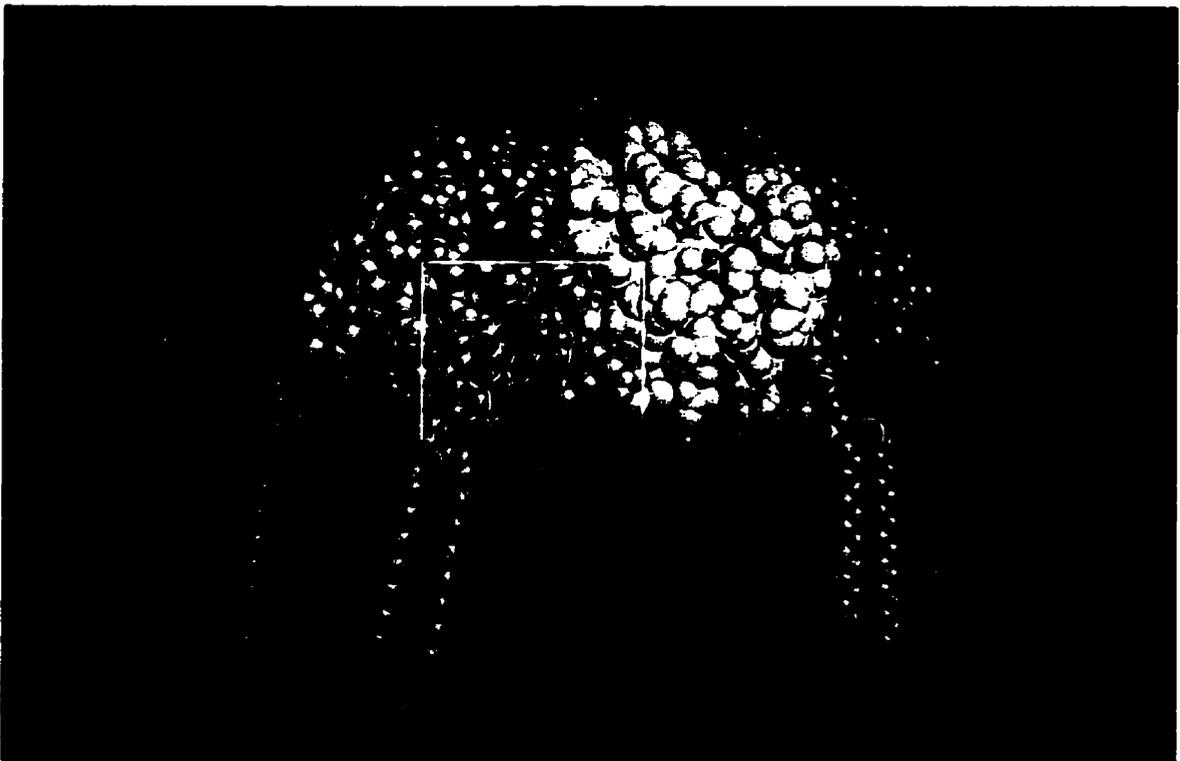
Figure 1.3   Space filling model of VT1B (top) and Gb$_3$ conformers (A).

Gb$_3$/ VT1B binding (B).

1.1.5 Verotoxin interaction with the immune system

Gb$_3$ is a marker of B cell differentiation known as CD77 (Mangeney et al., 1991) and is expressed primarily on B lymphocytes in germinal centers (Gregory et al., 1987; Gregory et al., 1988). Such B cells are highly prone to apoptosis in vivo (Mangeney, et al., 1991). In vitro, verotoxin has been shown to selectively abrogate B cell, as opposed to T cell, responses and to cause a significant reduction in antibody production in vitro by human tonsillar B cells, in particular IgG and IgA-producing cells but not IgM producing cells (Cohen et al., 1990). In addition, IgG antitoxin antibodies are rarely observed following VTEC infections (Keusch et al., 1976; Karmali et al., 1994) unlike other bacterial toxemias. This suggests that verotoxemia in humans might result in the temporary compromise of the humoral immune response, such as has been described in pigs (Christopher-Hennings et al., 1993).

1.2 Glycolipids

1.2.1 Molecular Structure of Glycosphingolipids

Glycosphingolipids (GSLs) are amphipathic molecules composed of a polar carbohydrate moiety and a non-polar ceramide moiety consisting of a fatty acid tail attached by an amide bond to a sphingosine base. The addition of a fatty acyl group to the amino group of sphingosine produces a sphingolipid, the trivial name of which is ceramide, and further addition of one or more carbohydrate residues to the C-1 hydroxyl group of ceramide results in a glycosphingolipid. GSLs occur predominantly in the outer leaflet of the plasma membrane lipid bilayer, with the ceramide portion anchored in the membrane and the carbohydrate moiety facing the extracellular matrix, (Karlsson et al., 1987). Changes in the crypticity of GSLs in the plasma membrane are frequently associated with a change in cellular function (Gahmberg et al., 1973).

GSLs show cell type-specific composition not only in the oligosaccharide part, but also in the ceramide moiety. Each cell type seems to regulate its lipid composition of GSLs according to its stage of differentiation (Kannagi et al., 1982); (Sonnino et al., 1985) and the lipid moiety has a role in defining the carbohydrate conformation of the GSL (Kannagi, et al., 1982; Nyholm et al., 1995).

The great number of structural variations in fatty acids (chain length, hydroxylation, and saturation), sphingosines (chain length, hydroxylation), and carbohydrates result in a large number of chemically distinct GSLs (Stults et al., 1989). Due to variations in sugar type, number and linkage, as well as substitutions with sulphate, and sialic acid, glycosphingolipids represent a very complex group of molecules.

1.2.2   Classification of Glycolipids:

Classification of glycosphingolipids has been based on their carbohydrate structure rather than on the ceramide moieties. So far, over 360 molecular species of GSL have been identified, based on their carbohydrate differences. GSLs are classified as neutral glycolipids, sulfatides, and gangliosides, depending on the substituted groups of the sugar residues. Neutral GSLs are classified into four major series; globo (Galα1-4Galβ1-4Glcβ1- Ceramide), lacto (GlcNAcβ1-3 Galβ1-4 Glcβ1- Ceramide), neolacto (GlcNAcβ1-4 Galβ1-4 Glcβ1- Ceramide), ganglio (Galβ1-3 GalNAcβ1-4 Gal glucosyl- Ceramide), and two minor series; muco (Galα1-4Gal - Ceramide) and gala (Galα1-4Gal- Ceramide) (Kanfer et al., 1983). In all types (with exception of the gala series), ceramide is linked to glucose which is linked to galactose.

1.2.3   Biosynthesis and Degradation of GSL

GSLs are biosynthesized by condensation of palmitoyl coenzyme A and L-serine to dehydrosphingosine, followed by reduction to dihydrosphingosine and

dehydrogenation to sphingosine. Then, a long chain fatty acyl group is linked through an amide bond to the nitrogen atom on C-2 of sphingosine (N-acyl sphingosine) to form ceramide and an oligosaccharide is linked through a glycosidic bond to C-1 of the sphingosine portion of ceramide (Mandon et al, 1992). Cerebroside is the simplest glycolipid with only one sugar residue, either glucose or galactose. UDP-glucose or UDP-galactose is the sugar donor in the synthesis of cerebrosides.

The ceramide backbone of GSL is synthesized in the rough endoplasmic reticulum and then transported to the Golgi apparatus where glycosyltransferases are localized (Younes et al., 1992). Glucosylceramide (GC) is synthesised at the cytosolic surface of the Golgi complex and is translocated to the Golgi lumen where enzymes acting in the late steps of glycosphingolipid biosynthesis have their active centres. Translocation of GC can be uncoupled from their biosynthesis, suggesting that separate proteins are involved (Burger, et al, 1996). Lactosylceramide (Galβ1-4Glcβ1 Cer), which is formed by galactosylation of GC, is a common precursor of almost all the neutral glycosphingolipids and gangliosides. The pathway is illustrated in Figure 1.4. The enzyme involved in catalyzing the synthesis of lactosylceramide is UDP-galactosyltransferase, which is located in the Golgi (Younes, et al., 1992). In the Golgi lumen, the sequential addition of monosaccharide residues is catalyzed by respective glycosyltransferases and each reaction involves the transfer of a sugar residue from a sugar nucleotide donor to an acceptor. The addition of sugar residues is strictly ordered since each glycosyltransferase is highly specific. However it is possible to get rapid shifts in the expression of GSLs by glycosyltransferase gene regulation, or by enzyme competition for available substrate. Thus the structure of cell surface GSLs is not strictly genetically defined but rather results from a complex interplay between genetically defined glycosyltransferases, substrate availability, subcellular localization, and intracellular membrane traffic (Schwarzmann et al., 1990). The

SPHINGOSINE + FATTY ACID

CERAMIDE

glcβ1-1cer (GC)

UDP-glc   UDP

galβ1-4glc-cer
lactosyl ceramide, LC

UDP-gal   UDP

UDP-gal   UDP

PAPS   PAP

gal SO₄ β1-4glc cer
sulfogalactosylceramide
SLC

CMPSA   CMP

SA.α2-3galβ1-4-glc cer
GM₃

UDP galNAc   UDP
GM₂

galNAcβ1-4galβ1-4-glc cer

UDP-gal   UDP
GM₁

galβ1-3galNAcβ1-4galβ1-4glc cer
  2   2
  SAu   SAu

GANGLIOSIDES

galα1-4galβ1-4glc-cer
Gb₃

UDP galNAc   UDP

galNAcβ1-3galα1-4 galβ1-4glc

GLOBO SERIES

UDP-gal   UDP

galNAcβ1-4 galβ1-4-glc cer
Gg₃

UDP-gal   UDP

galβ1-3 galNAcβ1-4galβ1-4glc cer
Gg₄

GANGLIO SERIES

UDP-glc NAc   UDP

galβ1-3glcNAcβ1-3galβ1-4glc cer

LACTO SERIES

glcNAcβ1-3galβ1-4glc cer

UDP-gal   UDP

galβ1-4glcNAcβ1-3galβ1-4glc cer

UDP-gal   UDP

galα1-4galβ1-4glcNAcβ1-3galβ1-4glc cer
P₁

NEOLACTO SERIES

CERAMIDE

UDP-gal   UDP

galα1-1cer

UDP-gal   UDP

galα1-4gal cer

galβ1-3galNAcβ1-4galβ1-4-glc cer

PAPS   PAP

galSO₄ ceramide
sulfatide, SGC

SYNTHETIC PATHWAY FOR THE
COMMON CORE SEQUENCES OF GSL'S

Figure 1.4   Biosynthesis of Glycosphingolipids

vesicular traffic through the Golgi apparatus is directed towards the plasma membrane, resulting in the insertion of newly synthesized glycolipids on the external leaflet of the membrane (van Meer, 1993).

GSLs are catabolized in lysosomes by sequential actions of glycosidases into ceramide which is further degraded to sphingosine base and free fatty acid by ceramidase (Sugita et al., 1972; Sweely and Siddiqui, 1977). Glycosidases cleave GSLs to ceramide and sugars in stepwise form as in glycosyltransferase action. Some of the GSL hydrolases require the assistance of small heat-stable-nonenzymatic proteins, sphingolipid activator proteins, or saposins. Saposins are a group of four heat-stable glycoproteins with molecular weights of 12-15kDa, named A to D, with high sequence homology and are compact and extraordinarily stable (Mehl et al., 1964; Li et al., 1976; Gartner et al., 1983, Hiraiwa et al, 1996). The first saposin to be discovered was saposin B, which stimulates the hydrolysis reactions of arylsulfatase A, GM1 ganglioside β-galactosidase, and α-galactosidase A (Mehl and Jatzkewitz, 1964; Li and Li, 1976; Gartner, et al., 1983). Saposin C was proposed to stimulate the activity of glucocerebrosidase and galactocerebrosidase (Wenger et al., 1982). Saposin A was shown to have a similar specificity to that of saposin C, although with less activity (Morimoto et al., 1989). Saposin D was shown to stimulate activities of sphingomyelinase and ceramidase (Morimoto et al., 1988; Azuma et al., 1994). However recent studies show that saposins may have nonspecific stimulative effects on lipid hydrolysis (Li et al., 1988; Hirawa et al, 1996). All saposins are equally capable of forming water-soluble complexes with all sphingolipids examined and make them available to enzymatic hydrolysis (Soeda, 1993; (Wenger et al., 1984). Deficiencies in glycosidase enzymes, saposin or prosaposin lead to lysosomal storage diseases (Sandhoff and Klein, 1994; Hakomori, 1986; Suzuki, 1994).

1.2.4 Role of the membrane lipid composition and fatty acid moiety of GSL on exposure of carbohydrate portion of GSLs in VTs binding

Individual GSLs are characterized by the presence of a single oligosaccharide chain attached to a ceramide moiety that is heterogeneous both in fatty acid and long chain base composition (Sonnino, et al., 1985). Despite extensive structural and functional studies on glycolipid carbohydrate, little attention has been paid to a possible functional role of the lipid moiety of these glycoconjugates. The ceramide has been thought to act simply as an anchor in the plasma membrane. However, there is increasing evidence that the ceramide portion of GSLs is important in carbohydrate/ligand binding. It has been proposed that the ceramide moiety of GSLs may influence the exposure of the carbohydrate portion, on the membrane surface can significantly affect the binding of the glycolipid with various ligands (Kannagi, et al., 1982; Kannagi et al., 1983; Crook et al., 1986; Stewart et al., 1990; Stewart et al., 1993). Monoclonal antibodies against lactosyl ceramide discriminate between lactosyl ceramide containing different fatty acids in the ceramide moiety (Symington et al., 1984).

The length and unsaturation of the alkyl chain present on GSLs can influence membrane fluidity and permeability through hydrophobic interactions with lipid and protein components in the membrane. GSLs can give rise to a number of ceramide species which differ with respect to the number and location of their hydroxyl groups. Free hydroxyl content and the presence of an amide group make the ceramide capable of acting as both an hydrogen bond donor and acceptor whereas glycerolipids lack hydrogen bond donor activity (Pascher et al., 1977).

The increase in the number of lateral hydrogen bonds through the addition of hydroxyl groups to GSLs creates a surface membrane of higher stability which promotes a close packing in the hydrocarbon chain matrix and create a barrier to polar and nonpolar molecules. This network of interlipid hydrogen bonds has an effect on membrane stability and permeability and may be regulated to fit specific requirements of different cell types by variations in ceramide structure and lipid composition (Boggs, 1987). In this regard, hydroxylation of gangliotriaosyl ceramide fatty acid was found to promote the binding of monoclonal antibody against Gg3 to lymphoma cells (Kannagi, Stroup et al., 1983), but reduced binding of anti-SGC antibody to SGC (Crooke et al, 1989). Moreover, antigalactosyl ceramide antibodies require hydroxylation of the fatty acids in the antigen for reactivity (Nakakuma et al., 1989).

Verotoxins recognize the terminal Galα1-4Gal disaccharide of Gb3 for specific binding, since α-galactosidase removes receptor binding, but the same terminal Galα1-4 Gal sequence as Gb3 linked to a glycerolipid moiety shows no VT1 binding (Lingwood, 1979; Waddell et al., 1988; Kiarash et al., 1994). In addition, the binding of VT1 to its GSL receptor in a lipid environment is affected by differences in receptor fatty acid content, which demonstrates that Gb3 fatty acid microheterogeneity plays an important role in VT-binding and may thus influence the possible clinical consequence of VTEC infection (Pellizzari et al., 1992). Also, the influence of fatty acid chain length and degree of saturation on VT1 and VT2c binding to Gb3 was demonstrated (Kiarash, et al., 1994). For most Gb3 isoforms, the presence of an unsaturated fatty acid significantly increased VT-binding in membrane immobilized on microtitre plate. Minimal binding was observed to Gb3 containing the short chain fatty acid (C12, C14) as compared to Gb3 with middle and long chain fatty acids (C16, 18, 20, 22, 24) (Kiarash, et al., 1994).

Moreover, a recent study has indicated that the alkyl chain length within a phospholipid bilayer also affects the relative ability of Gb3 to bind VTs (Arab et al.,

1996). For VT1 and VT2 the ability to bind Gb3 in liposomes or in the immobilized phospholipid matrix (microtiter plate), was inversely related to the phospholipid chain length. This is consistent with the finding that the plane of the phospholipid bilayer impinges upon the carbohydrate moiety affecting its conformation (Nyholm et al., 1993).

Thus, the presentation of lipid bound carbohydrate on a cell surface involves considerably more than the synthesis of the appropriate carbohydrate sequence. Lipid composition and biological membrane microenvironment can influence not only the exposure of the carbohydrate portion of GSLs, but also can play a dramatic modulatory role.

1.2.5 Glycolipid biological functions

Membrane GSLs are thought to perform many functions due to the wide variety of GSL structures. GSLs usually constitute only a minor (5%) fraction of the total lipid in the cellular membrane (Sweeley et al, 1985), leading to the conclusion that they are not simply used as membrane building units, but rather perform specific cell biological functions. They are thought to have roles in providing membrane structural rigidity, in regulating membrane protein functions and binding ions, in cell-cell recognition, and in regulation of cell growth through functional modulation of key molecules that are essential in transmembrane signaling (Curatolo, 1987; Hakomori, 1993; Liscovitch et al., 1994). In some cells, GSLs are also abundant in intracellular membranes (Symington et al, 1987), but little is known about the exact distribution and organization of GSLs in these cells. Some intracellular cytoplasmic GSLs are closely related with cytoskeletal proteins (Gillard et al., 1994). Electron-microscopic studies have revealed that GSLs are presented in a large cluster form at the external surface of the lipid bilayer with undefined function as of yet (Tillack et al, 1983; Rock et al, 1990; 1991). In addition, molecular modeling of GSLs and their organization in membranes suggest that a

GSL "patch" provides binding sites for interaction with ligands and adjacent cells, and that GSLs or their catabolites modulate transmembrane signaling (Hakomori, 1990).

GSLs are also known to be present as antigens in normal red blood cells (Thorn, 1992). Gb3 and Gb4 are antigens in the P blood group system. Gb3 has been shown to be a marker in normal B-cell differentiation (Wiels et al., 1991). Gb3-deficient cells, which were resistant to the growth inhibitory effects of α2 interferon, were cross-resistant to VT. Mutagenized Daudi cells, which had lost their high affinity binding to α-interferon showed a significant decrease in Gb3 and Gb2 levels (Cohen et al., 1987). These results suggest that these glycolipids may be involved in α2 interferon signal transduction (Ghislain et al., 1994).

Glucosylceramides are the most widely distributed glycosphingolipids in cells serving as precursors for the biosynthesis of over 200 known glycosphingolipids. In addition to their role as building blocks of biological membranes, glycosphingolipids have long attracted attention because of their putative involvement in cell proliferation (Hannun et al., 1989), differentiation (Schwarz et al., 1995); Harel et al, 1993), and oncogenic transformation (Hakomori, 1981; Morton et al., 1994). Moreover, some metabolites of glycosphingolipids, such as sphingoid bases, ceramides, and lysosphingolipids are suggested to have a second messenger function in signal transduction pathways involved in growth (Hannun et al., 1993), apoptosis (Obeid et al., 1993), and the action of tumor necrosis factor-α (Dressler et al., 1992). Moreover, there is increasing evidence for the role of fatty acids in signal transduction and protein lipidation (Casey, 1995; Wedegaertner et al., 1995).

1.2.5.1   GSLs as tumor associated antigens

GSLs are found to be associated with oncogenic transformation. Dramatic changes have been documented over many years in association with

differentiation, development and oncogenesis (Hakomori, 1990). Aberrant GSL expression is a ubiquitous phenotype of most types of tumors, which can lead to aberrant adhesion favoring metastasis and invasiveness of tumor cells, and aberrant catabolism leading to altered transmembrane signaling and loss of growth control. Many tumor-associated antigens have been identified as GSLs (Lee et al., 1981; Hakomori et al., 1983; Hakomori, 1984; Prokazova, 1989; Inokuchi et al., 1990; Mannori et al., 1990; Ohyama et al., 1990; Calsen, 1993; Wenk et al., 1994; Sing et al, 1994; Kingsley et al, 1995). Some tumors display a highly diverse patterns of glycolipid changes (Hakomori and Kannagi, 1983). The antigenicity of cellular GSLs is defined not only by their primary carbohydrate structure, but also by their organizational status and the structure of ceramide (i.e, length, hydroxylation of fatty acids and sphingosine) (Yoshino, 1982; Kannagi, et al., 1983).

1.2.5.2 GSLs as ligands in cell-cell recognition

GSLs have roles in cell-cell interactions (Hakomori, 1981) and are also closely linked to cell growth and differentiation. The expression of GSL on the cell surface changes with these events. For example, there is a definite stage-dependent appearance of certain GSLs in embryonic development (Muramastu, et al, 1988) and the appearance of stage-specific embryonic antigen-1 (SSEA-1) or Le^x, correlates approximately in time with the onset of compaction of the embryo, which occurs after the third cleavage division (Fenderson et al., 1990). Moreover, carbohydrate moieties of Le^x at the embryonic cell surface may specifically interact with Le^x on an opposing homotypic cell surface in the presence of a bivalent cation, suggesting that carbohydrate-carbohydrate interactions may play an important role in controlling cell recognition during embryonic development (Eggens et al., 1989).

1.2.5.3   Regulation of cell growth and transmembrane signaling by GSLs or their derivatives

The growth of most eukaryotic cells is regulated by three major transmembrane signaling mechanisms: protein kinases associated with growth factor receptors, protein kinase C, and the G-protein family. Many studies have shown that the former two mechanisms are modulated by GSLs or their derivatives (Bermer, 1982; Hannun et al., 1987; Igarashi, et al., 1987; Hannun and Bell, 1989; Casey, 1995; Wedegaertner, et al., 1995; Nudelman, 1992). The mechanisms whereby GSLs participate in cell growth and development are unclear, however Gb_3 recognition by VT provides a useful tool to study the role of this glycolipid in various normal processes of cellular physiology such as cell growth. For epithelial cells, exponentially growing cells are more sensitive to VT than are stationary phase cells. Synchronized cultures of Vero cells were found to be 10-fold more sensitive to the cytotoxicity of this toxin at the G1/S boundary than elsewhere in the cell cycle (Pudymaitis and Lingwood, 1992). The G1/S boundary is a major point of control within the cell cycle (Norbury et al., 1989), when environmental stimuli can determine whether or not a cell will enter S phase and divide. Perhaps Gb_3 is involved in the transduction of such signals, since elevation of specific Gb_3 isoforms were observed at this point (Chapter 5; Pudymaitis and Lingwood, 1992).

However the role of GSLs in the control of cellular proliferation is not understood. Evidence has been presented demonstrating that these molecules are potent pharmacological regulators of cell growth and differentiation. In addition GSLs are bimodal regulators of the epidermal growth factor (EGF) receptor tyrosine kinase activity (Hakomori, 1990; Zheng et al., 1993; Lee, et al, 1994). GSLs (GM1 and GM3) inhibit PDGF binding and PDGF-induced protein phosphorylation on tyrosine (Bremer 1994; Yates et al., 1995). GM3 has also been found to inhibit EGF-induced phosphorylation without inhibition of EGF binding (Bremer, 1984; Song et al., 1993). In addition, the hydrolysis of cell surface GSLs by endoglycoceramidase

reduces EGF receptor phosphorylation (Ji, et al, 1995). Therefore inhibition of growth by these GSLs appears to involve direct interactions with the PDGF and EGF receptors to modify signal transduction. The role of Gb3 in signal transduction will be addressed in section 1.3.5.

1.3 Globotriaosylceramide (Gb3)

1.3.1 Structure and Tissue Distribution

Globotriaosylceramide (Gb3) is a member of the globo-series of neutral glycolipids and contains a trisaccharide (Galα1-4Galβ1-4Glcβ1) linked to ceramide (Figure 1.5). Various fatty acyl groups may be attached to the amino group of sphingosine, but the carbohydrate sequence is specific. This glycolipid was originally purified from human spleen (Makita, 1964) and then from kidney of patients with Fabry disease (Sweely and Klionsky, 1963) and normal human kidney (Makita, 1964). Gb3 is a major glycolipid of human kidney, particularly the cortex (Boyd et al., 1989) where lesions in HUS are primarily seen (Richardson, et al., 1988). Gb3 is found on a number of cell lines such as cultured cells of epithelial morphology, endothelial cells, and Burkitt lymphoma (Daudi) cell lines.

In addition, there are a number of human conditions which have been reported to demonstrate an elevation or accumulation of Gb3 in different cell types. One such condition is Fabry's disease, which is the only X-linked genetic disorder of glycosphingolipid metabolism, as opposed to other forms of sphingolipidoses which are inherited as autosomal recessive mutations. Patients with Fabry's disease produce inadequate levels of the enzyme α-galactosidase and thus, Gb3 is not degraded and accumulates in most organs and body fluids (Kanfer and Hakomori, 1983). The clinical manifestations of this accumulation include joint pain, impaired sweat production, skin and myocardial lesions (Hakomori, 1986). Deficiency in glycosidases and ceramidase lead to other lysosomal storage diseases such as Tay-Sach's, and Gaucher's disease. Also, up to a threefold increase of Gb3

Gb₃

Galα1-4Gal

Sphingosine

fatty acid
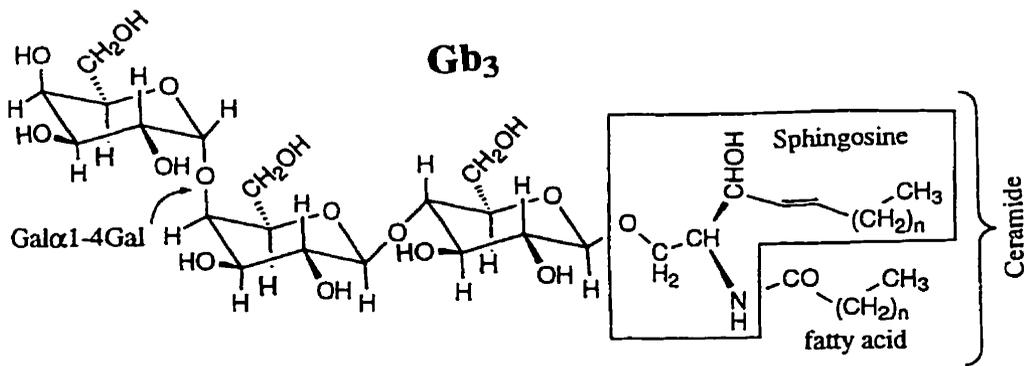
Ceramide

Figure 1. 5   Molecular structure of globotriaosylceramide (Gb3)

level in fibroblasts and lymphoblasts was seen in patients with Familial Dysautonomia, which is an autosomal recessive disease of unknown etiology (Strasberg et al., 1992). These patients are neurologically impaired, with deficits primarily in autonomic and sensory functions. Elevation of Gb3 in cancer will be discussed in Section 1.3.2.

1.3.2 Gb3 as a tumor marker

Gb3 is expressed on Burkitt's lymphoma cells and was identified as the Burkitt Lymphoma-associated Antigen (BLA) (Wiels et al., 1984). During human B cell differentiation, differentiation (CD) antigens are sequentially expressed on the surface of B cells and Gb3 now defines the CD77 cluster (Mangeney, et al., 1993). Gb3-positive Burkitt lymphoma cells and germinal B lymphocytes are highly susceptible to entering programmed cell death (apoptosis); VT1 and VT1B have been shown to kill cells not only by protein synthesis inhibition, but also by inducing apoptosis (Mangeney, et al., 1993; Inward, Williams et al., 1995; Arab, et al., in press). Moreover, the VT1-B subunit also appears to induce apoptosis, likely by signal transduction through Gb3 binding and translocation to the nucleus (Mangeney, et al., 1993; Arab et al., submitted)

Gb3 is specifically elevated in cells from several types of cancer when compared to the normal tissue counterparts (Mannori, et al., 1990; Farkas-Himsley et al., 1995; Arab, et al., submitted) and has been proposed as a marker for testicular tumors and germ cell tumors (Ohyama, et al., 1990; Wenk, et al., 1994). Surface exposure of Gb3 can be correlated with metastatic potential (Mannori, et al., 1990; Arab, et al., submitted).

1.3.3 Gb3, as a Functional Cellular Receptor for VT1, VT2, and VT2c

The glycolipid-binding specificities of VT1 were first described by Lingwood et al(1987). There is strong evidence showing that Gb3 is the functional receptor, for

VT1, VT2, and VT2c (Lingwood, et al. 1987; Lindberg, et al, 1987). Gb3 is present on the surface of the cells susceptible to VT but absent on the resistant cell lines (Cohen, et al., 1987; Jacewicz, et al., 1989; Pudymaitis, et al., 1991). Reconstitution of the cell lines deficient in Gb3 with this glycolipid restores VT susceptibility (Waddell, et al., 1990; Boyd, et al, 1992).

Binding to this glycolipid receptor (Gb3) on cells, mediates the cytotoxicity of VT both in vitro (Waddell, et al., 1990) and in vivo (Boyd et al., 1993). The glycolipid binding specificity of the pig edema toxin (VT2e) was altered from Gb4 to Gb3 using site-directed mutagenesis. The change in glycolipid binding specificity altered in vivo tissue targeting. This glycolipid binding specificity of VTs was shown to be the primary determinant in the site of toxin localization and toxin-induced lesion in vivo (Boyd, et al., 1993).

1.3.4   Gb3 as pk blood group antigen

Gb3 is the pk blood group antigen of the P blood group series (Naiki et al., 1974). VT also binds to the P1 antigen ($gal\alpha1$-$4gal\beta1$-$3glcNAc\beta1$-$4gal\beta1$-$4glc$ cer) present on P1 red cells. It has been speculated that red cells can bind systemic VT and that this may result in red cell fragmentation which could be responsible for the hemolytic anemia observed in HUS. Alternatively red cells have been suggested to provide an inert receptor pool to bind toxin in the circulation and thereby prevent systemic toxin reaching the more crucial targets in the renal endothelium. An inverse correlation between the frequency of HUS following VTEC infection and the P1 blood group status (P1 blood cells should express the maximum $gal\alpha1$-$4gal$ terminating glycolipids) has been claimed by Taylor et al., (1990) but disputed by others (Kaplan, 1994; Orr, et al., 1995). It was shown that under physiological conditions VT does not bind to human red cells to any significant degree, irrespective of P blood group status. P blood group dependent VT binding to red cells can be detected at 4°C (Bitzan et al., 1994) which is of

questionable relevance in normal cell physiology. Unlike VT1, which binds only to Gb$_3$, the pig edema disease toxin-VT2e, binds Gb$_4$ in addition (DeGrandis, et al., 1989). VT2e binds strongly to pig red cells (Boyd, et al., 1993) and mutation of the binding specificity from Gb$_4$ plus Gb$_3$ to Gb$_3$ alone (Tyrrell et al., 1992) results in the loss of red cell binding (Boyd, et al., 1993). The extensive binding of VT2e to red cells certainly does not protect the host against the vascular pathology of toxin induced disease. Thus the role of red cells and P blood group status remains an enigma in the etiology of HUS.

1.3.5 Gb$_3$ in signal transduction

It was found that CD19, a B cell specific antigen , has a putative Gb$_3$-binding domain as determined by sequence similarities with the B-subunit of VT's (Maloney et al., 1994). It was proposed that the interaction between Gb$_3$ and CD19 is important in B cell differentiation and germinal cell targeting. In addition, similarities in amino acid sequences between verotoxin B-subunit and the α2-interferon receptor have been reported (Lingwood et al., 1992). These sequences include several amino acids suspected to be involved in glycolipid binding. Recently ligation of CD19 was able to induce Gb$_3$ dependent apoptosis (Khine et al., submitted). In addition, CD19 induced apoptosis was shown to be independent of Bcl-2 and p^{53} in human B-lineage lymphoma cells (Myers et al., 1995).

Reduced sensitivity to the growth inhibitory effects of α2-interferon has been demonstrated for the mutagenized Daudi cell line, which is both Gb2- and Gb3-deficient (Cohen, et al., 1987). This growth inhibition by α2-interferon was restored in Gb$_3$-deficient Daudi mutants after reconstitution with Gb$_3$ (Ghislain et al., 1992). The earliest event after binding of interferon to its receptor is the rapid phosphorylation of a cytosolic transcription factor, IFN-stimulating gene factor (ISGF3α), which results in complex formation with ISGF3γ and translocation of the ISGF3αγ complex to the nucleus where it binds to the ISRE of interferon-sensitive

genes, thereby activating their transcription (Fu, 1992; Velazquez et al., 1992). ISG transcriptional activation is mediated by the protein tyrosine kinase (PTK)-dependent phosphorylation of latent cytoplasmic transcriptional activators, termed the STAT proteins for Signal Transducers and Activators of Transcription. Treatment of Daudi cells with a ceramide analogue, PDMP results in a dramatic reduction of Gb_2 and Gb_3 synthesis, and prevents interferon-induced ISGF3 activation and a concomitant reduction in interferon signaling is observed (Ghislain, et al., 1994). This suggests the importance of Gb_3 for interferon signal transduction.

1.4   Glycosphingolipid trafficking

The definition of cellular organelles has evolved over the last hundred years largely driven by morphologic obsevations, but more recently has been supplemented and complemented by functional and biochemical studies (Palade, 1975). Essential to the many cellular functions that take place within the central vacuolar system (which consists of ER, Golgi apparatus, secretory vesicles, endosomes and lysosomes) is membrane traffic which mediates the exchange of components between different organelles (Fig. 1.6). Without regulation, these processes would randomize the lipid composition of cellular membranes. There are two critical characteristics of membrane traffic. First, only certain sets of organelles exchange membrane and the patterns of this exchange define what is called membrane pathways. Second, multiple pathways intersect at specific points within the central vacular system. For specific components to "choose" the correct pathways at such points of crossing, mechanisms exist to impose choices on specific molecules. This process is called sorting (van Meer, 1993; Fig, 1.6) . This sorting may also occur at the plasma membrane level (chapter 8).

The concept of a coordinated glycolipid precursor-product relationship in the multi-glycosyltransferase system was first formulated by Roseman (1970). This was subsequently elaborated to show different pools of ganglioside for different functions (Caputto et al., 1976). At present, the localization and trafficking of several glycosyltransferases in the ER, Golgi network and plasma membrane axis are being elucidated (Trinchera and Ghidoni, 1990; Young, et al., 1992; van Meer, 1993; van der Bijl et al, 1996). The description of the endocytotic GSL trafficking pathways is based largely on studies carried out with C6-NBD derivatives of the simple neutral GSL, glucosylceramide (GC). In figure 1.6 schematic pathways of cellular organelles connected by vesicular traffic is demonstrated (Helvoort and van Meer, 1995). It has been concluded that glycolipids are internalized by coated or non-(clathrin)-coated endocytic vesicles (Evered, 1982). From peripheral early endosomes and beyond the early stage of endocytosis, GC follows several routes and partly segregates from the lysosomal marker transferrin. The peripheral early endosomes are apparently capable of sorting these molecules (Schmidt et al., 1985). A small percentage of the GC is transported to the Golgi area. The involvement of the Golgi apparatus in endocytic trafficking routes, in particular the role of the trans Golgi network (TGN), is not resolved completely (Griffiths et al., 1986). However, it has been proposed that GSLs are sorted in the TGN by self-aggregation into the apical precursor domain (Simons and van Meer, 1988; van Meer, 1993). In addition to a well established transport cycle of biosynthetic lysosomal enzymes between the TGN and late endosomes (Kornfeld et al., 1989), an input from the early endocytic pathway into the TGN may exist, followed by a recycling to the plasma membrane, thus constituting a transport cycle between the plasma membrane and the TGN (Kok et al., 1994). A part of the GC fraction is found to be transported to late endosomes which are the merging point of the clathrin-dependent and the clathrin-independent pathways (Kok et al., 1992). Moreover, newly synthesized GC was shown to be transported to the plasma membrane through a mechanism
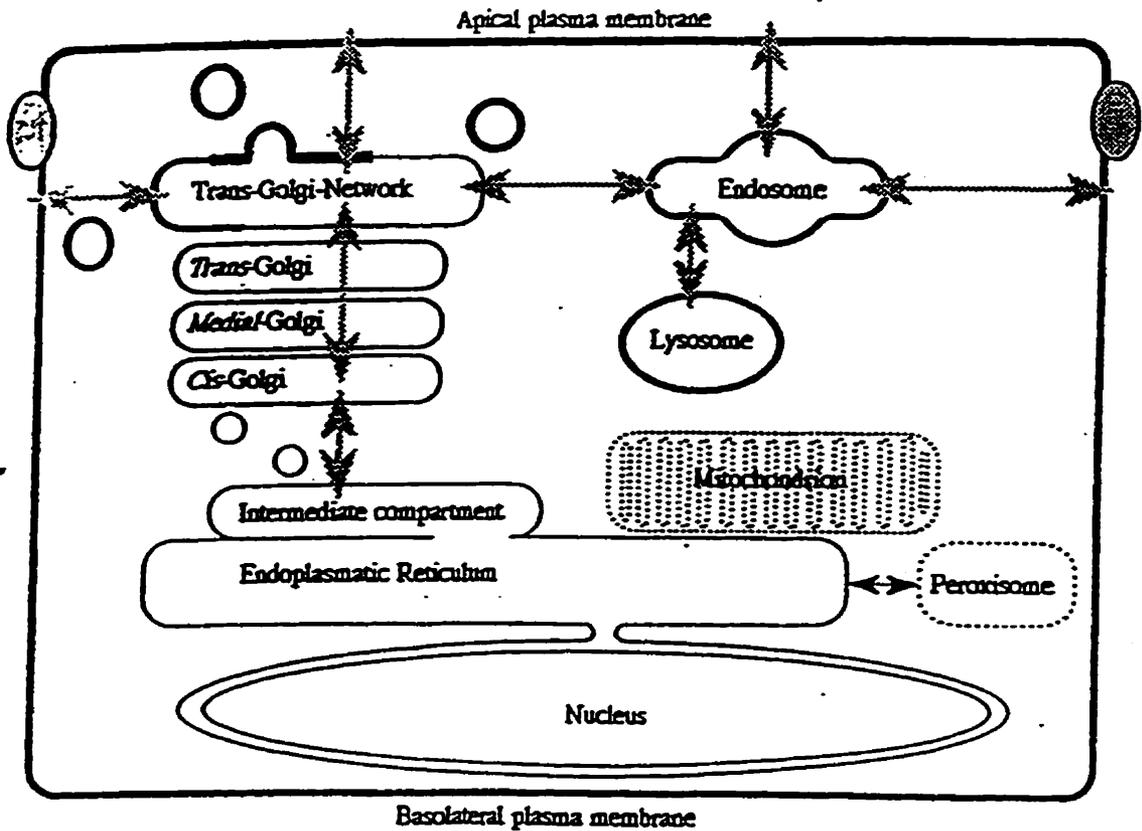
Figure 1.6 Schematic representation of cellular organelles connected by vesicular traffic. Dotted lines indicate organelles not connected by this type of transport. Thickness of the lines reflects the concentration of (glyco) sphingolipids and cholesterol.

independent of the vesicular pathways (Warnock, et al., 1994). It is possible that VT pathway is comprised of a reverse retrograde transport for intracellular targeting.

Some proteinaceous ligands move further from the late endosomes to lysosomes (Kok, et al., 1992). However, GC is not found in lysosomes and apparently can escape from this organelle. Prior to reaching the lysosomes, the lipid recycles to the plasma membrane (Kok and Hoekstra, 1994). It is important to note that the morphology and the mechanism of functioning of the endosomal system may depend on the cell type studied and the movement of molecules from peripheral early endosomes to central endosomes or late endosomes clearly depends on the microtubular system (Sakai et al., 1991; Kok, et al., 1992). Moreover, recent study has shown that the sorting of GSL appears to depend on lipid structure (van der Bijl et al, 1996). It was proposed that a different ratio of GSL synthesis in various epithelial cells governs the lipid sorting in the membrane of the TGN by dictating the composition of the domain from where vesicles bud to the apical and basolateral cell surface (Van der Bijl, et al, 1996). The identification of an endoplasmic reticulum-Golgi intermediate compartment (ERGIC) defined by the 53-KDa transmembrane marker protein ERGIC-53 (Schweizer, et al., 1990) has added to the complexity of the exocytic pathways. However, synthesis of glucosylceramide mainly occurs beyond the ERGIC in the Golgi apparatus. (Schweizer, et al, 1994).

After decades of research and documenting of endocytotic trafficking pathways, there are still many unanswered questions concerning both glycosphingolipid (GSL) trafficking and protein sorting. Progress in this field is relevant to a number of important problems in biology. GSL and glycoproteins share many features of intracellular trafficking (Schwarzmann and Sandhoff, 1990). Both the ceramide backbone of GSL (Helvoort and van Meer, 1995) and the polypeptide of glycoproteins are synthesized in the ER. GSL and glycoprotein transport take place through the Golgi by similar if not identical mechanisms

(Wattenberg, 1990). These feature make the GSL the preferred lipid class for studying vesicular traffic or retrograde transport in nucleus-ER-Golgi-plasma membrane pathway. GSL are also an inherent part of the vesicles that are involved in endocytic uptake of protein.

1.5 Retrograde transport and nuclear targeting

Membrane traffic is now recognized as a carefully regulated process controlled by distinct anterograde (from ER to plasma membrane) and retrograde (from plasma membrane to ER or nucleus) pathways (van Meer, 1993). These pathways link organelles with different morphologies, structures, and locations within the cell (Rothman, 1992; Lippincott et al, 1993). The ER, which is involved in multiple cellular functions including protein synthesis and folding, extends to the cell periphery and forms a dynamic tubular reticulum. By contrast, the Golgi apparatus, which functions in membrane sorting and recycling events is localized at the center of the cell and is comprised of compact cisternal units (Lippincott et al, 1993). The existence of retrograde transport along the biosynthetic pathway was first demonstrated for a certain class of luminal resident ER proteins carrying the carboxyl-terminal tetrapeptide KDEL (in mammals), or HDEL (in yeast) and KDEL receptor protein has been identified in mammals and yeast (Lewis et al., 1990, 1990; Semenza, 1990; Lewis et al., 1992). At steady state, the KDEL-receptor is found in the Golgi apparatus (Griffiths et al., 1994), but overexpression of substrate protein leads to its re-localization to the ER, suggesting that the KDEL-receptor functions as a shuttle from the Golgi to the ER (Lewis and Pelham, 1992).

Golgi resident proteins may also undergo retrograde movement. For instance, the medial-Golgi protein MG160 is sialylated, suggesting that it cycles between late-Golgi/TGN compartments (in which sialylation occurs and medial Golgi (Johnston et al., 1994). Retrograde transport has recently been documented for other yeast and mammalian Golgi proteins, such as cis-Golgi

mannosyltransferase Och1p, medial/trans Golgi N-acetylgucosaminyltransferase I, and Golgi localized Emp47p (Hoe et al., 1995; Schroder et al., 1995; Harris et al., 1996).

Other evidence for the existence of a retrograde pathway comes from work on bacterial toxins which seem to enter the cytosol of higher eukaryotic cells after reaching ER compartments (Pelham et al., 1992). Particular examples of these toxins are Shiga toxin and Verotoxins (Shiga -like toxins). VT cytopathology requires toxin internalization (Khine et al., 1994). Both the A and B subunits are internalized at 37°C following VT binding to Gb_3 receptors distributed over the cell surface. VT bound Gb_3 enters the cell via a process of receptor mediated endocytosis (RME) via clathrin-coated pits (Sandvig et al., 1989). Internalization of VT does not involve the classical toxin internalization pathway into lysosomes and translocation of the A subunit into the cytosol (Hudson et al., 1987). Rather, the holotoxin is internalized into an endosomal compartment and then is transferred to the Golgi, to the ER (Sandvig, Garred et al., 1992) and finally as we have shown, to the nuclear envelope (Chapter 5). This process is termed retrograde transport of VT. This internalization pathway is observed for both the holotoxin and B subunit (Khine and Lingwood, 1994; Sandvig et al., 1994) suggesting that these components may not separate, or separate late, during cytopathology and thus the pathway is defined by Gb_3 binding. Internalization of Gb_3 by verotoxin is inhibited in the presence of monodansyl cadaverine, which is a potent inhibitor of transglutaminase mediated ligand-receptor complex clustering (Khine and Lingwood, 1994). Prevention of internalization protects cells against VT induced cytopathology. Electronmicroscopical analysis has shown that Shiga toxin can be detected in the ER of Vero cells, of butyric acid treated A431 cells, of Daudi cells, and a VT1-B fragment in the Golgi apparatus of Vero cells (Sandvig, et al., 1992; Sandvig, et al., 1994; Garred et al., 1995; Kim et al., 1996).

Information on molecular mechanisms underlaying retrograde transport between the Golgi apparatus and the ER is still scarce. However, COPI proteins have been shown to play an essential role in the retrieval of di-lysine tagged proteins to ER (Cosson et al., 1994; Letourneeur, 1994). SNARE proteins, that are involved in vesicle docking and fusion at multiple steps of intracellular transport (Rothman, 1994), may also play a role in retrograde transport between the Golgi apparatus and the ER as well as in intra-Golgi transport (Banfield et al., 1994; Lewis et al., 1996; Nagahama et al., 1996; Subramaniam et al., 1996). In contrast, the involvement of Rab proteins, that are regulators of vesicular transport (Zerial et al., 1993; Pfeffer, 1994), in intra-Golgi or Golgi-to-ER retrograde transport remains enigmatic. A good candidate, however, for a Rab protein governing intra-Golgi retrograde transport is Rab6 (a small GTP-binding protein). Rab6 is associated with medial-and late-Golgi cisternae as well as with the membranes of the TGN (Antony et al., 1992) and it has previously been suggested that Rab6 could be a constituent of an intra-Golgi pathway (Martinez et al., (1994).

The data available to date suggest that proteins larger than 45 KDa require a nuclear localization sequence (NLS) in order to be targeted to the nucleus (Jans, 1994). However, glycosylated proteins, which lack nuclear localization signal, upon their injection into the cytosol, enter the nucleus in a sugar-dependent manner (Duverger, 1995). Through mutation and deletion analysis and /or by their ability to target a normally cytoplasmically localized protein to the nucleus, many NLSs (Jans, 1994) have been defined (Miller et al., 1991; Silver, 1991). The best-characterized NSL is that of the simian virus SV40 large-T antigen (PKKKRKV). A single amino acid substitution abolished its NLS function, resulting in complete cytoplasmic localization (Miller, et al., 1991; Silver, 1991). Additional putative NLSs have been proposed, including human α-interferon receptor with a RKIIEKKT sequence (Jans, 1994). Even though, theoretically, small proteins do not require an NLS for nuclear entry, some small proteins such as histone 2B contain

functional NLS (GKKRSKAK) capable of targeting large proteins such as β-galactosidase to the nucleus (Miller, Park et al., 1991). Shiga toxin localization to ER was reported in spite of the lack of KDEL (ER targeting signal) sequence (Sandvig, Garred et al., 1992). The mechanism of verotoxin retrograde transport is not fully understood. It might be speculated that its retrograde transport is achieved in association with its receptor Gb_3, or by the existence of an as yet unknown retrograde transport signal in the sequence of the B-fragment. The present study demonstrated the targeting of VT into the nucleus without an NLS (see chapter 5).

1.6 Tumor biology and genetics

Cancer, like many other chronic diseases, is a multi-step, multi-stage process in which multiple genetic alterations must occur during the course of a disease that develops over many years. At its most fundamental level cancer is a problem of aberrant cell cycle and apoptotic control. This axiom has been spectacularly borne out by basic research on cell cycle division. Because cell cycle machinery is so highly conserved, many fundamental insights have come from tractable genetic and biochemical systems. Recently, exciting insights into the role of cell cycle checkpoints in cancer progression and predisposition have emerged (Hartwell et al., 1994; Zakian, 1995). Many checkpoints are deregulated in oncogenesis (Hunter et al., 1994). One of the most important checkpoints is START (restriction point in mammalian cells) in late G1.

Complexes of cyclins with cyclin-dependent kinases (CDKs) play a central role in the control of the eukaryotic cell cycle (Pines, 1995). The discovery of proteins that bind to and inhibit the catalytic activity of cyclin-CDK complexes has identified kinase inhibition as an intrinsic component of cell-cycle control (Pines, 1995; Peter and Herskowitz, 1994).

Several " transforming genes" or oncogenes, whose products are positive regulators of cell growth and whose activation leads to transformation, have been

identified (Vicent et al, 1993). In recent years, a new class of so-called tumor suppressor genes, whose products are negative growth regulators and whose inactivation results in the expression of a malignant phenotype, have emerged (Vicent et al, 1993). The genetic alterations leading to the activation of oncogenes or the knockout of tumor suppressor function are acquired in the form of chromosomal translocations, deletions, inversions, amplifications, or point mutations (Baserga, 1993; Perkins, 1993) or as epigenetic alteration or gene imprinting (Morison et al., 1996).

Invasion of cancer cells into the host connective tissue, the neovascularization of the tumor mass, and the metastatic spread of malignant cells are the hallmarks of malignant neoplasia (Liotta et al., 1991). Evidence indicates that most of human malignancies are the end result of an accumulation of mutations within growth regulatory genes.

1.7   Cancer as a multi-resistant phenotype.

Many cytotoxic drugs, being lipid soluble, enter the cell via diffusion down a concentration gradient. However, in many type of cancer cells, overexpression of an ATP-driven drug efflux pump, p-glycoprotein (Juliano et al., 1976; Bosch et al., 1996), is believe to transport these drugs out of the cytosol or to act as a flippase/translocase (Higgins et al., 1992) to expel them from the bilayer. Consistent with the latter hypothesis the p-glycoprotein mdr2 gene product exhibits phosphatidylcholine translocase activity (Ruetz et al., 1994). Further, p-glycoprotein, as a member of the ABC (ATP-binding cassette) superfamily of transporters, shares a number of structural and sequence similarities (Hyde et al., 1990); Riordan, 1989] with the cystic fibrosis transmembrane regulator (CFTR) and may exhibit similar functional properties. Consistent with this hypothesis, mdr1 exhibits ATP-dependent chloride channel activity (Miguel et al., 1992). These

observations suggest that over-expression of p-glycoprotein in neoplastic tissues may contribute to decreased accumulation of cytotoxic drugs.

In addition to increased efflux, a decrease in drug influx has been observed in many multi-drug resistant cancer cell lines (Ling et al., 1973; Carlsen et al., 1976; Beck, 1983). Again, since many anticancer drugs are lipid soluble and diffuse freely into the membrane, it is not surprising that alterations in membrane permeability would contribute to multi-drug resistance. Consistent with this hypothesis, changes in membrane architecture have been observed in multi-drug resistant cells. A higher degree of structural order in the lipid phase of the plasma membranes, elevated intracellular lipid content, higher cholesterol level and lower level of phosphatidylserine was observed in multi-drug resistant murine leukemia cells (Ramu et al., 1983; Escriba et al., 1990). Moreover, changes in the phospholipid composition of the plasma membrane (sphingomyelin, cardiolipin, phosphatidyl ethanolamine and phosphatidyl choline) can affect membrane fluidity as well as the efficiency with which membrane transporters function (Bruzik, 1988). Interestingly, changes in the lipid composition of the membranes of tumor cells with respect to the ratio of saturated/unsaturated fatty acids have been reported (Tamiya-Koizumi et al., 1985). Another phenomenon consistently observed with the multi-drug resistant phenotype is change in intracellular pH. In multi-drug resistant cells the intracellular pH often increases with increased resistance (Keizer et al., 1989) and accordingly, drugs which acidify the cytosol reverse the multi-drug resistant phenotype (Espand et al., 1991). The pH of a tumor is often more acidic than in normal tissue, as a result of increased glycolytic activity associated with tumor cells (Warburg, 1955).

Since most chemotherapeutic agents are directed toward DNA in the tumor cells, a multi-drug resistant phenotype can also be achieved by preventing drug accumulation within the nucleus. Consistent with this hypothesis many chemotherapeutic drugs have been shown to accumulate in cellular organelles of

multi-drug resistant tumor cells including the Golgi and lysosomes (Beck, 1987; Simon et al., 1994) as well as within endocytic/exocytic pathways (Beck, 1987). Such intracellular compartmentalization provides an additional line of defence protecting the tumor cells against the cytotoxic effects of chemotherapeutic drugs.

In this thesis MDR refers to the cancer cells expressing P-glycoprotein and all other mechanisms for multi-drug resistant phenotype.

Several possible mechanisms for multi-drug resistant phenotype are schematically presented in figure 1.7.
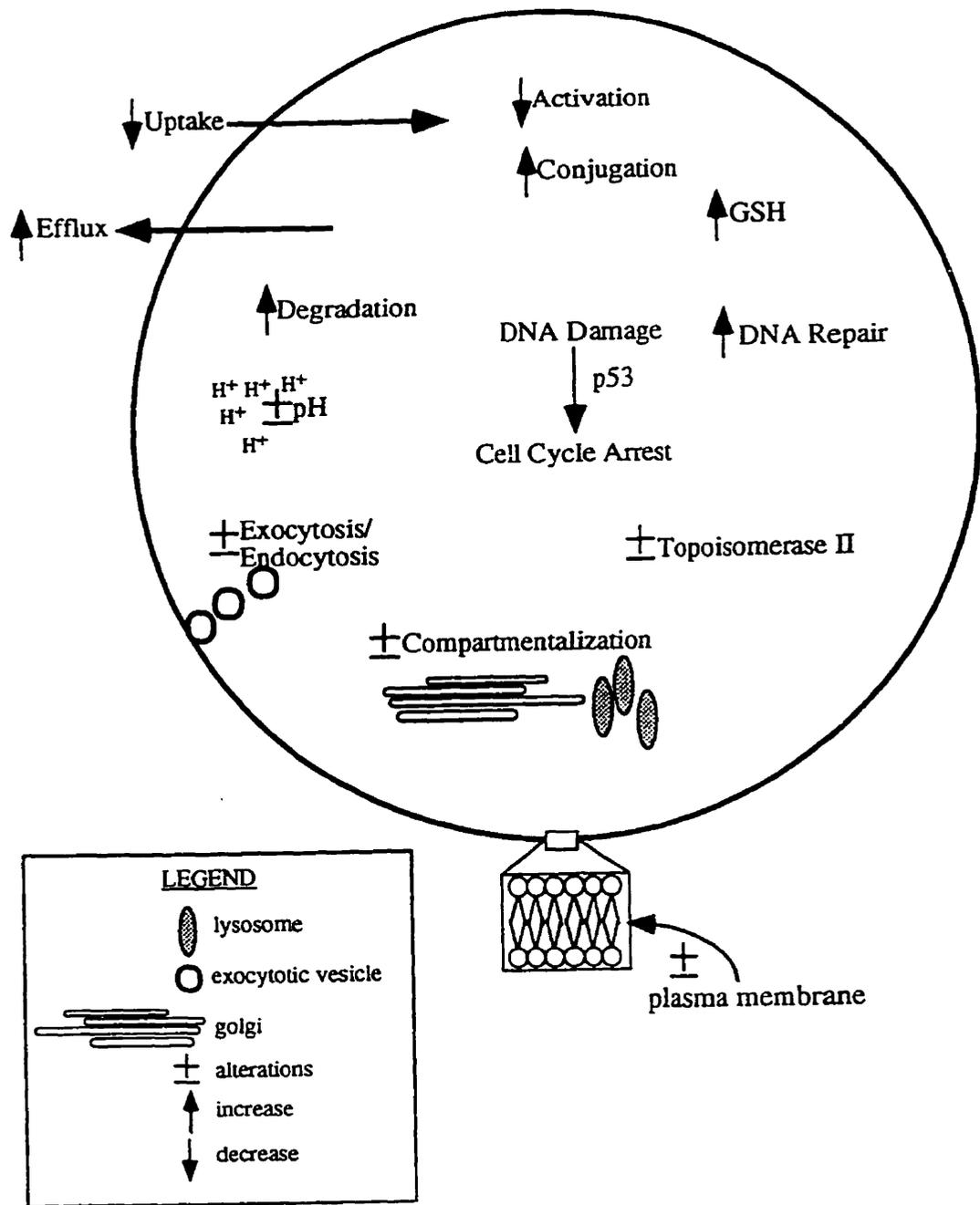
Figure 1.7   Schematic representation of several possible mechanisms for development of a multi-drug resistant phenotype

(a)　　Decreased uptake;

(b)　　increased efflux, mediated by expression of proteins such as p-glycoprotein;

(c)　　decreased mixed function oxygenase system, such as the cytochromes P450 required for the metabolic activation of xenobiotics to electrophilic reactants;

(d)　　increased conjugating systems such as, glutathione-S-transferase, or UDP-glucuronyl transferase;

(e)　　increased degradation;

(f)　　increased competing nucleophiles, such as glutathione (GSH);

(g)　　increased DNA repair capacity;

(h)　　decreased or mutations in genes like P^{53} whose products have the ability to recognize DNA damage and arrest, or to slow down the cell cycle;

(i)　　compartmentalization, including sequestration of xenobiotics in cytosolic organelles, such as Golgi or lysosome;

(j)　　decreased acidity, limiting drug protonation thereby preventing drug activation;

(k)　　alterations in the plasma membrane, leading to decreased membrane permeability;

(l)　　alterations in exocytosis/endocytosis of xenobiotics;

(m)　　alterations in topoisomerase II.

1.8　Angiogenesis

Angiogenesis is a fundamental part of a number of normal and pathological processes including wound healing, proliferative retinopathies, connective tissue disorders and neoplasia (Folkman et al., 1992; Barinaga, 1995). Growth and metastatic dissemination of solid tumors requires vascular support to provide nutrient supply (Liotta, et al., 1991; Folkman, 1992). Moreover, angiogenesis has been proposed to be the rate limiting factor for tumor growth, playing an important

role in metastasis (Folkman, 1995). The number and density of microvessels in different human cancers directly correlates with their potential to invade and produce metastasis (Weidner et al., 1991; Weidner et al., 1993). Angiogenesis was proposed as a prognostic indicator in advanced ovarian tumors and in several tumors increased vascularization has been directly correlated with poor prognosis (Hollingsworth et al., 1995). Consistent with this, highly malignant gliomas have shown higher VEGF (vascular endothelial growth factor) expression than tumors of lower malignancy (Folkman, 1995; Hatva et al., 1995). Current concepts of angiogenesis suggest a dynamic regulation involving both positive and negative modulatory influences. Stimulatory factors include TGFs, FGFs, Tumor necrosis factors and VEGF, prostaglandins E1, E2, interleukin-8, angiogenin, heparinase, hepatocyte growth factor, placenta growth factor, PDEF, and pleotropin while inhibitory molecules include angiostatin and thrombospondin, cartilage-derived inhibitor, heparinase, IFNs, platelet factor 4, prolactin fragment, protamine, and tissue inhibitor of metalloproteinase (Bouck, 1990; Fidler, 1994).

1.9 The molecular basis of ovarian cancer

Epithelial carcinomas of the ovary are thought to arise by neoplastic transformation of the surface epithelium. It has been hypothesized that with continuous ovulation, entrapment of the germinal epithelium within the stroma occurs, resulting in the formation of an epithelial inclusion cyst (Cramer et al., 1983). Subsequently, under the influence of oncogenic stimuli, this entrapped benign epithelium is thought to undergo malignant transformation (Fig. 1.8).

The findings of several investigators support the concept that certain benign and borderline ovarian tumors are indeed precursors to invasive ovarian carcinomas (Stenback, 1981; Plaxe et al., 1990).

Ovarian Surface Epithelium

Inclusion Cyst

Benign Tumor

Borderline Tumor

Invasive Tumor
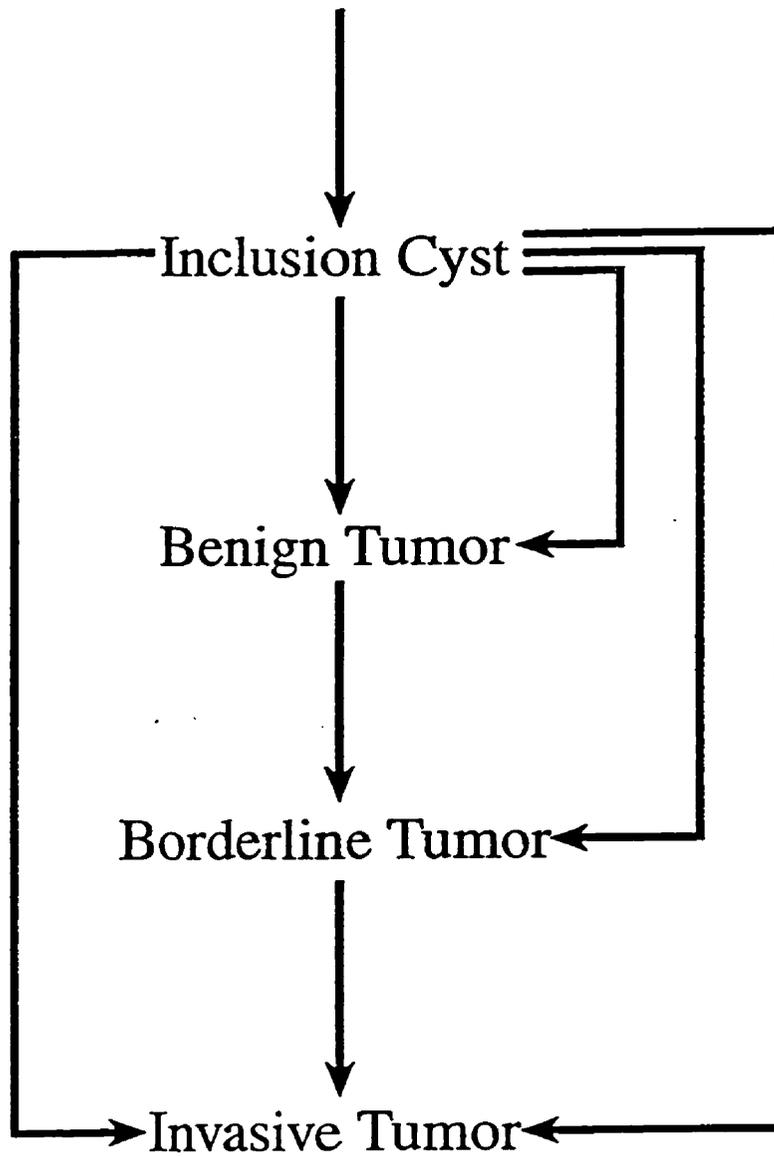
Figure 1.8   Three possible routes of ovarian carcinogenesis.

(Holly et al, 1995)

Although ovarian cancer is the leading cause of death from gynecologic malignancies (in USA), very little is known about the underlying molecular genetic mechanisms responsible for the development of this biologically aggressive malignancy (Boring et al., 1994). Of the little that is known derangements in proto-oncogenes and tumor suppressor genes appear to play a critical role in the development of ovarian cancer. Ovarian cancer occurs in both familial and sporadic forms (Barber, 1993), although much remains to be learned in this area. Oncogenes (HER-2/BRCA2, neu, K-ras), tumor suppressor genes (P[53], BRCA1), and mismatch repair genes (MSH2, MLH1, PMS1, PMS2) are reported to be altered in ovarian cancer (Holly et al, 1995).

Diagnosis at a late stage and resistance to chemotherapy rank among the most important therapeutic problems in management of ovarian cancer. Approximately half of the patients presenting with the advanced-stage disease are resistant to conventional chemotherapy (Ozols et al., 1994). Moreover, resistance to chemotherapy in these patients appears to be a complex and multifactorial problem (Perez et al., 1993). Since the introduction of the platinum chemotherapy agents twenty years ago (Barber, 1993), there has been little progress in its treatment. This is reflected in the fact that the survival rate in 1991 was only slightly better than in the past two decades (Barber, 1993). These statistics indicate the need for alternate approches. Since large scale screening for early detection of ovarian cancer is not financially feasible, the need for improved treatment becomes more vital.

1.10 The molecular basis of brain tumors

Central nervous system (CNS) neoplasms can be classified as primary and secondary tumors. Glial tumors account for about half of all intracranial tumors and malignant gliomas are the most common malignant brain tumors which are considered incurable (Kay and Laws, 1995). The infiltrative growth pattern of these

tumors precludes curative neurosurgery, and these tumors fail to respond to irradiation, chemotherapy, or immunotherapy (McCormack et al., 1992). Malignancy has been recognized to result not only from enhanced cell proliferation, but also from decreased cell death (Muller et al., 1977). Very little is known about the etiology of brain tumors. However, several associations with genetic, occupational, and environmental factors have been reported (Kay and Laws, 1995).

In considering chromosomal alterations in CNS tumors, it is important, but sometimes difficult, to differentiate primary etiological factors such as chromosomal changes from secondary alterations occurring as the result of genetic instability of cancer cells. Glioblastoma multiforme (GBM) generally shows a greater number of cytogenetic changes than other types of brain tumors. Thus, cytogenic abnormalities in human glioma can be considered a function of histological grade (Rasheed et al, 1991). The most common cytogenetic abnormalities in gliomas are gains or structural alterations of chromosome 7, and losses of chromosome 10, 22, and the sex chromosomes. The most frequently rearranged chromosomes are chromosomes 1>9>7>>>3, and 11 (Bigner, 1990). Mutation in the tumor suppressor gene, p53, located on the short arm of chromosome 17, has been reported in certain glial neoplasms, but it is still unclear whether it is an early or late event in the pathogenesis of gliomas (Sidransky et al, 1991). A multi-step theory of the pathogenesis of human glioma is presented in Fig. 1.9. Moreover, there is a spectrum of CNS tumors in which oncogenes have been activated, amplified or overexpressed (Kaye and Laws, 1995).
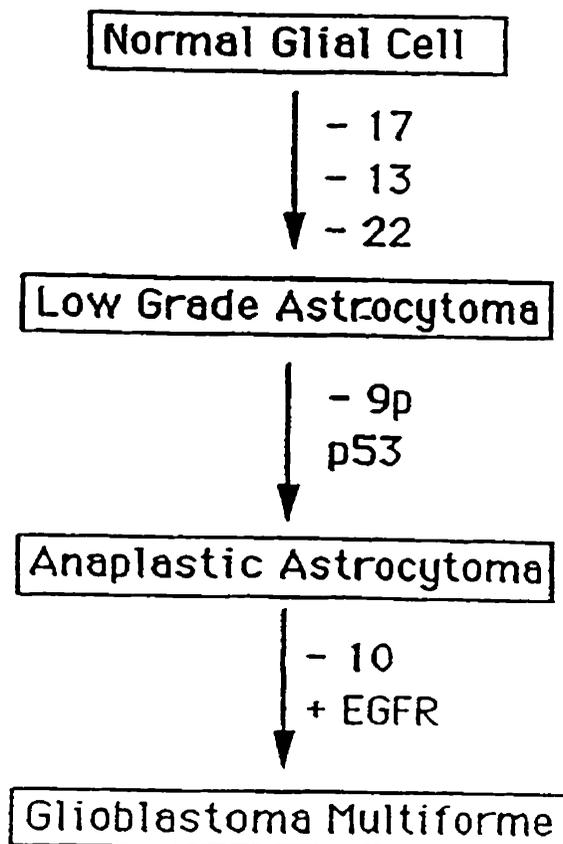
Figure 1.9   Multi-step theory of the pathogenesis of the human glioma. Early steps transforming a normal glial cell to a low grade precursor would include the loss of 17p (possibly p53 gene), 13q (possibly Rb gene), and chromosome 22. Transition of a low grade glioma to an anaplastic astrocytoma may be facilated by loss of 9p. Subsequently evolution to GBM may be the result of deletion of 10q ('glioma suppressor gene') and amplification of other oncogenes (e.g. +EGFR).

For reasons which are poorly understood, most primary brain tumors do not metastasize systemically (Cerame et al, 1985). Rather, spread of tumor occurs locally through extensions of infiltrating tumor cells into normal brain (Rutka et al, 1987; 1988).

Astrocytomas, arising from astrocytes, constitute the majority of all primary brain tumors and are the most common gliomas. Astrocytomas occur at any age, but are most common in adults (Laws et al., 1984) and can be classified into five subtypes: fibrillary, gemistocytic, protoplasmic, pilocytic, and subependymal giant-cell tumors. Almost all adult gliomas are fibrillary astrocytomas. Fibrillary astrocytomas display a wide range of growth characteristics and their classification remains somewhat controversial. Pathologically, these tumors form a continuum ranging from the low-grade, well-differentiated lesion that is barely distinguishable from normal brain, to the intermediate, moderately differentiated tumors commonly known as anaplastic astrocytomas, to the highly anaplastic, bizarre-appearing, malignant neoplasm known as glioblastoma multiforme (Burger et al., 1991). Angiogenesis is a prominent feature of anaplastic astrocytomas and is an essential feature of the histological diagnosis and grading of these tumors (Burger, et al., 1991). In patients who undergo serial biopsies over time, the evolution from well-differentiated, to anaplastic astrocytoma, to glioblastoma multiforme can be followed (Muller, et al., 1977).

Malignant astrocytomas are highly infiltrative tumors which have a marked prospensity to invade normal brain. This particular feature of astrocytic neoplasms makes them difficult to treat clinically. Despite the advent of promising adjuvant therapies and drugs which have impacted positively on patient survival in other tumor types in recent times, no such promising therapy has yet been found for the patient with a malignant astrocytoma. The median survival for patients with glioblastoma multiforme is approximately 12 months (Muller, et al., 1977; Laws, et

al., 1984; McCormack, et al., 1992). In this context, it is imperative that new therapeutic strategies continue to be explored for malignant astrocytomas.

1.11 Apoptosis

Apoptosis is an active cellular process that is involved in a variety of physiological events where rapid elimination of unwanted cells must occur. Apoptosis or programmed cell death (Kerr et al., 1993) represents the subtractive component of the physiological regulation of tissue mass (originally referred to as regressive or shrinkage necrosis) (Kerr et al., 1972), and thus is viewed as a natural counterpart of mitosis. For the proper development and maintenance of the multi-cellular organism, cellular proliferation, differentiation and programmed cell death (apoptosis) must be closely regulated. Apoptotic cells classically exhibit condensed nucleoplasm and cytoplasm, degeneration of nucleus into membrane-bound apoptotic bodies, formation of membrane blebs and a pronounced decrease in cell volume (Wyllie, 1981).

Apoptosis is physiologically triggered by extracellular signals of different nature, which appear to be cell type specific. The mechanism of cell death is still unclear. The interleukin-1β converting enzyme (ICE) family are candidates for regulators or components (or both) of the effector arm of apoptosis (Martin et al, 1995). Both positive and negative apoptotic signals exist which can, respectively induce or prevent apoptosis (Wyllie, 1981; Drago, 1990; Michael et al., 1990; Raff, 1993; Oltvai et al., 1994; Vaux et al., 1994; Mitchell, 1994; Jarvis et al., 1994; Merlo et al., 1995). Similarly, cells can be dependent on contact with extracellular matrix components for their survival (Meredith et al., 1993; Schwartzman et al., 1993; Ruoslahti et al., 1994; Frisch, 1994]. An accumulating body of evidence suggests that generation of the lipid messengers constitute an important trigger in the induction of apoptosis (Kolesnick, 1991; Hannun, 1994; Liscovitch and Cantley, 1994). Ceramide represents the first of perhaps many intracellular messengers

capable of initiating apoptosis induced by cytokine, radiation, and antineoplastic drugs (Jarvis et al., 1996). Induction of apoptosis underlies the cytocidal actions of many therapeutical agents such as antineoplastic nucleoside analogues (Gunji, 1991), topoisomerase inhibitors (Kaufmann, 1989), platinum-based organometallic compounds (Barry et al., 1990).

The data available to date argue that Bcl-2 and a family of recently discovered dimerizing proteins (Bcl-Xl, Mcl-1, Bfl-1, Al, etc.) (Sedlak, 1995) and the proteins with which they interact, (like Bax, Bad, Bak, etc.) are important regulators of apoptosis (Yang et al., 1995) and that they constitute a novel mechanism of chemoresistance in cancer (Reed, 1995).

1.12 Hypothesis and Objective of the thesis

Hypothesis

Intracellular routing of verotoxin from the cell surface to the nuclear membrane by retrograde transport, is an important signal transduction pathway which is related to its cytotoxicity. Verotoxin routing in the cell is determined by its receptor, Gb3 isoform, with shorter fatty acid chains targeting to the nuclear membrane and inducing rapid apoptosis. The specific elevation of Gb3 isoforms in certain neoplastic cells (MDR cells in particular) and activated endothelial cells which vascularize them in vivo may provide a ready system for verotoxin (VT1) to specifically target and kill receptor expressing cancer cells by inhibition of protein synthesis and extremely rapid induction of apoptosis.

Objectives

The overall objectives of the work described in this thesis were (i) to screen for verotoxin cytotoxicity on selected human cancer cell lines (ii) to determine the mechanisms of cell death and intracellular targeting of verotoxin within such tumor cells in relation to cell sensitivity and MDR and (iii) to investigate the potential anticancer activity of VT1 in human xenografts in vivo.

1.12.1 Anti-cancer activity of VT1 in vitro

A preparation of bacterial proteins with anticancer activity (ACP) from a strain of *Escherichia coli* (HSC 10) had long been shown to have antineoplastic activity in vitro and in vivo (Farkas-Himsley et al., 1975). VT1 was identified as the active component in this bacterial extract (Farkas-Himsley, et al., 1995). Thus, sensitivity of human tumor cell lines to VT1 and the mechanism of cell death was investigated. Strategies employed in this part were:

(i) to investigate the sensitivity of selected human tumor cell lines and

   MDR cell lines to VT1;

(ii) to determine the level of VT1 glycolipid receptor expression in selected

tumor cell lines and MDR cell lines;

(iii) to identify the mechanism of cell death induced by VT1; and

(iv) to measure the expression of the VT1 glycolipid receptor in human

tumor samples and to study their targeting of VT1 within the cell.

1.12.2 Studies of the basis of differential sensitivity to VT1

As outlined above it became important to investigate the basis for differential sensitivity to VT1. Since butyrate affected intracellular routing and sensitivity to VT1, it was important to determine whether differential targeting of this toxin correlates with the sensitivity of a given tumor cell to VT1 treatment and to investigate the molecular determinants involved in differential targeting of the toxin inside the cell lines. Strategies applied in this section were:

(i) to determine the targeting of VT1 and methods of altering its targeting

inside the tumor cell with respect to VT1 sensitivity; and

(ii) to examine the heterogeneity of receptor fatty acids and its contribution

in toxin targeting.

1.12.3 Study of the anticancer activity of VT1 in vivo.

The last objective of these studies was to address the feasibility of VT1 therapy in human tumors by determining the effect of VT1 treatment on human astrocytoma and ovarian tumor xenografts in nude mice. Since the cell lines from two aggressive human tumors; ovarian and astrocytoma without a known effective therapy are used in previous studies, the same tumor cell lines were used for representative tumor inoculation in nude mice and examined for their response to VT1.

Chapter 2

Materials and Methods

2.1   Verotoxin purification

Recombinant VT1 from pJLB28 (Petric et al., 1987), VT2 from R82, and VT2c from E32511and VT1 B subunit (Ramotar, et al., 1990) were purified by a recently developed affinity chromatographic technique (Boulanger et al., 1994). VTs were aliquoted in PBS and stored at -70° C. The appropriate dilution for the treatment of cell lines was prepared freshly in media (α-MEM or RPMI) and added to the cells.

2.2   Verotoxin cytotoxicity

Verotoxin cytotoxicity was measured by adding increasing doses of VTs (VT1, VT2 and VT2c) or B subunit (0, 0.1, 0.5, 5, 50, 100 ng/ml) to confluent cell monolayers in microtitre wells in triplicate.   Cell survival was monitored after 72hr (incubation at 37 C°, 5% CO2) by staining with 0.1% crystal violet and measuring the optical density at 570nm using a Dynatek microtiter plate reader (Kueng et al., 1989). Control cells under the same conditions without VT treatment were used for comparison and calculation of the cells sensitivity to VTs. The absorbance of control cells was taken as 100% live cells. Percentage of viable cells were plotted against the various concentrations of VTs or B-subunit. This method under estimates VT cytotoxicity, since some of the dead cells or their debries may stain with crystal violet and represent as viable cells.

2.3   Glycolipids and Gb₃ Extraction from Human tumor tissues

Frozen tumour tissue samples were thawed, weighed, homogenized in a minimum volume of PBS and extracted overnight with 20 volumes of 2:1 chloroform:methanol (v/v). The suspension was filtered and the tissue was re-extracted using 10 volumes of 1:1 chloroform: methanol. The combined extracts were partitioned against water (Folch et al., 1957). The resulting upper phase was partitioned again against theoretical lower phase consisting of 86:14:1 (v/v/v) chloroform: methanol: water. The combined lower phases were dried down and

resuspended in 1 ml of 98:2 chloroform:methanol. The samples were then loaded onto silica A columns and washed with 3 column volumes of chloroform and eluted with 10 volumes of 9:1 acetone: methanol. The acetone:methanol fraction containing neutral glycolipids was then dried and resuspended in 0.1 - 0.5 ml of 2:1 (v/v) chloroform: methanol and stored at -20 °C.

2.4   Gb3 quantitation

Gb3 extracted from tumor samples was quantitated with orcinol and VT1 overlay on TLC against various concentrations of known Gb3 standards by visual comparison. These Gb3 standards were calibrated by HPLC.

2.5   Binding of VT1 to the Gb3 by TLC Overlay

Glycolipid from human tumor samples or purified Gb3 from tumor cell lines were separated by TLC (chloroform, methanol, water-65:25:4 (v/v/v). The plates were dried and blocked with 1% gelatin in water at 37 °C overnight. They were then washed three times with 50 mM TBS for 5 min and incubated with 0.1mg/ml toxin for 1 hour at R.T. After further washing with TBS, plates were incubated with mouse monoclonal anti-VT1 antibody (2 mg/ml) for VT1 or rabbit polyclonal anti-VT2c antiserum, followed by peroxidase-conjugated goat anti-mouse antibody or peroxidase conjugated goat anti-rabbit polyclonal antibody (1:2000). Finally, the plates were washed with TBS, and toxin binding was visualized with 4-chloro-1 naphthol peroxidase substrate. A similar plate was prepared and sprayed with orcinol for comparison (Lingwood, et al., 1987).

2.6   Cell lines and Cell Culture Conditions

Four cervical cancer cell lines (SIHA, ME180, CASKI, and HT-3) were kindly provided by Dr. R. Hill. Six permanent human malignant astrocytoma cell lines (SF-126, SF-188, SF-539, U 87-MG, U 251-MG, and XF-498) selected for this study

(Westermark et al., 1973; Ponten et al., 1986; Rutka et al., 1987) were kindly provided by Dr. J. Rutka. All the cell lines except XF-498 (which was grown in RPMI media) and SKVLB (grown in the presence of 1μg/ml vinblastine) and SKVLC (grown in the presence of 0.1μg/ml vincristine), which are multiple drug resistant variants of the parental SKOV3 ovarian carcinoma cell line, kindly provided by Dr. Helen Chen (Bradley et al., 1989) were cultured in alpha-MEM, nonessential amino acids, glutamine, gentamycin, and 10% heat-inactivated fetal bovine serum (FBS). The cultures were incubated at 37°C and equilibrated with 5% CO_2 and air. Cells were harvested with 0.25% trypsin in Ca^{++}- and Mg^{++}-free Hank's balanced salt solution and were subcultured weekly. Astrocytoma cell line, SF-539 and ovarian carcinoma cell line, SKOV3 were used for tumor inoculation in nude mice. Cell lines were *Mycoplasma* free.

2.7   Tumor Inoculation and Verotoxin treatment

Cells in their exponential growth phase, were harvested using 0.05% trypsin for 2 min at 37C°. The trypsin was neutralized with media containing 10% FCS and centrifuged at 800x g to obtain a pellet. Cells were resuspended in sterile media at concentration of 5 $x10^7$ cells /ml. Six to eight week-old, athymic nude mice(CD1, nu/nu; Charles River Breeding Laboratory, Canada) were kept in sterile isolators under specific pathogen-free conditions at the animal facility of The Hospital for Sick Children in Toronto. Each athymic nude mouse was given an injection of 0.2 ml of cell suspension (1 $x10^7$ cells) subcutaneously (s.c.) on the back (to facilitate the monitoring and measuring of the tumors) with a 25-gauge needle. Mice were housed in a closed colony and provided sterile food and water. Animal health and tumor growth were monitored daily. Mice were tagged and tumors were measured with callipers twice a week. Mice with tumors of 50 mm^2 or larger (completely visible tumor) were injected i.t.(intratumor) with VT1 at two concentrations of 4 or 8 μg/Kg mouse in 50 ul volume of   sterile PBS using 27 gauge needle.

2.8 ^{125}I-Radiolabeling of VT1/VT1B

Purified toxin (50-100 ug) was iodinated using Iodo-beads (Pierce). Two iodo-beads were incubated with 0.5 mCi Na^{125}I (Amersham) and 100 mg of VT1 or VT1-B in 500 ml of 0.1 M sodium phosphate, pH 7.2 for 10 minutes. Labelled VT1 was separated from unreacted iodine using a G-25 column equilibrated with PBS (?).

2.9 ^{131}I-Radiolabeling of VT1 B

B subunit of VT1 was labelled by Na^{131}I using the iodogen technique (Fraker and Speck, 1978). Briefly, 20 mg of iodogen was dissolved in 2.0 ml chloroform and further diluted to 1 mg/ml. A 50-ul aliquot of pure VT1 B-subunit was incubated with 5mCi of ^{131}I-sodium iodide in 100ul and labelling allowed to proceed for 10 mins. The labeled VT1 B-subunit was separated by a PD-10 column using 3.5 ml 1% HSA in saline as eluant. The labeled VT1-B was collected and activity assayed in a dose calibrator. The labeled VT1 subunit was used within 2 hrs of preparation.

2.10 ^{125}I-Verotoxin binding of Intact Cells

Cells were grown on 96 well plates (Falcon). Media was aspirated, and the cells were washed with PBS (pH 7.4) at room temperature. After equilibrating the cells on ice, 100uL of ice-cold PBS was added to each well. The radiolabelled toxin was then added and allowed to incubate for one hour on ice. The unbound toxin was then washed off with ice-cold PBS 3 times. The cells were solublized with 1 ml of 10% SDS added to each well and incubated at 37°C for 15 minutes. The contents of each well were then placed into gamma counter tubes and the amount of radioactivity associated with the cells was measured.

2.11 Radiolabeled VT1-B subunit Administration to Nude Mice and Biodistribution.

Before animal administration the ^{131}I-labeled toxin subunit solution was filtered through a 0.2 um sterile filter. The radiolabeled VT1 B was used within 2 hrs of preparation and delivered by i.p. injection in a total volume of 0.2 ml in PBS to nude mice carrying s.c. xenotransplants of human ovarian tumor. The normal tissues and the tumor xenograft were excised, weighed, and assayed for content of radioactivity in gamma counter (Reilly et al. 1993).

2.12 Whole-body gamma-camera imaging of nude mice

s.c. implanted ovarian tumor-bearing mice injected i.p. with ^{131}I-VT1 were sacrified postinjection and whole-body imaging was performed using gamma-camera.

2.13 Fluorescein isothiocyanate labeling of verotoxin

The VT1 or VT1-B was added to FITC, 1:1 (w/w; 1 mg/ml) in 0.5 M carbonate/bicarbonate buffer at the final pH of 8.5 to 9. Reaction was allowed to proceed for 1 hour with gentle rotation (Johnson and Holborow, 1989). Unreacted FITC was removed by dialysis and SDS-PAGE was performed to check the labeling. All procedures were done at 4 C°.

2.14 DABCO for anti-fading of fluorescent slides

0.22M DABCO (1,4-Diazabicyclo-Octane, Sigma) was prepared by solubilizing 2.5 g of DABCO in 90 ml of glycerol in a 37°C water bath for approximately 1 hour before adding 10 ml of 0.15 M NaCl containing 0.5 M carbonate-bicarbonate buffer, pH 8.6 (pH 8.6 is optimum for FITC fluorescence emission) (Krenik et al., 1989). DABCO solutions were stored at 4°C.

2.15 Fluorescent labeling of tumor ascites cells

The cells were pelleted (at 800 rpm, 4 min.) and transfered to Eppendorf tubes and cells were chilled on ice. After one wash with 1 ml cold PBS, cells were then pelleted at 800 rpm, 4 minutes. First incubation was done on ice for 30 min. RITC verotoxin-B-subunit was diluted to a concentration of approximately 5µg/ml. After gentle resuspension of the cells in the RITC-VT1B containing solution, cells were incubated for 1 h (protected from light during the incubation). After three ice-cold PBS washes, cells were mounted on the slide in 1 drop of DABCO, sealed and viewed using a fluorescent microscope.

2.16 FITC-VT1 B subunit staining of tumor sections

Samples of surgically removed ovarian and brain tumors or metastasis were embedded in OCT embedding compound, flash frozen in liquid nitrogen, sectioned and stored at -70 °C. Serial 5 µM cryosections of samples were thawed, dried, blocked with BSA and stained with FITC-VT1 B, in PBS (2 µg/ml) containing 0.1% BSA, for 1 hr in a humidifier chamber at room temperature. Sections were extensively washed with PBS, mounted with mounting media with antifading agent DABCO (Krenik, et al., 1989), and observed under incident uv illumination. If present, background autofluorescence was subtracted by computer graphics, from the FITC-VT1 B staining shown. Two adjacent serial sections were used, as a control for autofluorescence and stained with hemotoxylin/ eosin for comparison.

2.17 Fluorescence and Confocal Microscopy

To determine the subcellular targeting of VT1 in live tumor cells, astrocytoma and ovarian cell lines, SF-539, XF-498, XF+, SKOV3, and SKVLB, grown on cover slips were incubated for 1h in the presence of 10 ng/ml of FITC-VT1 at 37°C for VT1 internalization. Following extensive washing with 50 mM PBS, the cells were mounted with DABCO and examined with a fluorescence

microscope at a fluorescence excitation wavelength of 493.5 nm. Fluorescent images were recorded on Kodak TMAX 400 ASA film. For confocal microscopy, samples labeled as above were analyzed using a laser confocal microscope with 40x objective. A krypton/argon laser tuned to produce both 488 nm and 565 nm wavelength beams, was used for fluorescein and rhodamine excitation, respectively. The dual filter block, K1K2 allowed the simultaneous monitoring of the fluorescein and rhodamine emission for the simultaneous visualization of the two different molecules. The signals were recorded by optical sections of 1um thickness. Diaphragm and fluorescence detection level were adjusted to avoid cross-emission from the two channels (FITC and RITC). Pictures were recorded with a Kalman filter (average of six images) and transferred to a Kodak T-max 400 film.

2.18 Lysosomal Labeling

Internalization of FITC-dextran was used as a lysosomal marker. Cells grown overnight on cover slips were incubated with 0.5 mg/ml media FITC-dextran for 24 hrs at 37° C and were chased with normal media (a-MEM) for 2 hrs at the same temperature. RITC-VT1B (2-5 ug/ml)was added to the cells in the last 1 hr of chase. The cells were then washed five times with sterile PBS, fixed in 2% formaldehyde and mounted with DABCO and visualized under incident fluorescent light in a Polyvar fluorescent microscope.

2.19 Electron Microscopy

Targeting of VT1 in SF-539 and XF-498 cells was examined in detail by transmission EM. Cells were cultured on a transferable membrane (Falcon) inserted in 24 well tissue culture plates (Falcon) and allowed to form a confluent monolayer. For surface binding, the cells were incubated at 4°C in the presence of 5ug/ml VT1B for 30 min and washed with cold PBS. Then, cells were incubated

with anti-VT1B primary antibody followed by GAM-gold secondary antibody for 30 min at 4°C. For toxin internalization studies, cells were incubated with VT1B(5 ug/ml) at 37°C for 1 hr. Following either treatment, the cells were fixed in 2.5% gluteraldehyde, 2% paraformaldehyde in PBS for 30 min at room temperature. After washing with PBS, cells were postfixed in 1% osmium tetroxide (OsO4) in phosphate buffer for 30 min and washed with saline. The cells were further postfixed with 2% uranyl acetate in 30% ethanol for 15 min (Pulczynski et al., 1993), and dehydrated in graded ethanols from 50% to 100%, 10 min each for two times. Dehydrated cells were serially infiltrated with 50% to 75% Epon in 100% ethanol for 30 min each and finally with 100% Epon for 1 h with 2 changes of Epon. The membrane was removed from the holder and inserted vertically into a gelatin capsule filled with 100% Epon and polymerized at 65° C.

After sectioning of the polymerized block on an ultramicrotome, the sections for surface binding studies were ready for counterstain while the sections for studies of internalization were immunolabeled. These sections were treated with saturated sodium metaperiodate for 30 min to unmask antigenicity (Stirling et al., 1995) and washed in distilled water. The sections were next blocked by floating on a drop of 0.1% BSA, 0.2% fish skin gelatin in 50 mM TBS (150mM NaCl, 50 mM Tris, pH 7.4) for 30 min and immunolabeled with 1:50 dilution of anti-VT1B antibody, followed by 1:50 dilution of GAM-15 or -10 nm gold for 1 h each at room temperature, with thorough washing after each step. Finally, the sections were counterstained with 5% uranyl acetate and then with Reynold's lead citrate for 6 min each and analyzed with a Philips 300 EM microscope at 60kV. Photographs were taken with fine grain positive film (Kodak) and printed on Polycontrast III RC (Kodak) at X120, magnification.

2.20   Ultrastructural Analysis of VT-treated Astrocytoma Cells

Cells were cultivated on a transferable 9 mm cyclopore membrane (0.45um pore size, Falcon) to form a confluent monolayer and were incubated at 37°C with VT1 (10 ng/ml). The cells were fixed at room temperature by addition of 1.6% glutaraldehyde to the well and then incubated in 0.066 M Sorensen buffer (pH 7.4) containing 1.5% glutaraldehde for 1 h at 4°C. After 2 h of washing with 0.1 M phosphate buffer, the cells were post-fixed in 2% osmium tetroxide in the same buffer.   After dehydration in graded ethanols and propylene oxide, Epon embedding, thin sectioning and uranyl-lead counterstaining on grids were performed. Thin sections were examined in a Philips EM 400 electron microscope and ultrastructural features of apoptosis was analysed (Falcieri et al., 1994; Anderson et al., 1994).

2.21   Nuclear staining with propidium iodide

SF-539 cells grown on cover slips overnight were incubated at 37 C° with VT1B-subunit (50 ng/ml) for 1.5 hrs or 10 hrs and fixed (with 1% paraformaldhyde for 3 minutes), permeabilized with 0.1% Triton X-100 in 100 mM PBS for 5 min, and stained with 5 μg/ml propidium iodide (Sigma). After extensive washing with 50 mM PBS, the fixed cells were mounted with DABCO (1,4-Diazabicyclo-Octane, Sigma), and nuclear staining observed under incident uv illumination.

2.22   Flow Cytometry

Apoptosis of astrocytoma cells, incubated with 10 ng/ml of VT1 for 24 - 36 hrs in the presence of 10% bovine fetal serum was analyzed by flow cytometry on an Epics Profile Analyzer (Coulter Electronics, Pathology, University of Toronto) according to published procedures (Tylor, 1990). After treatment, cells were trypsinized and centrifuged at 200g. To remove RNA prior to staining, cells were treated with 100 μl of 200 μg/ml DNase-free RNase A at 37° C for 30 min. The cell

pellet was suspended in 1ml of a hypotonic fluorochrome solution containing 50 ug/ml propidium iodide (Sigma) and stained for 30 min at 4° C. Cell cycle distribution was determined using the Coulter software. Flow cytometric quantitation of apoptotic cells within the propidium iodide- stained population was performed as described (Nicoletti et al., 1991). Debris and dead cells were excluded on the basis of their forward and side angle light-scattering properties. A standard was run before each experiment and astrocytoma cells grown simultaneously in the absence of VT1 served as controls.

2.23 TUNEL histochemistry

The TUNEL assay was performed using a modified procedure from Gavrieli et al, (1992). Five μm sections were cut from formalin fixed, paraffin embedded tissue blocks and mounted on coated slides (Fisher Scientific). The sections were deparaffinised through xylene and ethanol, washed, and then treated with proteinase K (Sigma), followed by further washing and blocking of endogenous peroxidase with 2% hydrogen peroxide (Sigma). After preincubation with terminal transferase buffer, the sections were incubated with digoxigenin-11-deoxyuridine triphosphate (dUTP) (Boehringer). The reaction was terminated with a solution of sodium chloride, sodium citrate and water. Detection of incorporation of dUTP was achieved by anti-digoxigenin staining and the section were counter stained with hematoxylin.

2.24 Cell sensitization to VT by butyrate treatment

XF-593 astrocytoma, ovarian and Vero cell lines were treated with Sodium butyrate at a dose of 2 mM. 50 μl of Sodium butyrate solution of 200 mM concentration, which was filtered through 0.2μ Millipore filter was added to 5 ml media (a-MEM or RPMI with 10%FCS) containing cells and incubated at 37°C. The

incubation time varied for different cell lines (e.g. 6-7 days for XF-498, 1 and 2 days for SKOV3 and Vero cells respectively).

2.25 Inhibition of glycolipids synthesis

XF-498 astrocytoma cells were incubated in the presence or absence of 2mM butyrate with various concentration of PPMP to determine the effective concentration of PPMP which is able to inhibit glycosphingolipid synthesis without inducing cell death, which was found to be at 27 µM. The cells were then incubated at 37°C with 27 µM of PPMP in the presence and absence of 2 mM butyrate for 6-7 days.

2.26 Analysis of Gb₃ Fatty Acid Content (FAME)

Analysis of fatty acids was performed as described by Myher et al., (1989). Briefly 1 ml of H_2SO_4:MeOH (6:94, v/v) was added to the lipid sample and heated at 80° C for 2h. After cooling the sample, 2 ml of hexane and 0.5 ml of 3M aqueous ammonia were added. The sample was vortexed vigorously, centrifuged, and passed through a prewashed pipette containing glass wool and anhydrous Na_2SO_4. The sample was dried under N_2 and redissolved in a minimum volume of hexane. The FAME (fatty acid methyl ester) was determined by Gas Liquid Chromatography using a 15 m x0.32 mm ID capillary column coated with sp 2380 (Supelco) in order to determine the percentages of fatty acid content in each Gb₃ sample. The carrier gas was hydrogen at an inlet pressure of 3 Psi. The sample was injected into a split injector at 260° C (split ratio= 7:1). The column was held at 100° C for 5 min. and then raised to 130°C at 20°C /min and finally to 240 ° C at 5° C/ min.

2.27 Preparation of the Data

Each experiment was repeated two to four times, and representive experiments are presented in the tables and figures. Values in the figures are the means of triplicate analyses with standard deviation given.

2.28 List of Materials   Source

Acetone	Caledon
Bovine serum albumin (BSA)	Sigma Chemical Co., St Louis, MO
chloroform	Caledon
4-chloro-1-naphthol	Sigma Chemical Co., St Louis, MO
DABCO (1,4-Diazabicyclo(2,2,2)Octane)	Sigma Chemical Co., St Louis, MO
dUTP	Boehringer
Epon 812	Polysciences Inc., Warrington, PA
Fetal calf serum	GIBCO, Grand Island, N.Y.
FITC	Sigma Chemical Co., St Louis, MO
FITC-dextran	Molecular Probes Inc
FITC-labelled VT	Lingwood's Laboratory
GAM-gold	Zymed Laboratories Inc.,SanFrancisco
Glutaraldehyde	Polysciences Inc., Warrington, PA
Goat anti-mouse IgG	Sigma Chemical Co., St Louis, MO
Goat anti-rabbit IgG	Bio-Rad
Methanol	Caledon
MEM	Sigma Chemical Co., St Louis, MO
Microtitre plates (96 well, flexible)	Falcon
OCT compound	Miles laboratories
orcinol monohydrate	Sigma Chemical Co., St Louis, MO
osmium tetroxide	Polysciences Inc., Warrington, PA

Phaloidin-RITC	Molecular Probes, Inc.
PH1 monoclonal anti-VT1	Lingwood's laboratory
paraformaldehyde	Polysciences Inc., Warrington, PA
PPMP	Matreya Inc., Pleasant Gap, PA
Proteinase K	Sigma Chemical Co., St Louis, MO
RPMI 1640	Sigma Chemical Co., St Louis, MO
RITC	Sigma Chemical Co., St Louis, MO
Sodium butyrate	Sigma Chemical Co., St Louis, MO
Sodium Capronate	Sigma Chemical Co., St Louis, MO
Sodium Propionate	Sigma Chemical Co., St Louis, MO
Thin-layer chromatography plates	Brinkmann
Trypsin	Gibco, Santa Clara, CA
VT1 and VT2c	Lingwood's laboratory
VT1 B subunit	Lingwood's Laboratory
Texas Red-con. A	Molecular Probes Inc
uranyl acetate	Polysciences Inc., Warrington, PA

Chapter 3

Identification of human tumor cell lines sensitive to VT1

3.1 INTRODUCTION

Bacteriocins are bacterial proteins produced to prevent the growth of competing microorganisms in a particular biological niche. An anti-neoplastic activity in an extract of E coli (HSC$_{10}$) was first described in 1975 (Farkas-Himsley, et al., 1975). Since that time, significant effort has been placed in attempts to purify the active component (Farkas-Himsley et al., 1985) but without consistent results. This bacteriocin preparation or ACP (anti-cancer proteins) was found to be active against a variety of human tumors in vitro (Farkas-Himsley, 1976; 1980) and a mouse metastasis model in vivo (Hill et al., 1991). HSC$_{10}$ E. coli is a Verotoxin producing strain, and it was found that VT1 is the active anticancer component in ACP and was able to mimic in vitro and in vivo the anticancer activity of ACP (Farkas-Himsley, et al., 1995). The present studies were designed to investigate the cytotoxic effect of VT1 and VT1B in vitro and to find a model system to investigate molecular mechanism of VT activity in cancer cells [Chapter 4].

(Parts of this chapter were previously published in Proc. Natl. Acad. Sci. USA. 92: 6996-7000, July 1995.)

3.2 RESULTS

The cytotoxic activity of VTs in vitro, was tested for eight cell lines in this chapter. These cell lines included human ovarian tumor cell lines and MDR variants, cervical cancer cell lines, and B-lymphocytes infected with Epstein Barr virus (high level of Bcl-2 expression). Experimental details are described in Chapter 2. All the cell lines tested for VT sensitivity were at confluency prior to VT addition.

3.2.1 Study of VTs cytotoxicity on human Ovarian carcinoma and MDR cell lines

To demonstrate the anti-cancer activity of VT in vitro, it was tested against ovarian carcinoma (SKOV3) and two MDR variants (SKVLB, SKOVC). Interestingly, it was found that multi-drug resistant variants of ovarian tumor cell lines were markedly more sensitive to VT1 (or VT2 or VT2c,) cytotoxicity in vitro than the drug sensitive parental cell line (Fig. 3.1A). This elevated sensitivity to VTs was consistant with elevated levels of receptor, Gb3 expression (Fig 3. 2). Other members of VT family, VT2 and VT2c showed significant levels of cytotoxicity (Figs. 3.1B & C), however VT1 was more effective than other members.

3.2.2 Expression of VT1 receptor glycolipid in human ovarian carcinoma and MDR cell lines

Receptor expression was determined by extracting the neutral glycolipids of the least (SKOV3) and the most (SKVLB) sensitive human ovarian cell lines, and was assayed by VT1 TLC overlay. Both cell lines show significant level of receptor expression (Fig. 3.2) SKVLB, the MDR variant expressed the highest level of VT1 receptor, and a prominent lower band, representative of the shorter fatty acid chain in Gb3, is depicted in Fig. 3.2.

3.2.3 SKVLB sensitivity to α-interferon

In comparison to SKOV3 cells, SKVLB the MDR variant of SKOV3 showed significant sensitivity to treatment with interferon-α (Fig. 3.3). Gb3 has been implicated in interferon signaling (Cohen, Hannigan et al., 1987; Ghislain, Lingwood et al., 1994). The specific isoform of Gb3, highly expressed in SKVLB cell can be responsible for its hypersensitivity to VT and its sensitivity to interferon. SKOV3 cells did not demonstrate any sensitivity to interferon at this dose level (data not shown).

3.2.4 Photomicrograph of SKVLB cell after VT1 treatment

Photomicrographs of SKVLB cells before and after VT treatment are presented in Fig. 3.4 as a sample illustrating the response of sensitive MDR ovarian tumor cells (SKVLB) to the cytotoxic effect of VT1. Panels B and A show confluent SKVLB cells before and after VT1 treatment respectively.

3.2.5 Cytotoxicity of VT1 on human cervical cancer cell lines.

Human cervical carcinoma cell lines, SIHA, ME180, CASK1, AND HT-3 were tested for VT1 sensitivity. SIHA, with a CD50 of 0.5 ng/ml and ME-180 cells with a CD50< 0.05 ng/ml, showed significant sensitivity to the cytotoxic effect and two other cell lines, HT-3 and CASKI cells were not sensitive (Fig. 3.5). HT-3 cells did not respond to sensitization with butyrate treatment at a concentration of 2 mM (data not shown).

3.2.6 Cytotoxicity of VTs on human White Blood cells infected with Epstein Barr virus.

Human B-lymphoma cells infected with Epstein Barr virus were tested for sensitivity to VT1. These cells, with elevated level of Bcl-2 expression (Dr. Dosch, Hospital for Sick Children, Toronto), were significantly sensitive to cytotoxic effect of VT1. Bcl-2 is an antiapoptotic protein (Vaux, 1993; Oltvai and Korsmeyer, 1994) and VT action is independent of Bcl-2 expression (personal communication, Joelle Wiels). This issue will be addressed more fully in Chapter, 6. All VT members (VT1, VT2, and VT2c) demonstrated cytotoxicity in these cells, however, as depicted in Fig. 3.6, VT1 was the most potent member of VTs, with CD50 of VT1, VT2, and VT2c being >0.5, >5, and <50 ng/ml respectively.
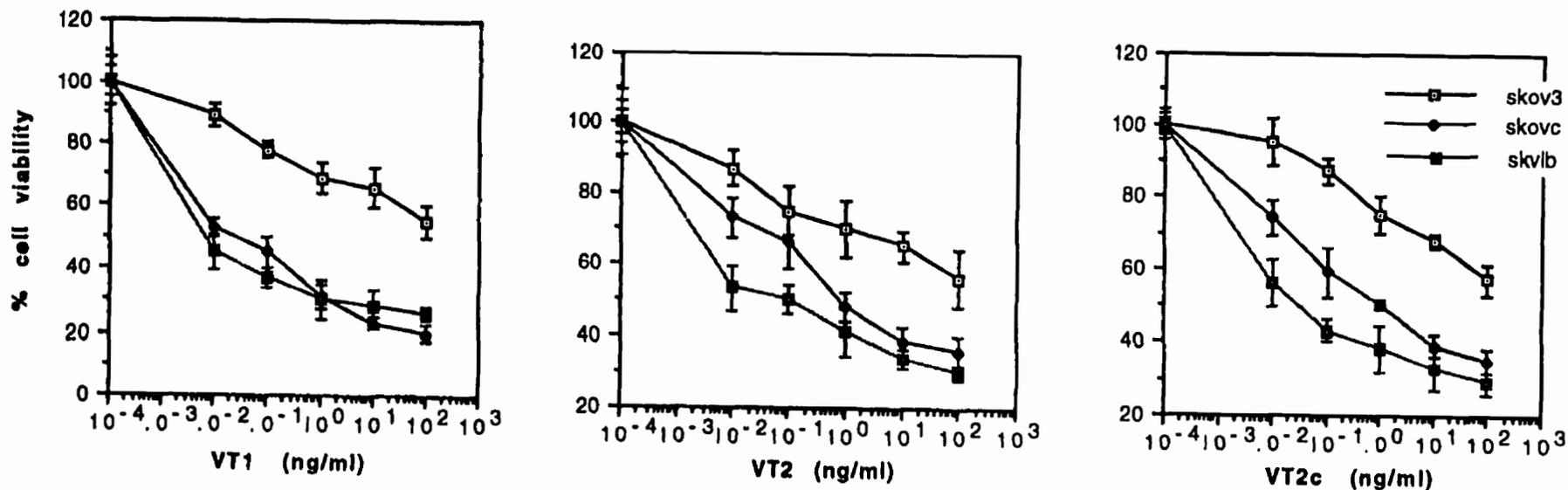
Figure 3.1   Cytotoxicity of VTs on human Ovarian carcinoma and MDR cell lines

Human ovarian carcinoma cell line, SKOV3 and MDR variants cell lines were seeded in 96-wells plates overnight. Cells were treated with serial dilutions of VTs in media, incubated for 3-days and cytotoxicity was determined as described in Chapter 2. SKVLB and SKVLC, the multidrug resistant variants were grown in presence of 1ug/ml vinblastine & .1 ug/ml vincristine respectively. Fig 3.1A, 3.1B and 3.1C represent the cytotoxic effect of VT1, VT2 and VT2c respectively.
Each value represents the mean ± S.D. of triplicate wells.
This experiment was repeated three times with a similar pattern of results.
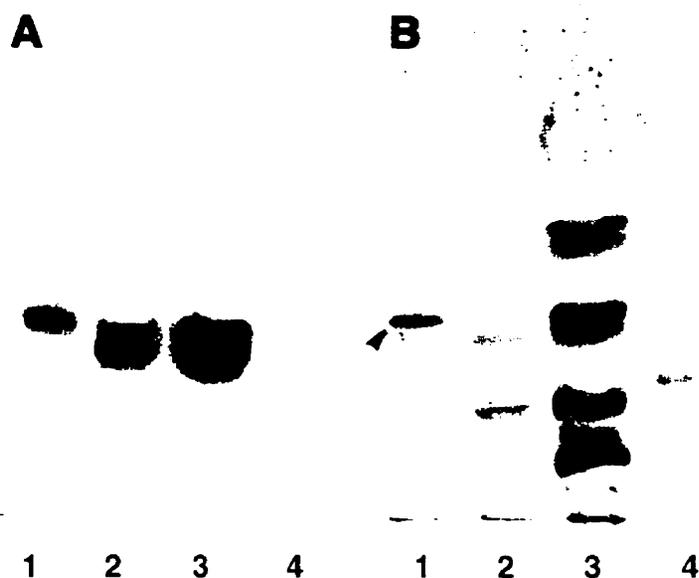
Figure 3.2   VT Receptor expression in human Ovarian tumor and MDR cell
lines

Gb3 expressed on human ovarian carcinoma (SKOV3) and MDR (SKVLB)
cell lines was determined, using the method outlined in Chapter 2. SKVLB, cells
expressed the higher level of VT1 receptor, and a prominent lower band
representative of a shorter fatty acid chain in Gb3. Gb4 (1 ug, lane 4) was used as
negative control and Gb3 (1ug, lane 1) as positive control band is depicted by arrow
head in orcinol (B) and VT1 overlay (A) in tlc plate respectively. Lanes 2 and 3
show glycolipid from SKOV3 and SKVLB cells respectively.
This experiment was repeated four times with a similar pattern of results.
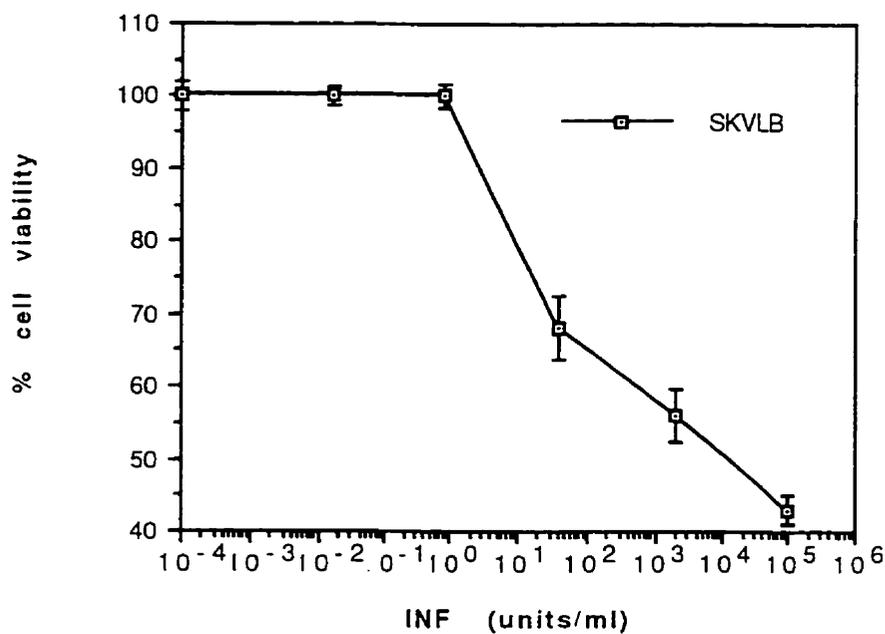
Figure 3.3   SKVLB sensitivity to α2-interferon

Same experimental procedure as outlined in Fig. 3.1 was used to assess the sensitivity of SKVLB to α2-interferon and showed significant sensitivity.
Each value represents the mean ± S.D. of triplicate wells.
This experiment was repeated twice with a similar pattern of results.
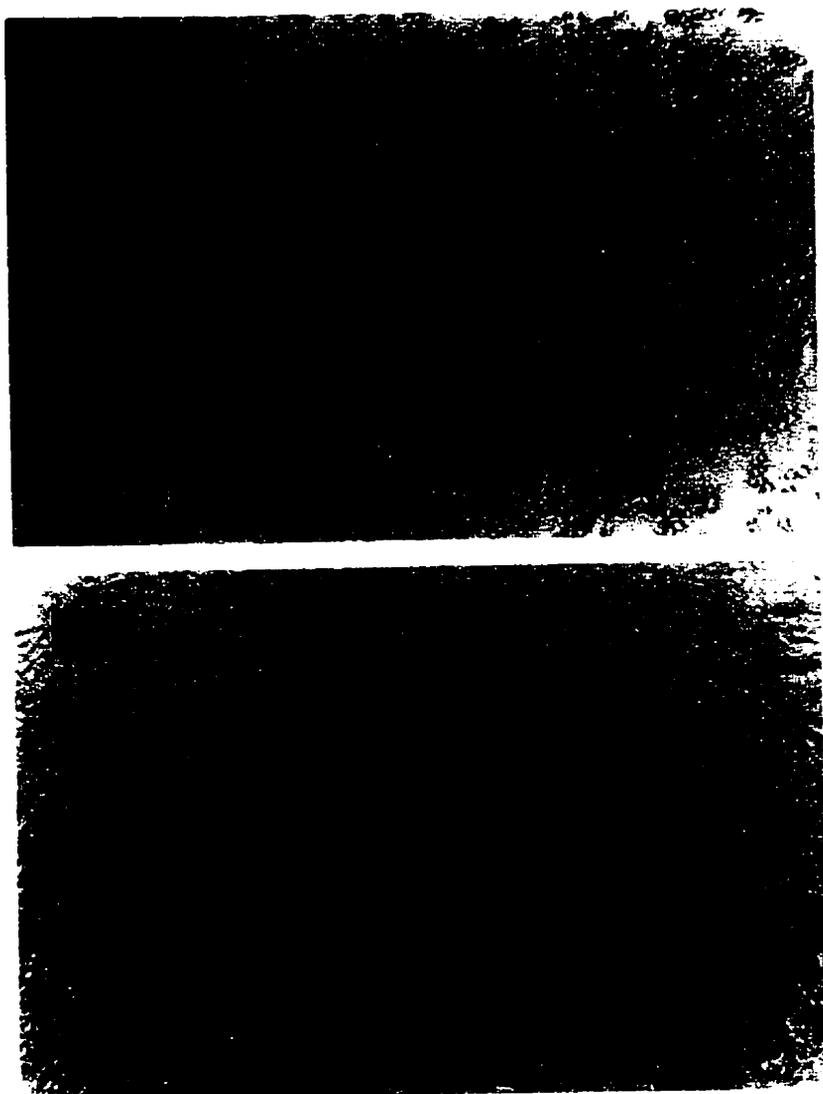
Figure 3.4   Light microscopy of SKVLB cell after VT1 treatment

Photomicrographs of SKVLB cells before (B) and after (A) VT1 (100 ng/ml, 37°C, 3-days) treatment are shown. Panel A, demonstrates cell death after VT1 treatment and panel B, shows confluent SKVLB cells before VT1 treatment.
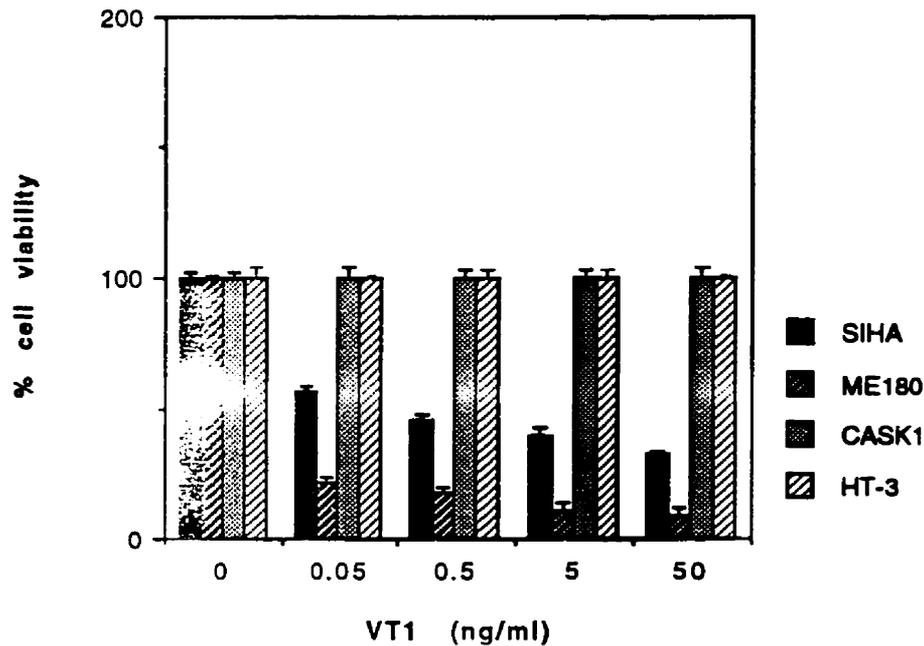
Figure 3.5   VT1 cytotoxicity on human cervical cancer cell lines.

Human cervical cancer cell lines were tested for VT1 sensitivity. Two cell lines, SIHA and ME-180 showed significant sensitivity to VT1 cytotoxicty, with a CD50 of 0.5 and <0.05 ng/ml respectively. Two other cell lines, CASK1 and HT-3 were not sensitive.

Experimental details are as outlined in legend to Fig. 3.1.

Each value represents the mean ± S.D. of triplicate wells.

This experiment was repeated twice with a similar pattern of results.
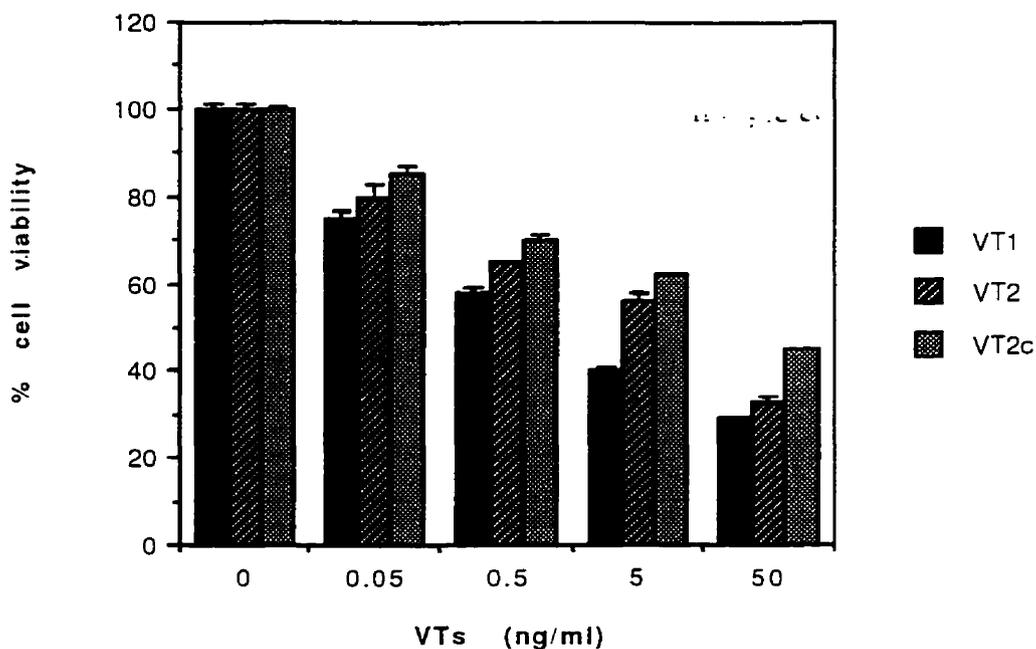
Figure 3.6   VTs cytotoxicity on human B-lymphocyte cells infected with Epstein
              Barr virus.

Human B-lymphocytes infected with Epstein Barr virus were tested for VT
sensitivity. These cells with elevated level of Bcl-2 expression, were significantly
sensitive to the cytotoxic effect of VTs particularly VT1 with CD50 of VT1, VT2, and
VT2c of >0.5, >5, and <50 ng/ml respectively.

Experimental details are as outlined in legend to Fig. 3.1.

Each value represents the mean ± S.D. of triplicate wells.

This experiment was repeated twice with a similar pattern of results.

3.3   DISCUSSION and CONCLUSIONS

This chapter encompasses the results of studies using *in vitro* systems to demonstrate VT cytotoxicity on selected cancer cells. VT1 was tested against ovarian carcinoma (SKOV3) and two MDR variants (SKVLB, SKOVC), and four cervical cancer cell lines. Interestingly, it was found that multi-drug resistant variants of ovarian tumor cell lines were markedly (~1000 fold) more sensitive to VT1 (or VT2 or VT2c,) cytotoxicity in vitro than the drug sensitive parental cell line (Fig. 3.1A). This elevated sensitivity to VTs was consistent with an elevated level of Gb3 receptor expression in SKVLB on VT TLC overlay. A prominent lower band, suggestive of a shorter fatty acid chain length isoforms of the receptor, Gb3 was observed in this cell line (Fig. 3.2). The receptor from SKOV3 and SKVLB was later analysed for fatty acids content (Table. 5.3). SKOV3 and SKVLB cells were chosen as a suitable model system to further explore the molecular mechanism of differential sensitivity (Chapter, 5). The Verotoxin receptor, the glycolipid globotriaosylceramide (Gb3) which mediates binding of this toxin to the surface of the Gb3 expressing cells, internalizes the toxin into specific locations in sensitive cells and is necessary to confer sensitivity to VTs. Interestingly only SKVLB cells showed sensitivity to interferon. Gb3 has been implicated as being involved in the α-interferon signal transduction pathway (Cohen, Hannigan et al., 1987; Ghislain, Lingwood et al., 1994). The specific isoform of Gb3 with a shorter fatty acid chain length can be important in this signaling pathway, both for its hypersensitivity to VT and sensitivity to interferon. This issue will be fully discussed in chapter, 5.

Human cervical tumor derived cell lines, SIHA and ME-180 showed significant sensitivity to VT1 cytotoxic effect but two other cell lines, HT-3 and CASKI were not sensitive (Fig. 3.5). SIHA and HT-3 cells showed comparable level of receptor expression and HT-3 cells did not respond to sensitization with butyrate treatment at a concentration of 2 mM (data not shown).

In addition, human white blood cells infected with Epstein Barr virus with elevated expression of Bcl-2 protein showed sensitivity to VT (Fig. 3.6). In all the cell lines tested, VT1 was the most potent member of the VT family and this finding is in agreement with the previous study (Head, et al., 1991).

VTs are all active on a number of cell lines such as Vero, HeLa, Daudi, MDCK, and HEp2 cells (Head et al., 1990; Tyrrell et al., 1991). Recently it was shown that human renal endothelial cells contain 50 to 100-fold higher levels of Gb3 than human umbilical vein endothelial cells (HUVECs) and are as sensitive as Vero cells to the cytotoxicity of VT *in vitro*. This correlates with cytopathology of VTs *in vivo* (Obrig, et al., 1993).

In summary, VT1, more effectively than other VTs, was able to induce cell cytotoxicity in the human tumor cell lines studied. Most importantly multi-drug resistant variants of an ovarian tumor cell line were hypersensitive with elevated expression of the Gb3 receptor. However,astrocytoma cell lines with a significant level of receptor expression did not show the same level of sensitivity to VT1 and this differential sensitivity is the basis for the experiments described in Chapter 5. In the next chapter, the mechanism of cell death induced by VT will be addressed.

Chapter 4

Studies on the mechanism of cell death induced by Verotoxin

4.1   INTRODUCTION

Astrocytoma is the most common primary human brain tumor (Laws, et al., 1984). The majority of astrocytomas are malignant neoplasms which infiltrate diffusely into regions of normal brain. The median survival for patients with glioblastoma multiforme, the most malignant form of astrocytoma, is approximately 12 months (Muller, et al., 1977; Laws, et al., 1984; McCormack, et al., 1992). In this context, it is imperative that new therapeutic strategies continue to be explored for malignant astrocytomas.

The results from the previous chapter have identified VTs to be the active agents against some human tumor cell lines. We have extended our studies to examine the antineoplastic effects of verotoxins on human astrocytoma cell lines, since $\alpha2$ interferon is used in the treatment of astrocytoma (Mahaley et al., 1985; Buckner et al., 1995) and the $\alpha2$–interferon receptor shows homology with VTs (Lingwood and Yiu, 1992). The present study illustrates the potent *in vitro* efficacy of VTs on a series of human astrocytoma cell lines and addresses the mechanism of cell death induced by VT.

(The results from this chapter are in press in the J. Neuropathol. & Exp. Neurol.)

4.2   RESULTS

4.2.1   Study of VT cytotoxicity on astrocytoma cell lines

To determine the cytotoxic effect of VT1 on human astrocytoma cell lines, the cells were grown to confluency and then tested for VT1 sensitivity as described in Chapter 2 (Section 2.2). Figure 4.1, shows the 72hr response of 6 astrocytoma cell lines treated with increasing levels of VT1. Although each cell line was sensitive to VT1, there was a significant difference in sensitivity between the most (SF-539) and least (XF-498) sensitive cell line.
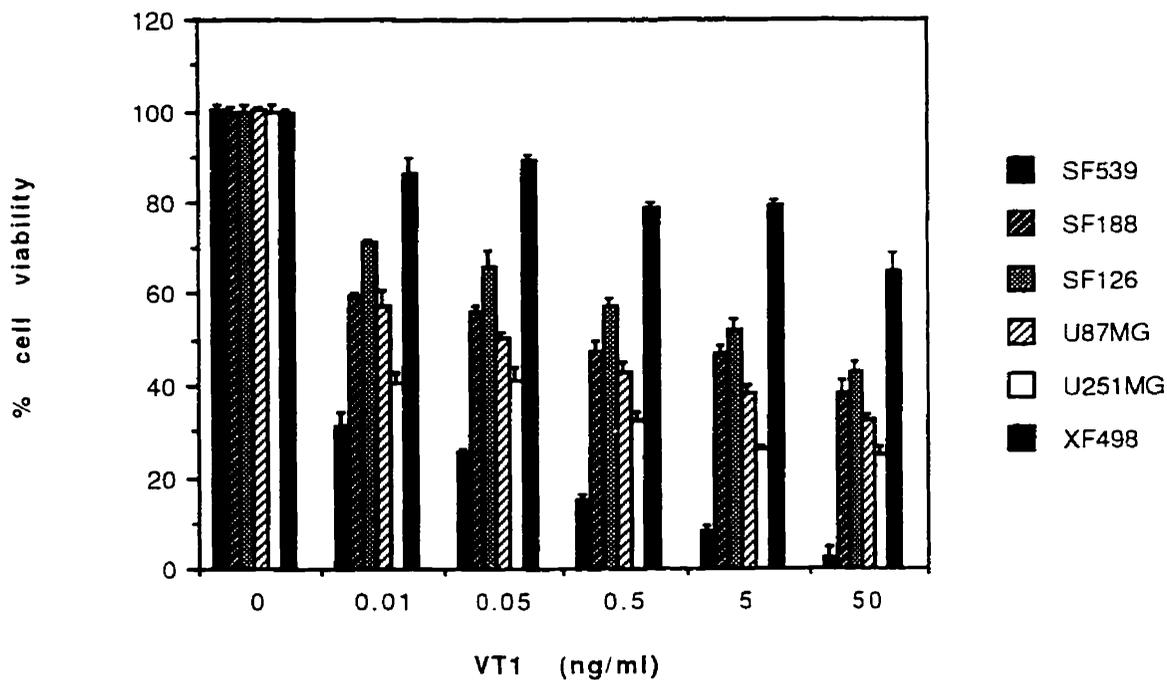
Figure 4.1 VT1 cytotoxicity in different human astrocytoma cell lines

Six astrocytoma cell lines were grown and seeded in 96-wells plates as outlined in Chapter 2. Cells were treated with serial dilutions of VT1 in media, incubated for 3-days and cytotoxicity was determined as described in Chapter 2. SF-539 is the most and XF-498 is the least sensitive of the cells to VT1 cytotoxicity. Each value represents the mean ± S.D. of triplicate wells.

This experiment was repeated three times with a similar pattern of results.

4.2.2 VT-Receptor Analysis of Human Astrocytoma Cells

Since the presence of Gb3 is essential to confer sensitivity to VT, the glycolipid profile of the 6 human astrocytoma cell lines analyzed for Gb3 content as detected with orcinol is shown in (Fig. 4.2A). All of the astrocytoma cell lines expressed significant levels of Gb3 and showed binding with VT1 in the overlay assay used (Fig. 4.2B). SF-539, the most and XF-498, the least sensitive cell lines, express maximal level of Gb3. The expression of receptor by all astrocytoma cell lines tested, shows that the level of receptor expression is not sufficient to explain the difference in sensitivity to VT in these cells. The question of how Gb3 expression levels relate to sensitivity to VTs for any given cell type or tumor cell system has not yet been fully elucidated, however some insight in to this issue is discussed in Chapter 5.

4.2.3 Study on the cytotoxicity of different VTs on astrocytoma cells

To investigate the sensitivity of SF-539 to other members of the verotoxin family, SF-539 cells were treated with other members of the VT family including VT2, and VT2c. SF-539 showed sensitivity to VT2 and VT2c (Fig. 4.3), however as expected from previous studies (Head, et al., 1991; Lindgren et al., 1994), VT-1 was the most potent species (Fig. 4.3).

4.2.4 Study on the cytotoxicity of VT1-B subunit on astrocytoma cell lines

A comparison between the sensitivity of SF-539 and XF-498 cells (with comparable level of Gb3) (Fig. 4.3) to VT1 B subunit was made (Fig. 4.4). SF-539 astrocytoma cells were significantly more sensitive to the B subunit alone than XF-498 astrocytoma cells, since over 40% cell death was observed in the presence of 50 ng/ml (Fig. 4.4). This compares to over 95% cell death in the presence of 50 ng/ml VT1 in SF-593 cells (Fig. 4.1).
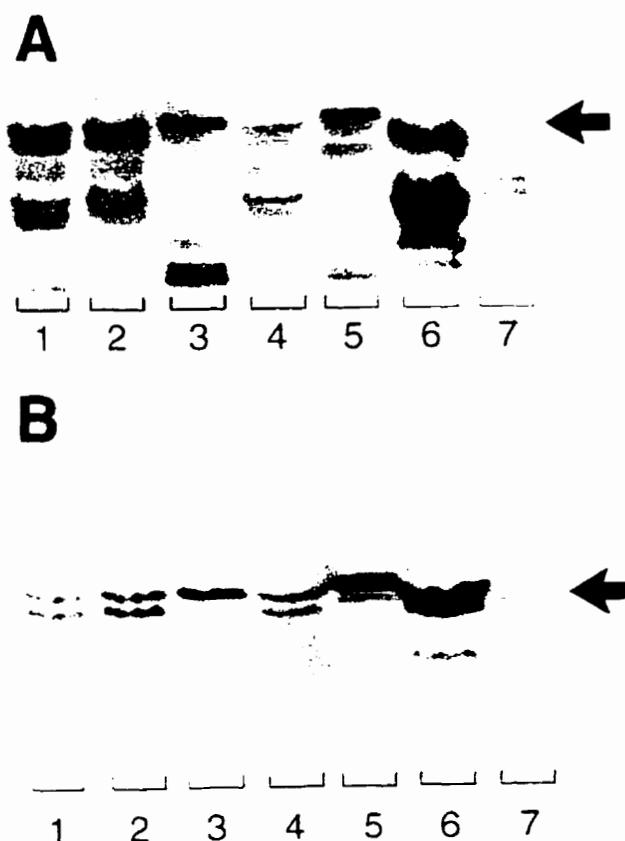
Figure 4.2   Detection of the VT-Receptor glycolipid, Gb$_3$ in human astrocytoma cell lines.

Astrocytoma neutral glycolipids were extracted from 1×10^6 cells and separated by TLC. (A) Glycolipids visualized by orcinol. (B) The same blot assayed by VT1 overlay. SF-539 and XF-498 express the highest level of VT1 receptor, Gb$_3$. Lane 1, U87 MG; lane 2, U251 MG; lane 3, SF-126; lane 4, SF-188; lane 5, XF-498; lane 6, SF-539; lane 7, standard Gb$_3$ (0.3 ug/ml). Gb$_3$ band is depicted by arrow head in orcinol and VT1 overlay in tlc plate in top and bottom panel respectively.
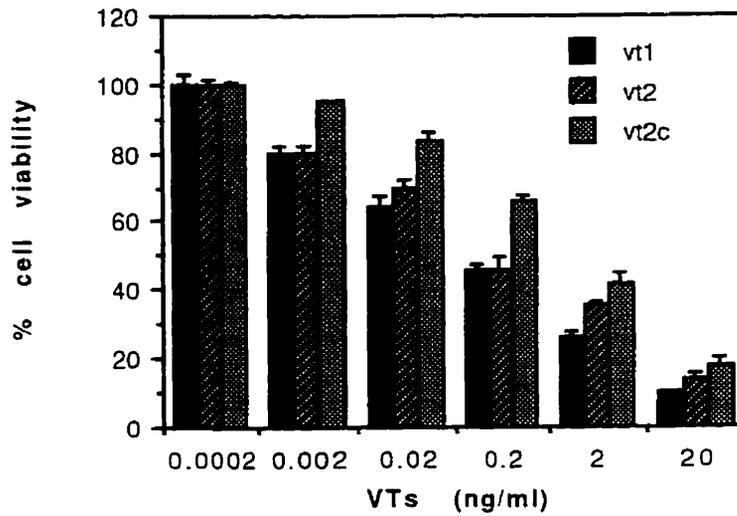
Figure 4.3   Sensitivity of SF-539 astrocytoma cells to VT1, VT2, and VT2c.

SF-539 cells were tested for sensitivity to three members of VT family. From this analysis, VT1 demonstrates the greatest cytotoxic effect against SF-539 cells. Experimental details as outlined in the legend to Fig. 4.1.
Each value represents the mean ± S.D. of triplicate wells.
This experiment was repeated twice with a similar pattern of results.
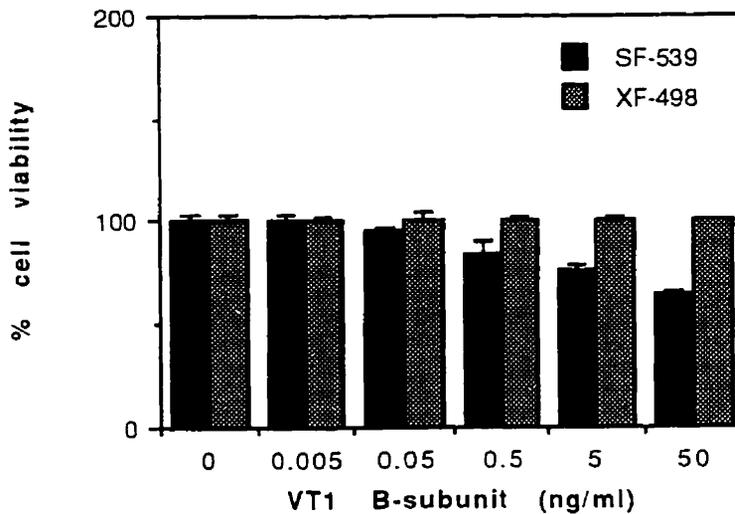
Figure 4.4   Comparison of SF-539 and XF-498 sensitivity to VT1-B subunit

Forty-eight hrs following the treatment of SF-539 and XF-498 cells in monolayer culture with VT1B, the percent cell survival was calculated. Experimental details as outlined in the legend to Fig. 4.1. Only SF-539 cells were sensitive to VT1B.
Each value represents the mean ± S.D. of triplicate wells.
This experiment was repeated three times with a similar pattern of results.

4.2.5 Mechanism of cell death induced by VT1 in astrocytoma cell lines

The next step in these studies was to determine the mechanism of cell death induced by VT1 in astrocytoma cells. Light microscopy study of VT sensitive astrocytoma indicated apoptosis in VT1 treated cells. Three methods were employed to confirm the mechanism of cell death induced by VT:

Propidium iodide staining of the nucleus, flow cytometry analysis, and ultrastructural analysis by Electron microscopy studies.

I. Propidium iodide stains

For detection of apoptotic morphology in cells treated with VT1 or VT1 B-subunit, permeablized SF-539 cells were stained with the DNA-intercalating agent propidium iodide and were analyzed by fluorescence microscopy. VT1 treated cells displayed characteristic features of apoptosis, such as marked reduction in diameter and condensed chromatin (Fig. 4.5). Nuclear shrinkage (A), nuclear segmentation and subnuclear bodies (C) were prominent in cells treated with VT1 for 1.5 (A) or 10 hours (C).

II. Flow Cytometric Analysis

To determine the extent of astrocytoma cell death by apoptosis and obtain cell cycle analysis of the non-apoptotic cell population, astrocytoma cells were analysed by flow cytometry. SF-539 and XF-498 astrocytoma cells exposed to VT1 (10 ng/ml) revealed the characteristic features of apoptosis. As a result of chromatin condensation and DNA cleavage, apoptotic cells show less propidium iodide fluorescence than viable cells and can be quantified as the "subdiploid" population (Nicoletti, et al., 1991) or pre-G1 position in cell cycle (Figure 4.6 arrow head). Presence of cells with fractional DNA content, typical of apoptosis was more marked in SF-539 than XF-498 cells. A cell cycle analysis of the non-apoptotic cell

marked in SF-539 than XF-498 cells. A cell cycle analysis of the non-apoptotic cell population revealed marked differences in the proportion of cells in the respective phases of the cell cycle. In VT1-sensitive SF-539 cells, a pronounced loss of S phase cells from 32 to 14 and 9% was seen from 0 to 24 to 36 hrs after VT1 treatment, whereas with the less VT1 sensitive XF-498 cells, the loss of S phase cells observed was only from 13 to 10 to 8%. Changes in the proportion of cells in G2-M phase and a shift in XF-498 cells after 24 hrs of VT1 treatment were also observed (Fig. 4.6).

III. Ultrastructural Analysis of VT-Treated Astrocytoma Cells

By electron microscopy, VT1-treated astrocytoma cells (SF-539) demonstrated characteristic features of apoptosis such as, blebbing of the cytoplasmic membrane, fragmentation of heterochromatin, condensation of the nuclear membrane, loss of cell junctions and microvilli, nuclear disintegration, and apoptotic bodies (Fig.4.7B).
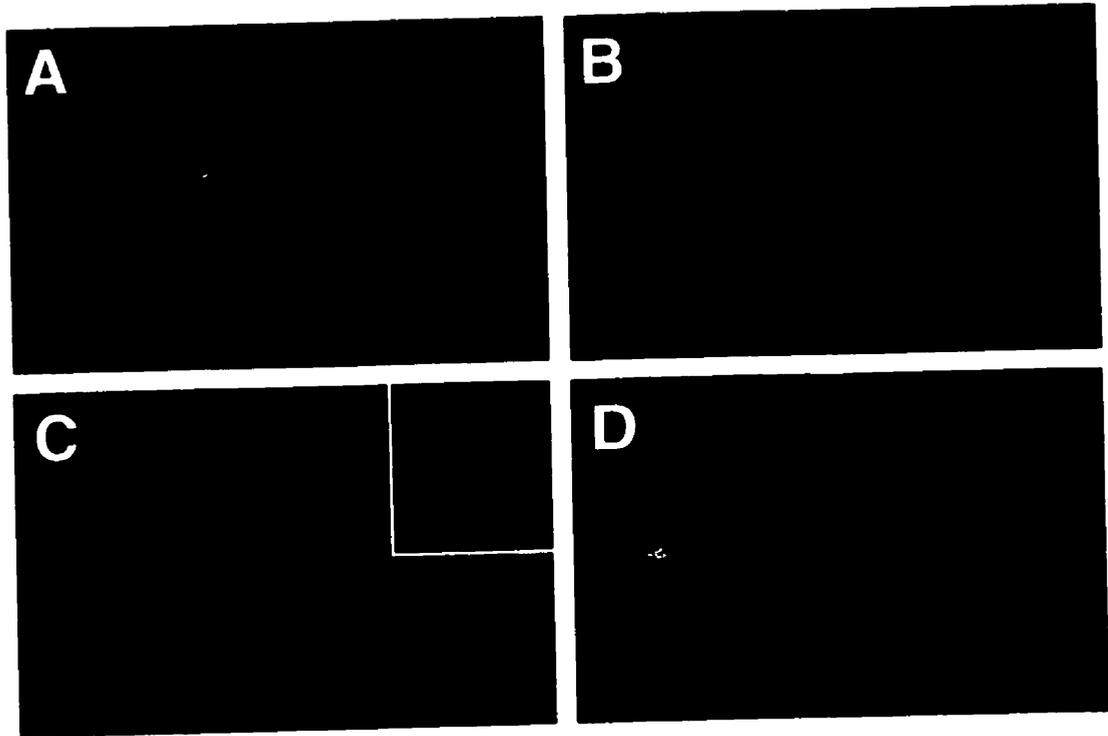
Figure 4.5   Fluorescence microscopy of propidium iodide-stained nuclei of SF-539 astrocytoma cells.

(A) SF-539 astrocytoma cells 1.5 hr after treatment with 50 ng/ml VT1 B-subunit showing rapid reduction in nuclear diameter (viewed at magnification of 100x). (B) Untreated astrocytoma cells showing homogeneity of nuclear staining with propidium iodide (same magnification as in A). (C) SF-539 astrocytoma cells 10 hr after treatment with 50 ng/ml VT1 B-subunit (viewed at magnification of 10 x). (D) Untreated astrocytoma cells (viewed at magnification of 10 x). In comparison to untreated astrocytoma cells (D), the toxin treated astrocytoma nuclei (C) show extensive nuclear blebbing. Insert shows higher magnification of nuclear blebbing. The insert shows the apoptotic nucleus viewed at 200x.

This experiment was repeated two times with a similar pattern of results.
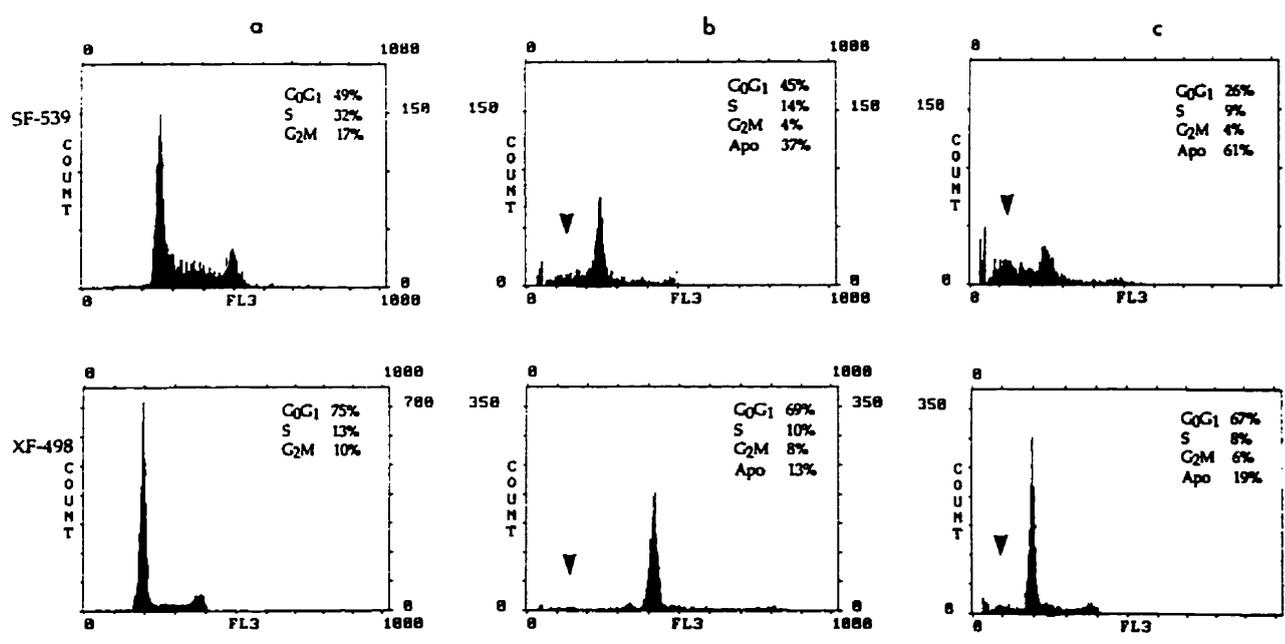
Figure 4.6   Flow cytometric analysis of apoptosis and cell cycle distribution in asynchronous population of cells with or without VT treatment.

Astrocytoma cells   were treated with 0 ng/ml (panel a) or 10 ng/ml VT1 for 24 hr (panel b) or 36 hr (panel c) as shown. Four discrete populations of cells representing G0/G1, G2/M, S-phase, and apoptotic cells (pre-G0/G1, arrow heads) are seen. A shift was observed for XF-498 cells (panel b). A marked increase in percent apoptotic cells was observed over time (61%) following treatment of SF-539 astrocytoma cells with VT1. A lesser number of apoptotic cells (19%) was observed in similarly treated XF-498 astrocytoma cells.

Figure 4.7   Ultrastructural morphology of SF-539 human astrocytoma cells

(A) Untreated SF-539 cells after 12 hr in monolayer culture showing the presence of cell surface microvilli, cytoplasmic organelles and an euchromatic nucleus. Original magnification x 12,000. (B) SF-539 astrocytoma cells 12 hr after treatment with 10 ng/ml VT1. Of particular note are the loss of microvilli, chromatin margination, significant cell and nuclear shrinkage, extensive cytoplasmic vacuolation and the formation of apoptotic bodies containing intracellular structures (Original magnification x 11000). These ultrastructural features are highly suggestive of apoptosis following treatment with VT1.

4.4   DISCUSSION and CONCLUSIONS

This chapter encompasses the results of studies demonstrating VT1 cytotoxicity towards a series of human astrocytoma cell lines, which is consistent with our previous correlation of sensitivity to α_2 interferon and VT (Cohen, et al., 1987). All cell lines which showed significant sensitivity to VT1, contained the Gb$_3$ receptor for VT, and demonstrated ultrastructural features indicative of apoptosis following VT treatment.

The data indicate that the B subunit is also capable of inducing apoptosis independent of the action of the A subunit. Therefore, in addition to cell death by inhibition of protein synthesis, VTs can cause programmed cell death. These studies show that the most toxin sensitive astrocytoma cell line, SF-539, originaly derived from a recurrent glioblastoma multiforme (Rutka, et al., 1987), is also sensitive to B subunit-induced apoptosis, suggesting that Gb$_3$ itself plays an important role in this signal transduction pathway (to induce apoptosis) in these cells. Definitive morphological evidence of apoptosis (nuclear shrinkage and chromatin condensation) was observed within 1.5 hrs of toxin (Fig. 4.5 & 4.7) administration to astrocytoma cells, which is a considerably more rapid induction of apoptosis than that induced by chemotherapeutic drugs (Barry, et al., 1990). Additional evidence in support of VT1 causing apoptosis in sensitive astrocytoma cells includes accumulation of VT1-treated astrocytoma cells in a pre-G1 position in cell cycle, nuclear staining with propidium iodide and ultrastructural alterations indicative of apoptosis.

Homeostatic control of cell number is the result of a dynamic balance between cell proliferation and cell death. VT treatment targets both compartments of this equation by inhibiting cell proliferation through protein inhibition and augmentation of Gb$_3$ signal, and increase in cell death by inducing apoptosis.

Previous studies (Pudymaitis and Lingwood, 1992) and results from flow cytometry analysis of the astrocytoma cells (Fig. 4.6), shows that cells are most

sensitive to VT1 treatment at G1/S. Therefore, VT1 treatment will target one of the most important checkpoints in the cancer cell cycle. Genotoxic insults such as radiation and chemotherapy are known to induce apoptosis (Clark et al., 1993; Lowe et al., 1993; Strasser et al., 1994). In fact, apoptosis has been recognized as the major mechanism in the action of many chemotherapeutic agents (Barry, et al., 1990). However, the overexpression of Bcl 2 renders the tumor cells resistant to the apoptotic activity of the anti-cancer drugs (Wang et al., 1993). Bcl-2 is known as an antiapoptotic protein, since mammalian cells destined to die by programmed cell death may be rescued by bcl-2 overexpression (Vaux, 1993; Vaux, et al., 1994). Expression of the bcl-2 gene, either as studied in transgenic mice or in cell lines transformed with a bcl-2 expression vector, renders cells resistant to a spectrum of stimuli and various chemotherapeutic agents. This includes agents which act independently of P^{53} (Strasser, et al., 1994)., supporting the idea that conserved downstream events leading to the cell death are being modulated. Interestingly, apoptosis induced by treatment of VT is not inhibited by bcl-2 expression (personal communication, Joelle Wiels). A newly proposed model of apoptosis suggests that only apoptotic signals received after the last cell death check point are able to trigger irreversible, programmed cell death. This check point is believed to be down stream of the action of bcl-2 (Oltvai and Korsmeyer, 1994). Since the signal for VT to induce apoptosis circumvents the inhibition of programmed cell death by bcl-2, VT may target the last checkpoint site of apoptosis.

Interestingly, human cerebral endothelial cells were largely resistant to the growth inhibitory and cytotoxic effects of VT1 (data not shown, Arab, in press). Further study to determine the receptor, Gb3 expression and isoforms in these cells would be useful when considering an in vivo test of VT1 anticancer effect in humans.

In summary, this chapter contains results that have described the Gb3 receptor status and the cytotoxic effects of VT on a series of astrocytoma cell lines. These results suggest that VTs may provide the basis of new agents active against some human astrocytoma cells. These encouraging preliminary results were further investigated in nude mice xenografted with astrocytoma tumor cells (Chapter 7). VT or the B subunit may provide the basis of a new approach to the treatment of these chemotherapeutically refractile tumors.

In the next chapter the molecular differences of Gb3 isoform and its contribution to subcellular targeting of VT will be addressed.

Chapter 5

VT sorting

Intracellular targeting of the ER/nuclear envelope by
retrograde transport determines cell hypersensitivity to
verotoxin

5.1 INTRODUCTION

The evidence presented in the preceding chapters indicates that in spite of comparable level of Gb3 expression in astrocytoma cell lines the level of sensitivity to verotoxin is significantly different. To explore further the mechanism of cell sensitivity to VT, the targeting of the toxin inside the cell was studied. The B subunit of verotoxin (VT) mediates the attachment to cell surface globotriaosyl ceramide(Gb3) to facilitate receptor mediated endocytosis of the toxin. The endocytotic pathway of many protein toxins comprising separate A(enzymatic) and B (receptor binding) subunits, involves cell binding, internalization, acid-mediated translocation from an intracellular compartment to the cytosol, and enzymatic inactivation of their intracellular targets (Middlebrook and Dorland, 1984; Olsnes et al., 1993). After translocation to the cytoplasm, the A subunits of ricin, abrin, modeccin, and verotoxins catalytically inactivate the 28 S RNA of 60 S ribosomal subunits, leading to an inhibition of cellular protein synthesis (Endo et al., 1988).

Gb3, the specific receptor for all verotoxins, at the plasma membrane mediates the internalization of verotoxin into susceptible cells by capping and subsequent receptor-mediated endocytosis (RME) (Khine and Lingwood, 1994). Verotoxin is the only known glycolipid binding ligand that is internalized into eukaryotic cells by means of RME through clathrin coated pits (Sandvig, et al., 1991; Sandvig, et al., 1994).

Recent studies indicate that in addition to receptor concentration, both heterogeneous fatty acid composition of Gb3 (Pellizzari, et al., 1992;Kiarash, et al., 1994) and phospholipid chain length within the phospholipid bilayer (Arab and Lingwood, 1996) play important roles in VT binding. Recent molecular modelling studies of the Gb3 binding site in the B subunit (Nyholm et al., 1996) have suggested that different conformers of membrane Gb3 may bind at different sites and it was speculated that such conformers may be related to the fatty acid content and phospholipid microenvironment (Kiarash, et al., 1994; Arab and Lingwood, 1996).

The requirement of retrograde transport for intoxication of cells to Shiga toxin has been demonstrated (Sandvig, et al., 1992). In butyric acid-treated A431 cells a Shiga-HRP conjugate was observed in Golgi cisternae, in ER and in the nuclear envelope and at the same time cells became sensitive to this toxin. In untreated cells the conjugate could be traced to the endosomes/lysosomes and the cells were not sensitive to the toxin (Sandvig, et al., 1992; Sandvig et al., 1993).

In addition to the N-glycosidase activity of the A subunit leading to inhibition of protein synthesis (Endo, et al., 1988; Saxena et al., 1989), both the holotoxin and the B subunit are capable of inducing programmed cell death (apoptosis) (Wiels, 1993; Inward, et al., 1995; Arab, et al., in press). Moreover, results in previous chapters demonstrated that human ovarian tumor cell lines are highly sensitive to VT1 (Farkas-Himsley, et al., 1995) and astrocytoma cell lines for example, show high sensitivity to VT1 and VT1B induced apoptosis (Arab, et al., in press).

5.2 RESULTS

The results from previous studies demonstrated that the expression of receptor level by the astrocytoma cell lines tested was not sufficient to explain the difference in sensitivity to VT1 in these cells. To explore this differential sensitivity and investigate the question of how Gb_3 expression levels and different isoforms relate to the sensitivity to VTs for any given cell type or tumor cell system, the internalization and targeting of FITC-VT inside the cells was studied.

5.2.1 Subcellular targeting of VT1B in astrocytoma cell lines

The intracellular routing of VT1 following RME was monitored in SF-539 and XF-498, using FITC-labelled B subunit and fluorescence microscopy (Fig. 5.1). Similar results were obtained using FITC-VT1.

The pattern of FITC-VT1 B localization was completely distinct in the SF-539 and XF-498 astrocytoma cell lines, despite comparable Gb_3 content (Figs 4.2, 5.6, & Table 5.1) and cell surface binding at 4°C (Fig. 5.1a & 5.1 b). In the more VT sensitive SF-539 cells, at 37°C, intracellular FITC-VT1-B accumulated around the nuclear membrane/ER (Fig. 5.1a, B-E) and within the nucleus (Fig. 5.11a). However, the intracellular localization of FITC-VT1 in XF-498 cells was less distinct and in a juxtanuclear location suggestive of Golgi localization (Figs 5.1b, B-E & 5.2a, A-F & 5.2b & 5.11c).

5.2.2 Subcellular localization of toxin in XF-498 cells by confocal microscopy

To further explore the possibility of VT1 targeting to Golgi in XF-498 cells, a colocalization study of Golgi marker concanavalin A with VT1B was performed. Concanavalin A (Con A) is a jackbean lectin known to bind to integral membrane glycoproteins of the endoplasmic reticulum and Golgi complex (Tartakoff et al, 1983). The identity of the compartment where FITC-VT1B accumulates following internalization in XF-498 cells was explored by comparison with the location of an acknowledged resident protein in Golgi, Con A. The Texas red labeled-lectin displayed diffuse staining, with some accumulation in a region near the nucleus which overlapped completely with FITC-VT1B only in Golgi region(Fig. 5.2). Confocal and fluorescence microscopy studies both are indicative of the sorting of VT1 or VT1B to the Golgi. FITC-VT1B and concanavalin A were colocalized only in the Golgi region (Fig. 5.2). However in order to identify in which compartment of the Golgi region VT accumulates, further colocalization with specific markers of each Golgi compartment is necessary.
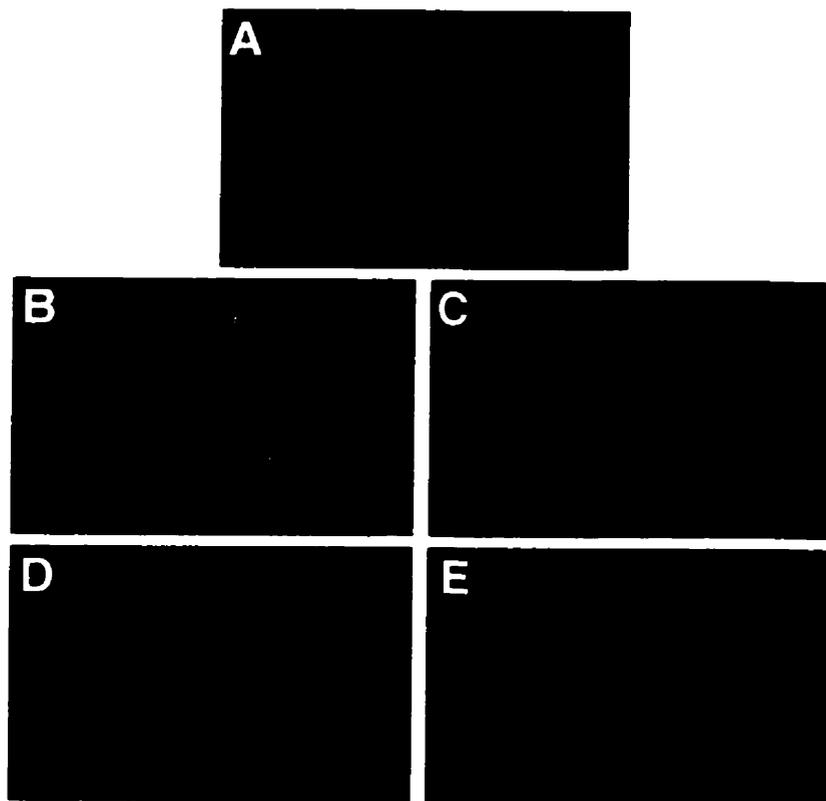
Figure 5.1a   FITC-VT1B subcellular targeting in human astrocytoma cell lines

Astrocytoma cells (SF-539) grown on cover slips were incubated with 10 ug/ml of FITC VT1B at 4C° or 37C° for 1 h. After extensive washing, cells were fixed and mounted with DABCO and visualized under fluorescence microscopy. FITC- (A,B,D,E) or RITC (C)-VT1B subcellular targeting in SF-539. A, at 4°C and B, C, D, E at 37° C. Nuclear targeting is shown in cells incubated with toxin at 37°C. This experiment was repeated four times with a similar pattern of results.
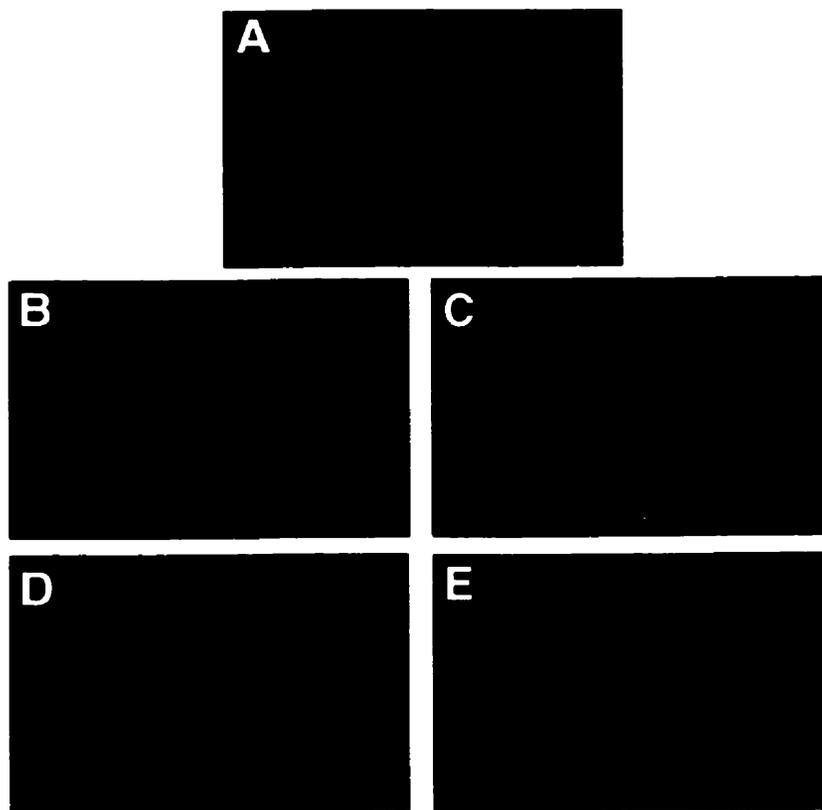
Figure 5.1b   FITC-VT1B subcellular targeting in human astrocytoma cells

Astrocytoma cells (XF-498) grown on cover slips were incubated with 10 ug/ml of FITC-VT1B at 4C° or 37C° for 1 h. After extensive wash, cells were fixed and visualized under florescence microscopy. FITC- (A,B,D,E) or RITC (C)-VT1B binding on XF-498. A, at 4C° and B, C, D, E, at 37 C°.
This experiment was repeated four times with a similar pattern of results.
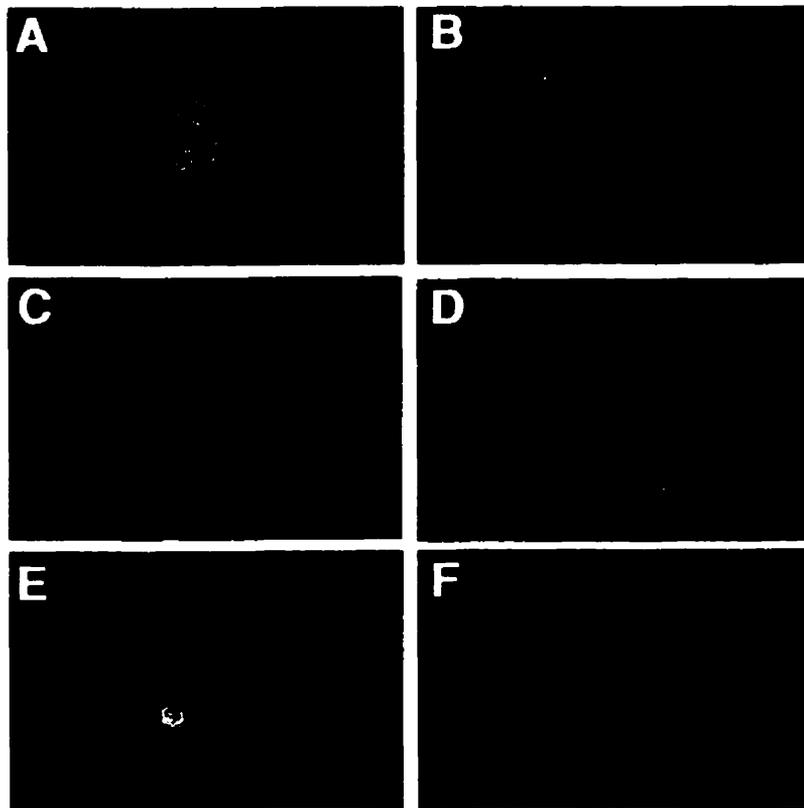
Figure 5.2a   Comparison of the subcellular localization of FITC-VTB with that of
Golgi marker in astrocytoma cells by fluorescence microscopy

XF-498 cells were prepared as in Fig. 5.1 and incubated with FITC-VT1B in
PBS at 37 C° for 1 h, fixed, permeablized and stained with Texas Red-labeled
concanavalin A (2-5 ug/ml; 30 min at 37 C°). Concanavalin A was clearly
colocalized with FITC-VT1B in Golgi area which is depicted both in Fig. 5.2a and
5.2b from fluorescence and confocal microscopy respectively.
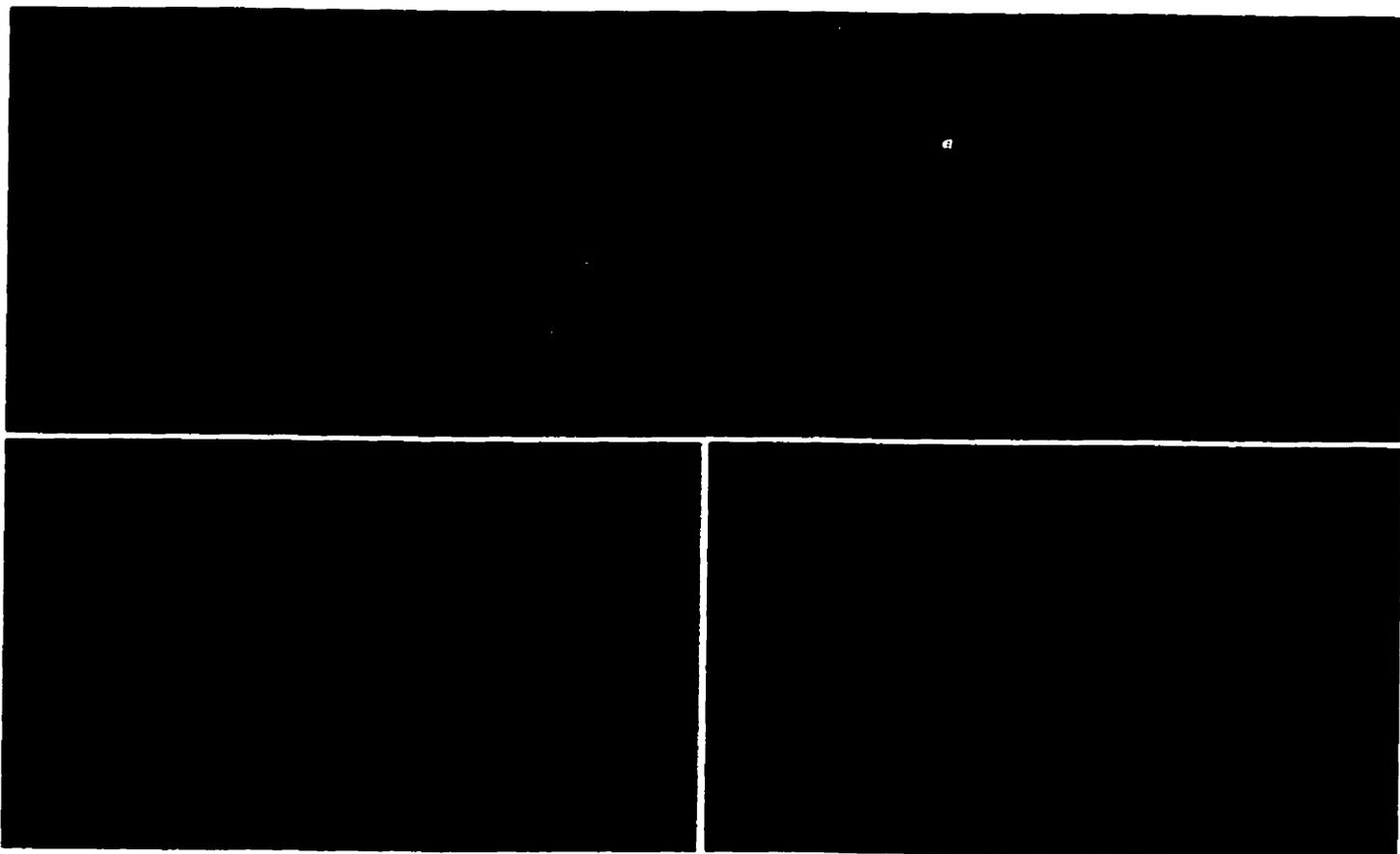
Figure 5.2b   Comparison of the subcellular localization of FITC-VTB with that of
Golgi marker in astrocytoma cells by confocal microscopy

XF-498 cells were prepared as in Fig. 5.1 and incubated with FITC-VT1B in
PBS at 37 C° for 1 h, fixed, permeablized and stained with Texas Red-labeled
concanavalin A (2-5 ug/ml; 30 min at 37 C°). Concanavalin A was clearly
colocalized with FITC-VT1B in Golgi area which is depicted both in Fig. 5.2a and
5.2b from fluorescence and confocal microscopy respectively.

5.2.3   Effect of Brefeldin A treatment on XF-498 cell sensitivity to VT1

Since toxin localization by fluorescence microscopy and colocalization studies of FITC-VT1 with a Golgi marker (Con A) by confocal microscopy were both suggestive of toxin targeting to the Golgi in XF-498 cells, the role of the Golgi in the activity of this toxin has been studied by using Brefeldin A (BFA). BFA, is a macrocyclic lactone synthesized from palmitate (C16) by a variety of fungi (Harri, et al., 1963). BFA has been shown to affect both structure and function of the Golgi apparatus, resulting in an inhibition of protein secretion and the redistribution of the Golgi proteins into the endoplasmic reticulum (Misumi et al, 1986; Oda et al, 1987; Orci et al, 1991). The earliest action of BFA (within 30 s) is to cause release of the 110K non-clathrin coat protein (β-COP) from the Golgi apparatus, an event which precedes any morphological changes (Donaldson, et al., 1990). Within minutes of BFA treatment, the Golgi enzyme markers, mannosidase II and thiamine pyrophosphatase appeared within the ER as well as within the nuclear envelope (Lippinocott-Schwartz et al., 1989). The effect of BFA on transport was rationalized to be a cessation of the forward flow of the membrane which is normally mediated by COP-coated vesicles (Misumi et al, 1986; Oda et al, 1987; Orci et al, 1991), caused by preventing assembly of the coat protein (COP) onto membrane (Serafini, et al, 1991).   It has been suggested that disappearance of a distinct Golgi complex upon treatment of cells with BFA is a consequence of halting the anterograde membrane transport, without disturbing retrograde traffic [Lippincott-Schwartz, 1990; Serafini, 1991]. Treatment with BFA increased the cytotoxicity of VT1 in human astrocytoma, XF-498 cells (Fig., 5.3a) and increased nuclear targeting of toxin in these cells (Fig. 5.3b). BFA treatment of SF-539 cells did not affect the level of VT1 cytotoxicity in these cells (data not shown). In SF-539 cells the toxin is targeted to the ER/nuclear membrane (Fig. 5.1a & 5.10b) as compared to the XF-498 cells which VT is targeted to the Golgi (Fig. 5.1b & 5.2b).
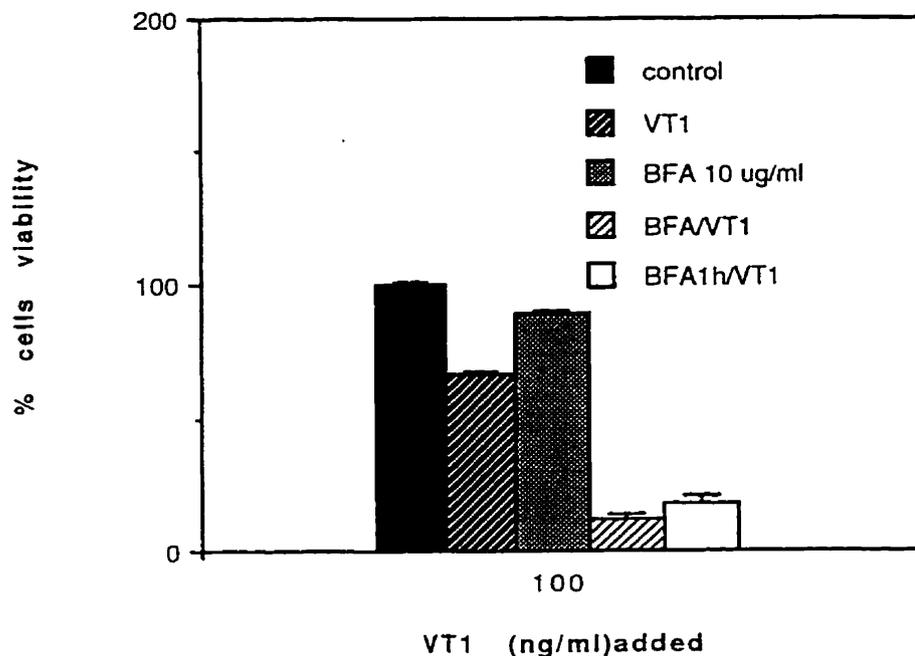
Figure 5.3a   Study on VT1 cytotoxicity on XF-498 treated with BFA

XF-498 cells were seeded in 96-well plates to form a confluent monolayer. VT1 (100 ng/ml) was added to the cells with or without BFA (10 ug/ml), incubated for 3-days and cytotoxicity was determined as described in Chapter 2. XF-498 show elevated level of cytotoxicity after only 1 h treatment with BFA prior to incubation with VT1(BFA 1h/VT1) or in the presence of BFA throughout incubation period (BFA/VT1). Each value represents the mean ± S.D. of triplicate wells.
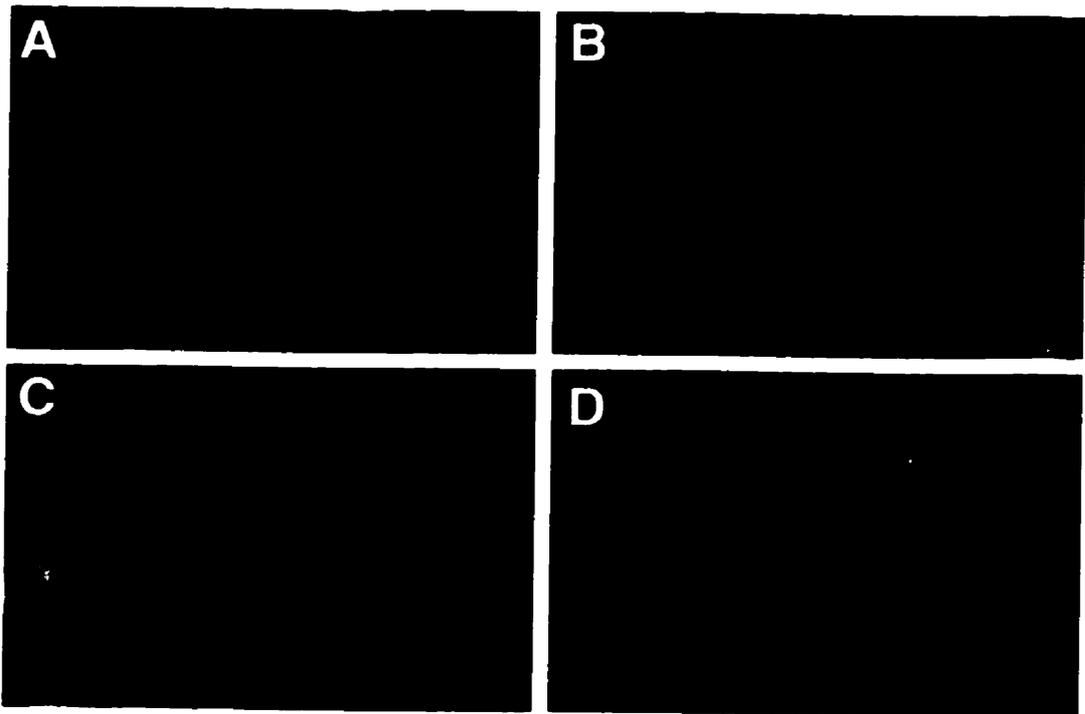
Figure 5.3b   Study on FITC-VT1B subcellular localization in XF-498 treated with
BFA

BFA treatment of XF-498 cells significantly increased the nuclear targeting in
these cells, concomitant with elevation of VT1 cytotoxicity Fig. 5.3a. Subcellular
targeting of FITC-VT1B is shown after BFA (1 ug/ml) treatment. A,B,C) FITC- and
RITC-VT1B (D). Experimental details are as outlined in legend to Fig. 5.1.
These experiments were repeated two times with a similar pattern of results.

5.2.4 Effect of sodium butyrate treatment of XF-498 cells on morphology and sensitivity to VT

Studies from many laboratories have indicated that inducers of differentiation frequently lead to an enhanced glycolipid synthesis (Hakomori, 1981; Burczak et al., 1984). These inducers (e. g., sodium butyrate, phorbol asters, various retinoid compounds) are known to alter the phosphorylation state of the cells, thus the activities of glycosyltransferases may be under phosphorylation-dephosphorylation control during cellular differentiation or proliferation. Sodium butyrate has been found to increase Gb_3 synthesis and thereby sensitivity to VT, in several cell lines (Sandvig, Garred et al., 1992) and was therefore tested for effect on XF-498 cells, since XF-498 cells show a significant level of receptor expression and yet are the least sensitive cell line to VT cytotoxicity (Fig. 4.1).

A) Morphology and growth: Sodium butyrate treatment of XF-498 cells causes profound morphological changes in these cells (Fig 5.4a). XF-498 cells are small cells with more rounded shape that pile up, and even upon confluency do not spread (Fig. 5.4a, A). The butyrate treated XF-498 cells (XF^+) (Fig. 5.4a, C)show similar morphology (Fig. 5.4a, B) (flat and extended) to SF-539 cells (the most sensitive cells to VT) as opposed to small round and piling up form of XF-498 cells (Fig. 5.4a, A).

B) VT1 sensitivity: Consistent with the morphological change, butyrate induced a significant increase in XF-498 sensitivity to VT1 (Fig 5.4b).

5.2.5 Subcellular VT targeting in butyrate treated astrocytoma cell

Since the growth of XF-498 cells in the presence of butyrate markedly increased the sensitivity of these cells to VT cytotoxicity (Fig. 5.4), the effect of butyrate on the intracellular targeting of VT1 in XF-498 cells was investigated. Interestingly, butyrate induced a marked change in the intracellular routing of VT1 B in XF-498 cells such that the FITC-VT1 was localized mainly around the nuclear membrane (Fig. 5.5) as for SF-539 (Fig 5.1) (see section 5.2.10 for confocal study).
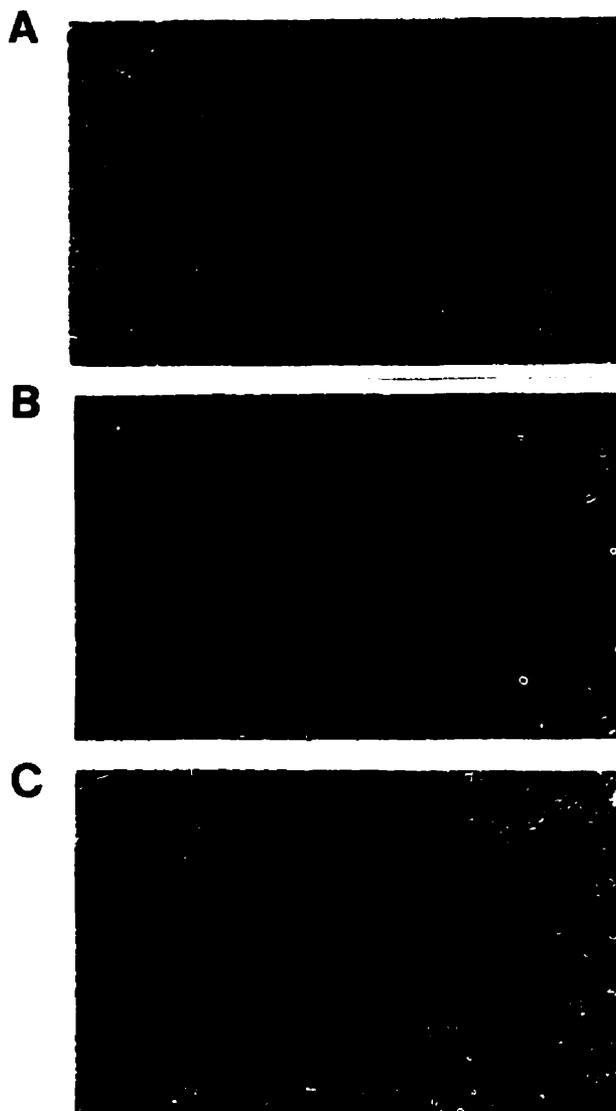
Figure 5.4a   Morphological changes in butyrate treated astrocytoma cells

XF-498 cells were grown in 25 ml Falcon flask in the presence or absence of butyrate,(2 mM) for 6-7 days. Significant morphological changes were observed in XF-498 cells treated with 2mM butyrate for 6 days. A) XF-498, B) SF-539, and C) XF+ cells (butyrate treated).
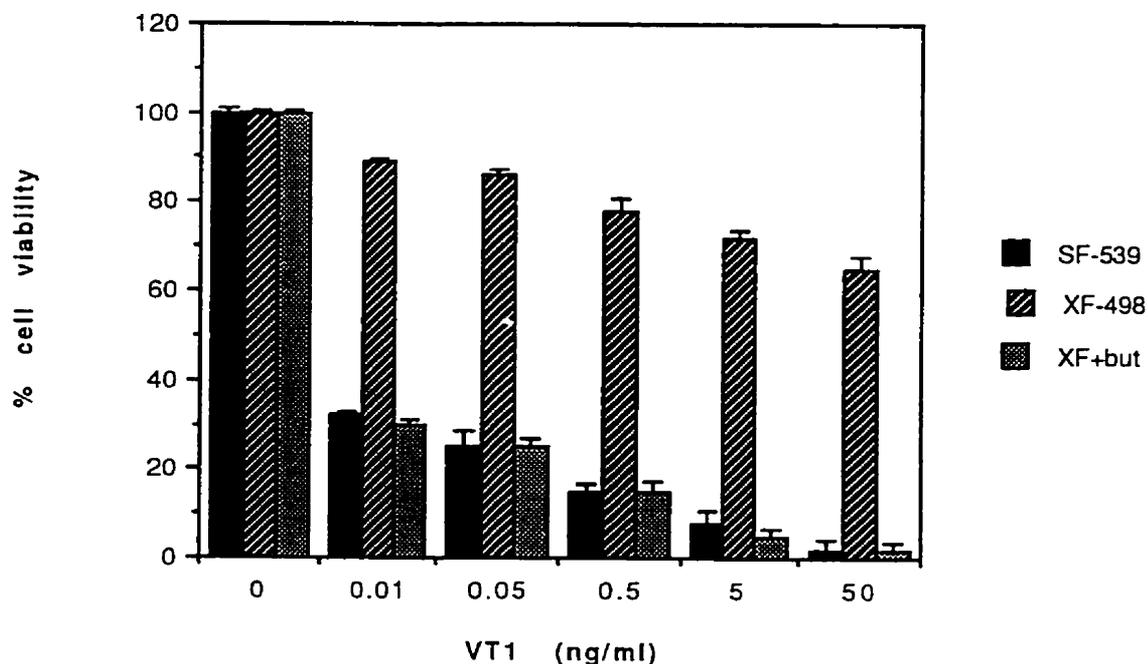
Figure 5.4b   VT1 cytotoxicity changes in butyrate treated cells

Astrocytoma cells, XF-498 were cultured in RPMI (10% FCS) containing a single dose of sodium butyrate (2mM, 7days). Cells were seeded in 96 well plates overnight (no butyrate) and incubated with serial dilutions of VT1 for 3-days and VT1 cytotoxicity was determined as outlined in Chapter 2. XF+ cells show elevated sensitivity to VT1 as compared to parental cell line, XF-498.

Each value represents the mean ± S.D. of triplicate.

This experiment was repeated three times with a similar pattern of results.
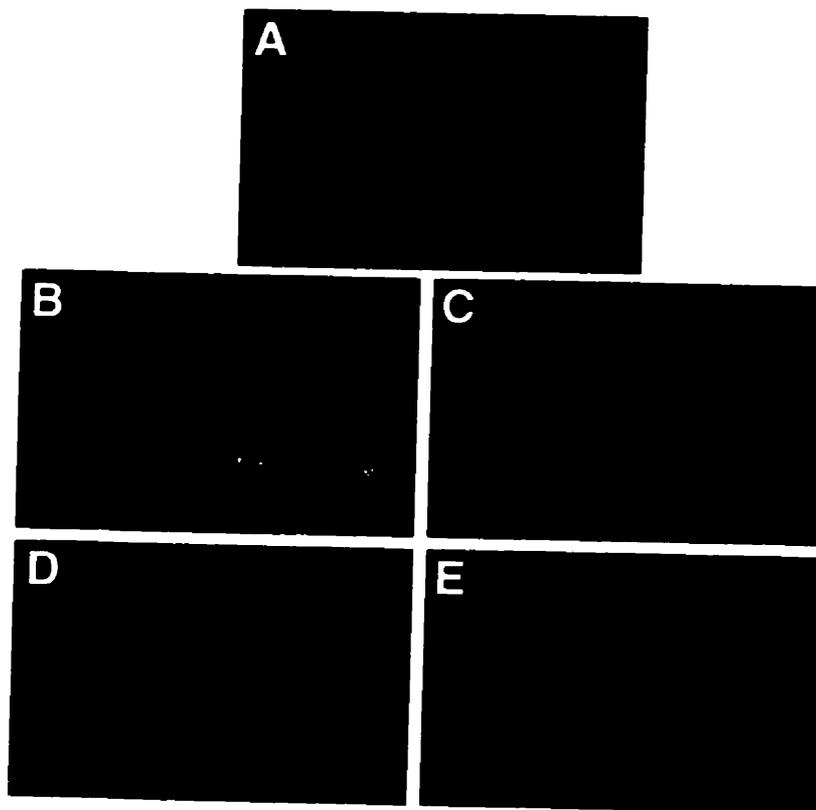
Figure 5.5   Subcellular localization of FITC-VT1B in XF+ cells

FITC-VT1B internalization was studied in XF+ cells which were grown on cover slip overnight (no butyrate) for studies on internalization of FITC-VT1B. Cells were incubated with 10 ug/ml of FITC-VT1 or FITC VT1B in PBS and 0.1% BSA at 4C° (A) or 37C° (B,C,D) for 1 h. Experimental details as oulined in legend to Fig. 5.1. A,B,D,E) FITC-and C) demonstrate RITC-VT1B nuclear targeting at 37°C in XF+ (butyrate treated) cells.

This experiment was repeated three times with a similar pattern of results.

5.2.6   VT overlay study of Gb₃ isoform expression in XF-498, SF-539 and XF⁺ cells

The next step in determining the basis for the differential targeting of VT, thus the differential sensitivity observed in these three astrocytoma cell lines (SF-539, XF-498 & XF+), was to investigate the difference in Gb3 isoforms expressed in these cells. The fatty acid heterogeneity of Gb3 was studied using a VT1 overlay method (Chapter 2) and is depicted in Fig. 5.6, with arrow pointing Gb3 band. The level of the shorter fatty acid chain length isoform of Gb3 was proportional to the sensitivity to VT and it was maximal in XF⁺ cells and the least in XF-498 (the least sensitive to VT). The result of this study is clearly suggestive of a relation between Gb3 with different fatty acid isoforms expressed and subcellular targeting, and thus the sensitivity to VT.

5.2.6.1   FAME analysis of Gb₃ isoforms expression in SF-539, XF-498, & XF⁺ cells

A fatty acid analysis was performed to confirm the finding of the VT overlay examination which clearly demonstrates the presence of a slower migrating Gb3 species being minimal in XF-498 cells and preferentially elevated following butyrate treatment (Fig 5.6). Fatty acid analysis of this species showed a decrease in the proportion of long chain and an increase in short chain fatty acid content. Notably the levels of C24 fatty acid are greatly diminished while C16 is markedly enhanced (table 5.1) in SF-539 cells and XF⁺ cells relative to untreated XF cells. The increase in expression of Gb3 containing shorter fatty acid chains is proportional to sensitivity to VT, XF-498 being the least sensitive astrocytoma cells with the lowest level of shorter chain fatty acids as compared to SF-539 and XF⁺ cells.

Figure 5.6   Comparison of Gb3 isoforms expression in SF-539, XF-498 and XF+ cells

Glycolipid extract of three different astrocytoma cell lines ; SF-539, XF-498, and XF+ (+butyrate) was compared for Gb3 isoform expression using VT1 overlay (panel A) as described in chapter 2. The significant increase in the lower band species of Gb3 is suggestive of shorter chain fatty acids expression in SF-539 and is particularly elevated in XF+ cells. Glycolipid extraction was outlined in Chapter 2. Standard Gb3 (3ug) lane 1, neutral glycolipid extract from equal number of cells (about 1x10^6), XF-498 (lane 2), SF-539 (lane 3), XF+ (lane 4) cells, and human kidney extract (lane 5, as a control) is depicted. Orcinol plate is shown on the panel B. This experiment was repeated four times with a similar pattern of results.

FAME	Gb₃ XF	Gb₃SF	Gb₃ XF + butyrate
14 : 0	3.0	0.8	5.05
15 : 0	0.9	0.0	2.2
16 : 0	17.5	24.0	57.8
16 : 1	1.6	1.1	0.0
16 : 1 ; 7	1.2	0.6	4.2
17 : 0	0.0	0.0	1.47
18 : 0	13.1	13.8	19.4
18 : 1 ; 9	6.8	11.1	10.2
18 : 1 ; 7	0.0	0.0	2.6
18 : 2 ; 6	3.2	5.5	7.8
20 : 0	1.2	0.8	1.65
22 : 0	10.7	8.8	5.05
23 : 0	2.7	1.4	1.92
24 : 0	32.0	22.7	18.6
24 : 1	7.0	9.4	13.1
Total Fatty Acids	100.0	100.0	100.00

			XF	SF	XF+ butyrate
Increased	{	16 : 0	17.5	24.0	57.8
	{	18 : 2	3.2	5.5	7.8
Decreased	{	22 : 0	10.7	8.8	5.05
	{	24 : 0	32.0	22.7	18.6

Table 5.1 Analysis of Gb3 fatty acids

FAME analysis of Gb3 isoforms from SF-539, XF-498, and XF+ demonstrate and confirms the results depicted in Fig. 5.6. Level of short chain fatty acids show significant increase, accompanied by a decrease in long chain fatty acids in Gb3 from XF+ cells as compared to XF-498 cells.

5.2.7 Study on I^{125}-labeled-VT1 Binding to astrocytoma cell lines at 4 C°

To examine if the hypersensitivity of XF$^+$ cells to VT1 is due to increased binding of these cells to VT1 or the low sensitivity of XF-498 is not due to decreased binding to the toxin, I^{125}-labeled VT1 binding experiments were performed. At 4°C, toxin is only able to bind the cells with no internalization (Khine and Lingwood, 1994). SF-539, XF-498, and XF$^+$ cells, were cultured in 96-well culture plates, were labeled with I^{125} VT1 and the amount of radioactivity associated with the cells is shown in figure 5.7. All three cell types demonstrate a comparable level of binding and thus the difference in cytotoxicity is not due to differential binding of the toxin to the cells.

5.2.8 Effect of PPMP treatment on butyrate induced morphological changes

To further explore the connection between elevation of Gb3 level and morphological changes due to butyrate treatment in XF-498 cells, these cells were incubated in the presence of 2mM butyrate with various concentration of PPMP (1-Phenyl-2-palmitoylamino-3-morpholin-1-propanol) to determine the effective concentration for maximum blockage of glycolipid synthesis without cell death. PPMP, is an effective inhibitor of glycosphingolipid synthesis which blocks the enzyme glucosylceramide synthase responsible for the production of the glucosyl ceramide (Abe, 1992) a precursor for Gb3. PPMP was found to be effective in XF-498 cells at 25-27 uM in the presence of butyrate. Morphological changes as a result of butyrate treatment were significantly blocked by the treatment of PPMP (Fig. 5.8). HPLC analysis demonstrated the profound decrease in the expression of Gb3 in XF-498 butyrate treated cells in the presence of PPMP (Table 5.2). This result demonstrates the link between morphology changes in butyrate treatment and the level of glycosphingolipid, specifically Gb3. To my knowledge this is the first evidence of a Gb3 effect on morphological changes, suggestive of cytoskeleton rearrangement .
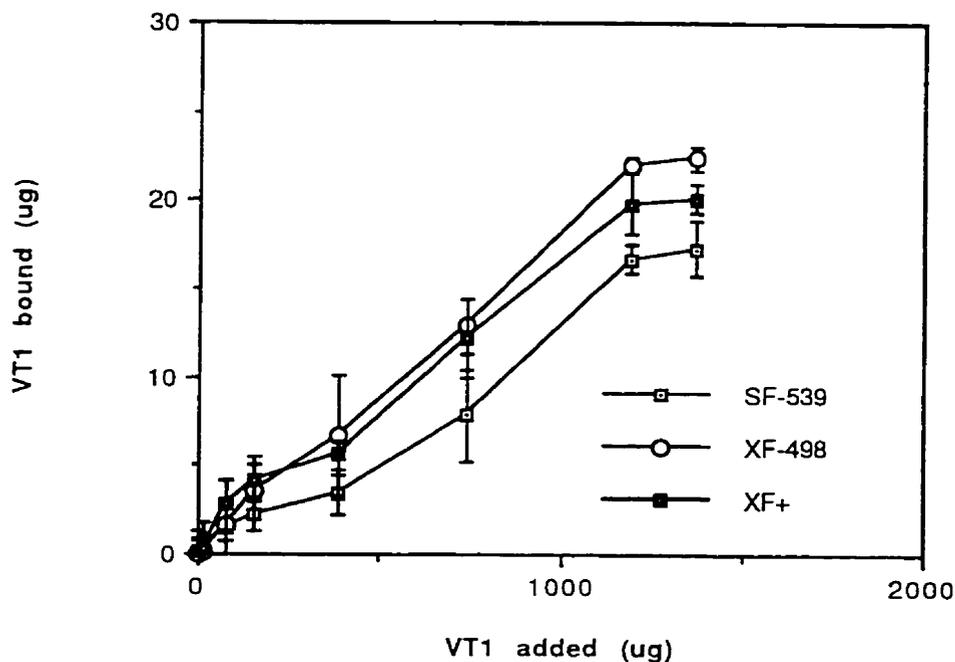
Figure 5.7   Study on I^{125}-labeled-VT1 Binding to astrocytoma cell lines at 4° C

Three astrocytoma cell lines (SF-539, XF-498; XF+) were grown as outlined in Chapter 2 and ^{125}I-VT1 binding to the cells was investigated. Cells were treated with serial dilutions of ^{125}I-VT1 in PBS at 4°C, incubated for 1h and binding was determined as described in Chapter 2. All three cell lines demonstrate similar levels of radiolabeled-VT1 binding.

Each value represents the mean ± S.D. of triplicate wells.
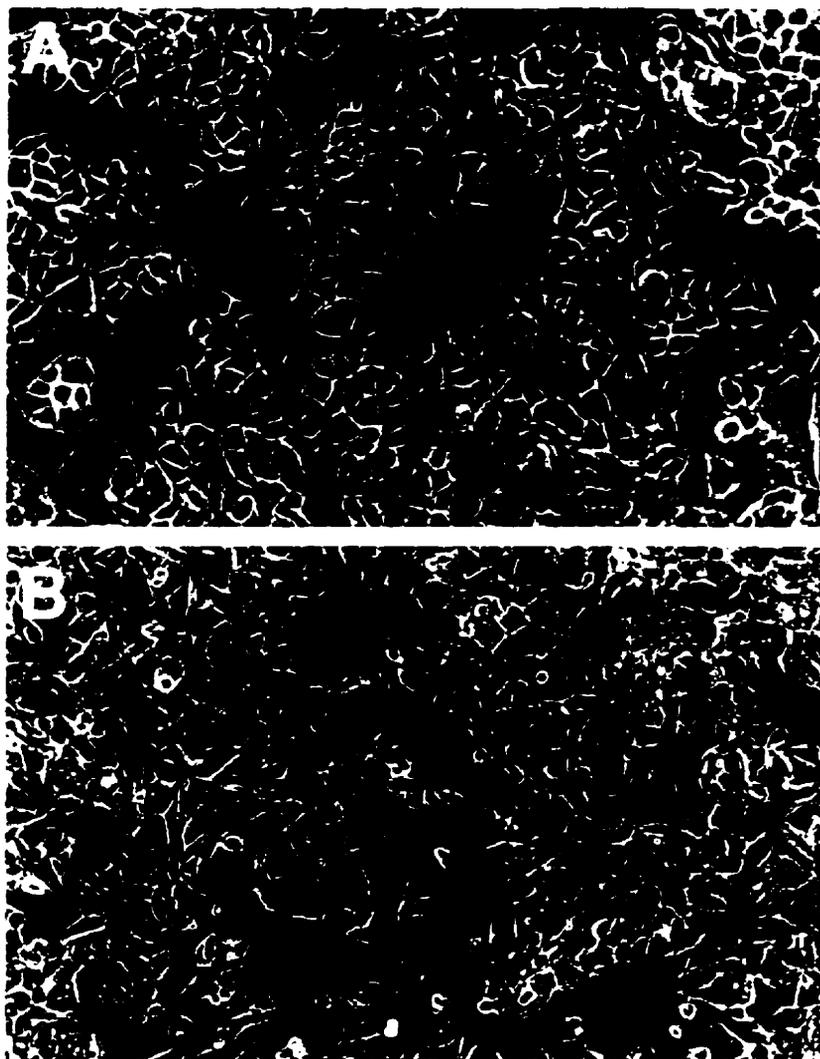
Figure 5.8   Butyrate induced morphological changes are blocked by PPMP
treatment

XF-498 cells were treated with or without butyrate (2mM) in the presence or absence of PPMP (27 uM).   Inhibitory effect of PPMP on butyrate induced morphological changes are depicted in panel B. Compare to Fig. 5.4a. Panel A, control cells treated only with PPMP.

Astrocytoma cells	GC (nmol/10⁶ cells)[a]	LC	Gb3
SF-539	1.3	2.4	.57
XF-498	1.2	.64	.90
XF+but	5.5	1.04	3.2
XF+PPMP	ND[b]	.48	.48
XF+but+PPMP	ND[b]	1.4	1.4

a-for Gb3 1 nmole is approximately 1 ug.

b-not determined due to interference of PPMP

-PPMP interfered also with the internal standard which was estimated for these compounds.

Table 5.2   Analysis of glycosphingolipid composition of astrocytoma cells

HPLC analysis of glycosphingolipids from SF-539, XF-498, XF+ (butyrate treated), XF+ PPMP treated and XF+ butyrate and PPMP treated astrocytoma cells are demonstrate.   Significant elevation of Gb3 level in responce to butyrate treatment and decrease in Gb3, in the presence of PPMP alone or PPMP and butyrate confirms   the   results   in   Fig.   5.4C   and   5.8   respectively.

5.2.9 Effects of other short chain fatty acid treatment on Gb3 isoforms expression, VT1 sensitization and morphology of astrocytoma cells

The effect of other short chain fatty acids on inducing morphological changes, elevating the expression of Gb3 isoforms with shorter fatty acid chain length, and inducing VT sensitivity in XF-498 astrocytoma cells, was examined. XF-498 cells were treated with 2mM propionate or capronate with three and six carbon chains respectively for 6-7 days. Fig. 5.9 shows the results of this study. Propionate was able to induce both significant morphological changes and sensitivity to VT1, but the effect of capronate was not significant (Fig. 5.9a &b). These results demonstrate that the effect of butyrate on sensitization of XF-498 cells can be mimicked by other short chain fatty acids, however butyrate has the greatest efficacy.

The effect of capronate and propionate treatment on Gb3 isoform expression in XF-498 cells was examined and the result is depicted in Fig 5.9c. Consistent with morphological changes and elevated sensitivity to VT1, the level of lower band (shorter chain fatty acids) Gb3 isoforms was increased only in propionate treated cells. These results further demonstrate the relation between cell shape, cell sensitivity to VT, and expression of Gb3 isoforms with shorter fatty acid chains.
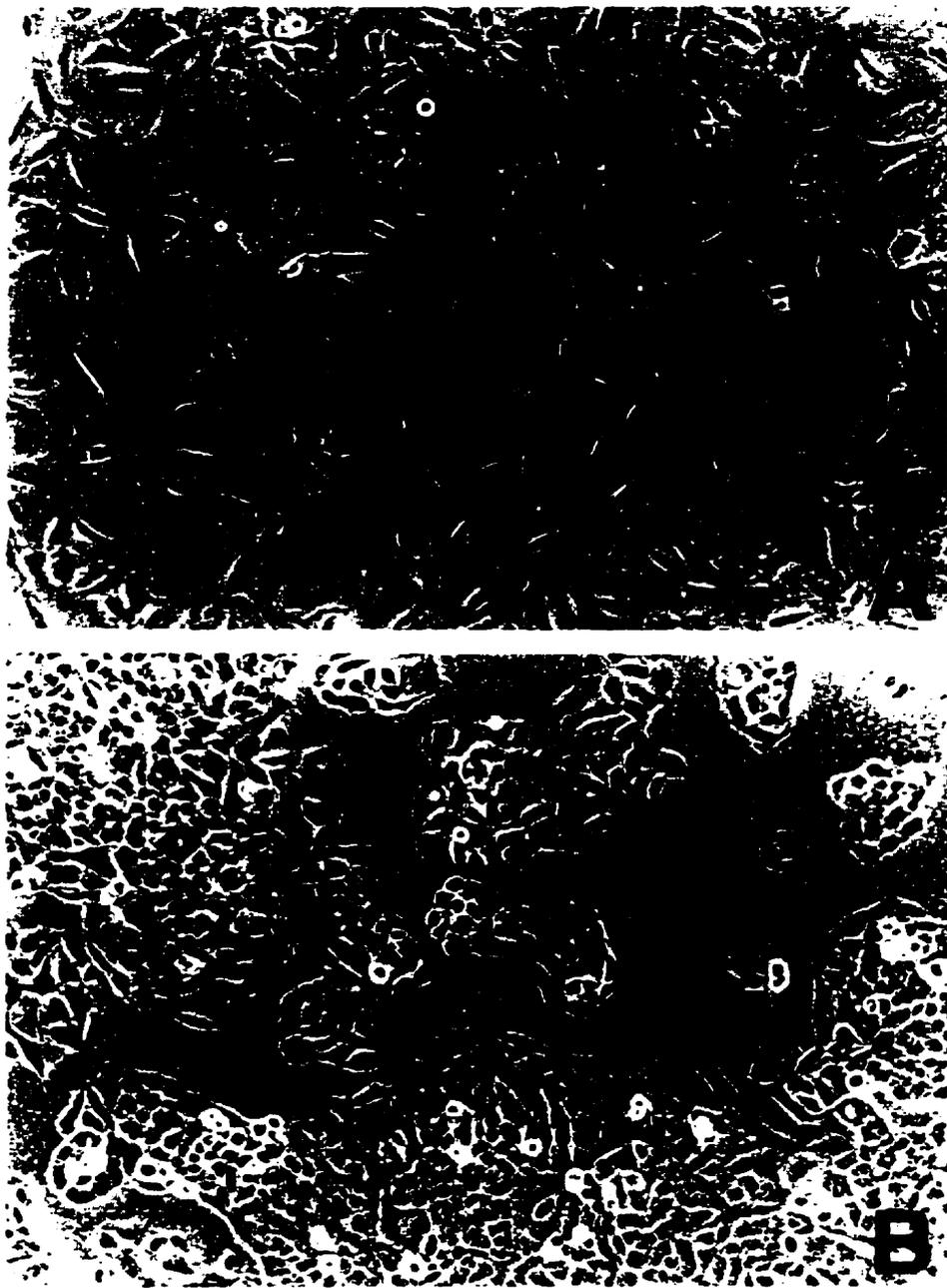
Figure 5.9a   **Effect of other short chain fatty acids treatment on morphology of XF-498 cells**

Effect of two other short chain fatty acids, sodium capronate (6-c chain) and sodium propionate (3-c chain) on morphological changes, expression of Gb3 isoform with shorter fatty acids, and sensitization to VT1 in XF-498 cells. This Figure demonstrates the morphological changes due to treatment with propionate (panel A) and capronate (panel B). Capronate was not able to induce significant morphological changes, but significant morphological changes consistent with elevation in sensitivity to VT1 was observed in propionate treated cells.
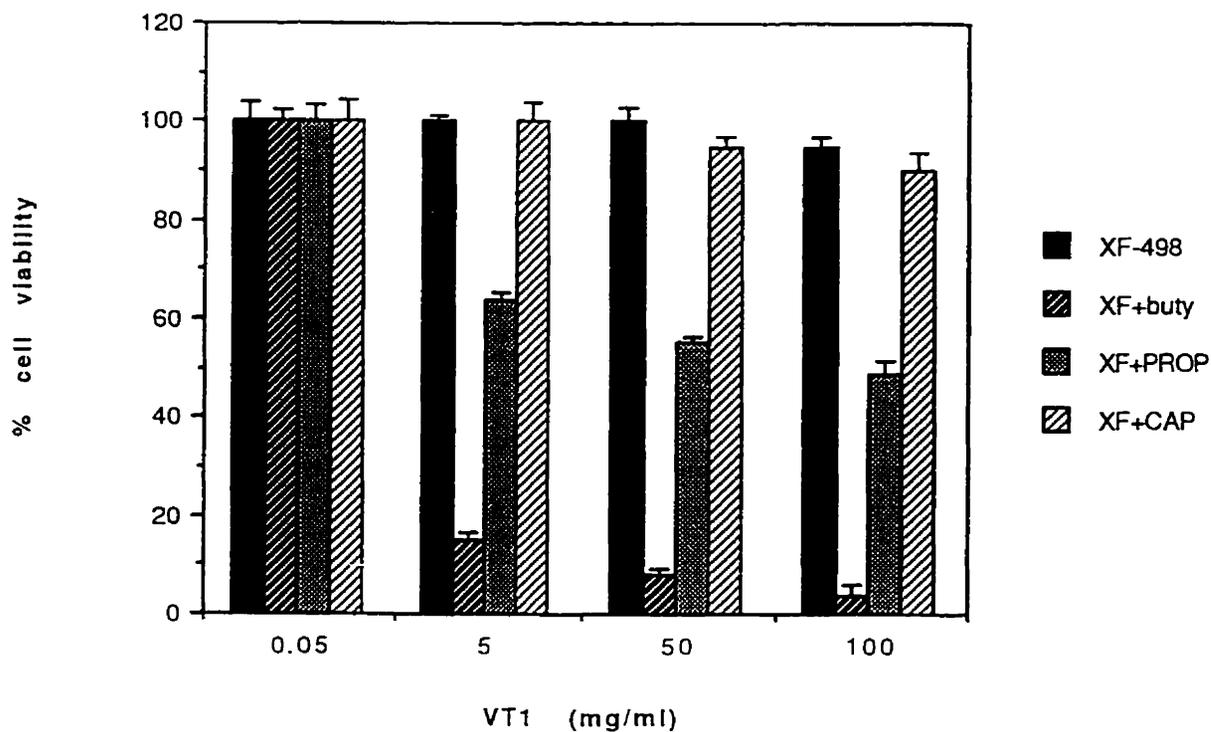
Figure 5.9b   Effect of other short chain fatty acids treatment on VT cytotoxicity of
              XF-498 cells

Propionate was able to mimic the effect of butyrate in increasing the cytotoxicity to VT1. Capronate treatment did not increase VT cytotoxicity significantly. Experimental details in chapter 2. XF-498 and XF+ are shown as control.

Each value represents the mean ± S.D. of triplicate.
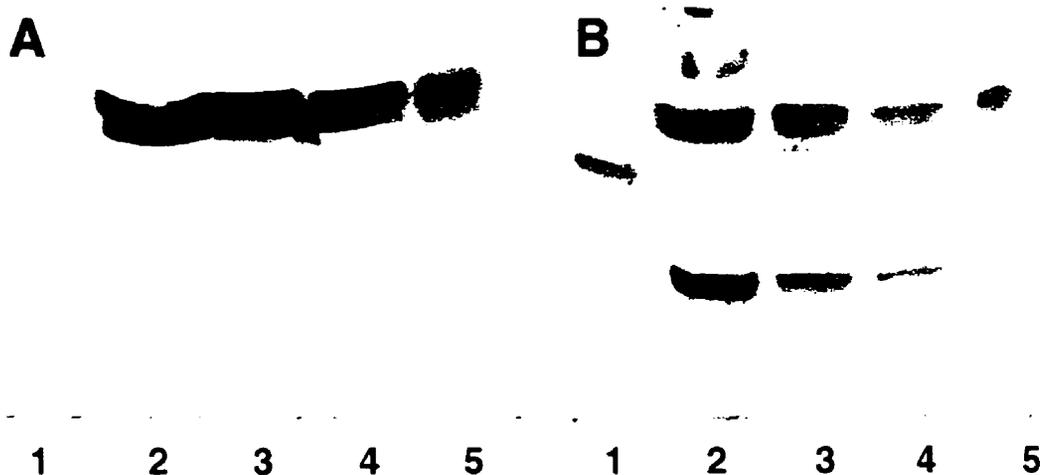
Figure 5.9c   **Effect of other short chain fatty acids treatment on Gb₃ isoforms expression in XF-498 cells**

Elevated level of Gb₃ isoform with shorter fatty acid chain was observed in propionate treated XF-498 cells   by VT1 overlay (A) and orcinol staining (B). Experimental details as outlined in legend to Fig. 5.4 except cells were treated with capronate and propionate.   Gb₃ standard as a positive control (1 µg, lane 5), Gb₄ standard as a negative control (1 µg, lane 1), glycolipid extract from butyrate, propionate, and capronate treated XF-498 cells (lane 2, 3, and 4 respectively). Capronate was not able to induce elevated expression of Gb₃ isoform with shorter chain fatty acids (lane 4).

5.2.10 Comparison of VT-B targeting in SF-539, XF-498, and XF+ by fluorescence and confocal microscopy

Fig. 5.10a demonstrates the differential targeting of FITC-VT1B in SF-539 (left panel), XF-498 (center panel), and XF$^+$ (right panel) cells by fluorescence microscopy. A similar pattern of nuclear membrane staining is depicted in SF-539 and XF$^+$ cells. The accumulation of VT within the nucleus of SF-539 and XF$^+$ is clearly visible in cross sections of SF-539 and XF+ cells, however further experimentation is required to exactly define the target inside the nucleus, since punctuated accumulation of FITC-VT1B is suggestive of nucleolar targeting. Cross-sectional or z scans corroborated the deep penetration of the toxin in the nucleus (in SF & XF$^+$ cells) and the Golgi (in XF-498 cells) as depicted in the confocal microscopy study of VT1B subcellular localization(Fig. 5.10b).

5.2.11 Comparison of VT targeting in SF-539, XF-498, and XF+ by Electron microscopy

The results from immunoelectron microscopy are supportive of the previous findings by fluorescence and confocal microscopy. Electron micrographs of VT1-B targeting in SF-539, XF+, and XF-498 are shown in Fig. 5.11a-c. Elevated levels of gold-labelled toxin are observed in nucleus of SF-539 and XF+ cells (Fig. 5.11a & b) as compared to the level of nuclear staining in XF-498 cells (Fig. 5.11c) which is at the level of nonspecific binding or background level.

5.2.12 Comparison of lysosomal labeling in SF-539, XF-498, and XF+ cells

Colocalization of RITC-labeled VT1B with FITC-dextran (lysosomal marker) was observed in XF-498 cells suggesting that the toxin is internalized through lysosomal/endosomal vesicles in these cells (Fig. 5.12c). In SF-539 and XF+ butyrate treated cells FITC-dextran was not colocalized with RITC-VT1B (Fig. 5.12a & b), which is indicative of different routing of VT1B in these two astrocytoma cell lines. It is possible that because of elevated level of Gb$_3$ isoforms with shorter chain of fatty acids, the targeting is affected.
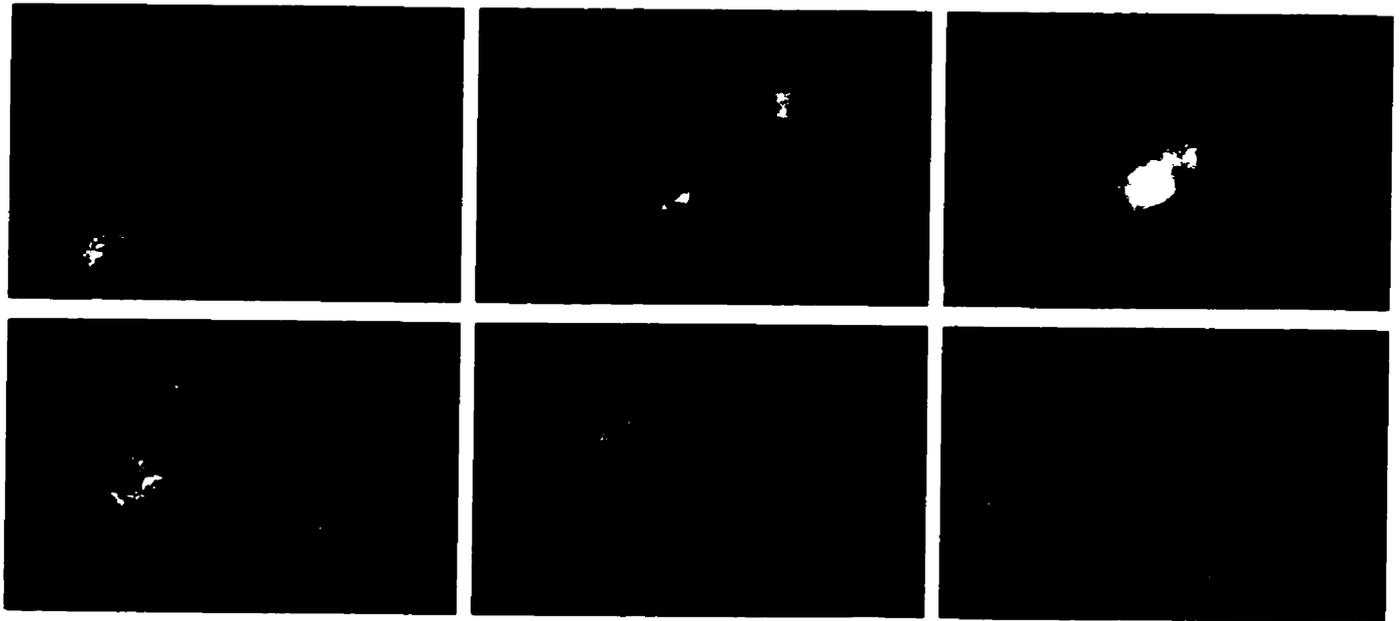
Figure 5.10a   Comparison of FITC-VT1B subcellular targeting in SF-539, XF-498 and
XF+ cells by Fluorescence Microscopy

Internalization and subcellular targeting of labeled-VT1B in astrocytoma
cells, studied by fluorescence microscopy are as follow: SF-539 cells, left panels; XF-
498 cells, center panels and XF+ cells, right panels.
This study was performed four times with a similar pattern of results.
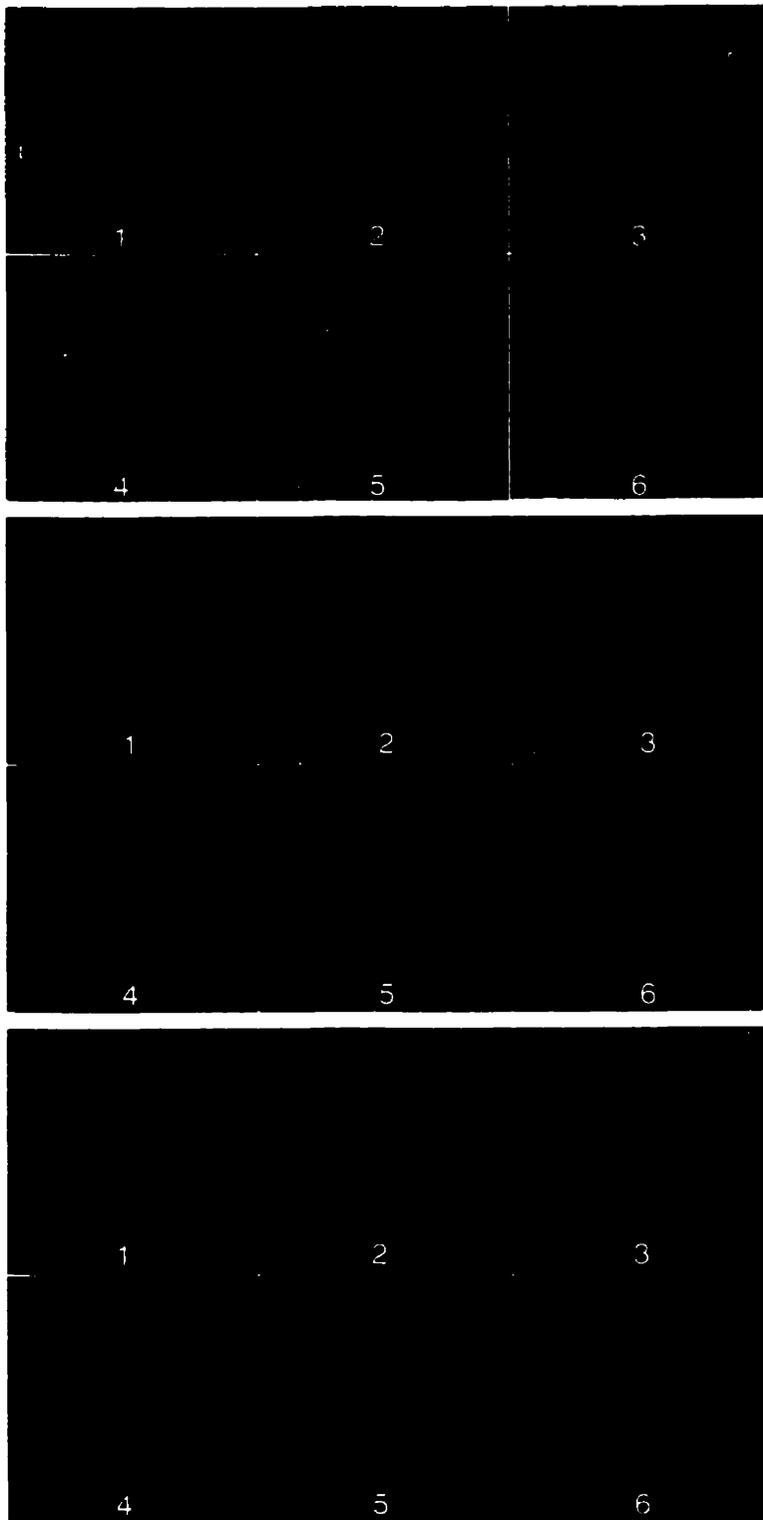
Figure 5.10b   Comparison of FITC-VT1B subcellular targeting in SF-539, XF-498 and
XF+ cells by Confocal Microscopy

In confocal microscopy, x vs z (cross-sectional) scan (1-6) are illustrated.
Subcellular targeting of labeled-VT1B in astrocytoma cells, studied by fluorescence
microscopy are as follows: SF-539 cells, top panels; XF-498 cells, center panels and
XF+ cells, bottom panels.
Experimental details are as outlined in legend to Fig. 5.1.
This study was performed two times with a similar pattern of results.

Figure 5.11a Immunogold-VT1B subcellular targeting in SF-539, cells by Electron
Microscopy

Post-embedding immunoelectron microscopy of astrocytoma cell lines,
treated with VT1 for 1h at 37 C°. Fixation and embedding were followed by anti-
VT1B and gold labeled (15 nm) secondary antibody. Some nucleus and nuclear
membrane gold labeling is shown by arrows (mag. 84000X). Experimental details
are presented in Chapter, 2. This experiment was repeated two times with a similar
pattern of results.

Figure 5.11b Immunogold-VT1B subcellular targeting in XF⁺ cells by Electron
Microscopy

Post-embedding immunoelectron microscopy of astrocytoma cell lines,
treated with VT1 for 1h at 37 C°. Fixation and embedding were followed by anti-
VT1B and gold labeled (10 nm) secondary antibody which is depicted by arrows in
the nucleus (mag. 35000X). Experimental details are presented in Chapter, 2. This
experiment was repeated two times with a similar pattern of results.
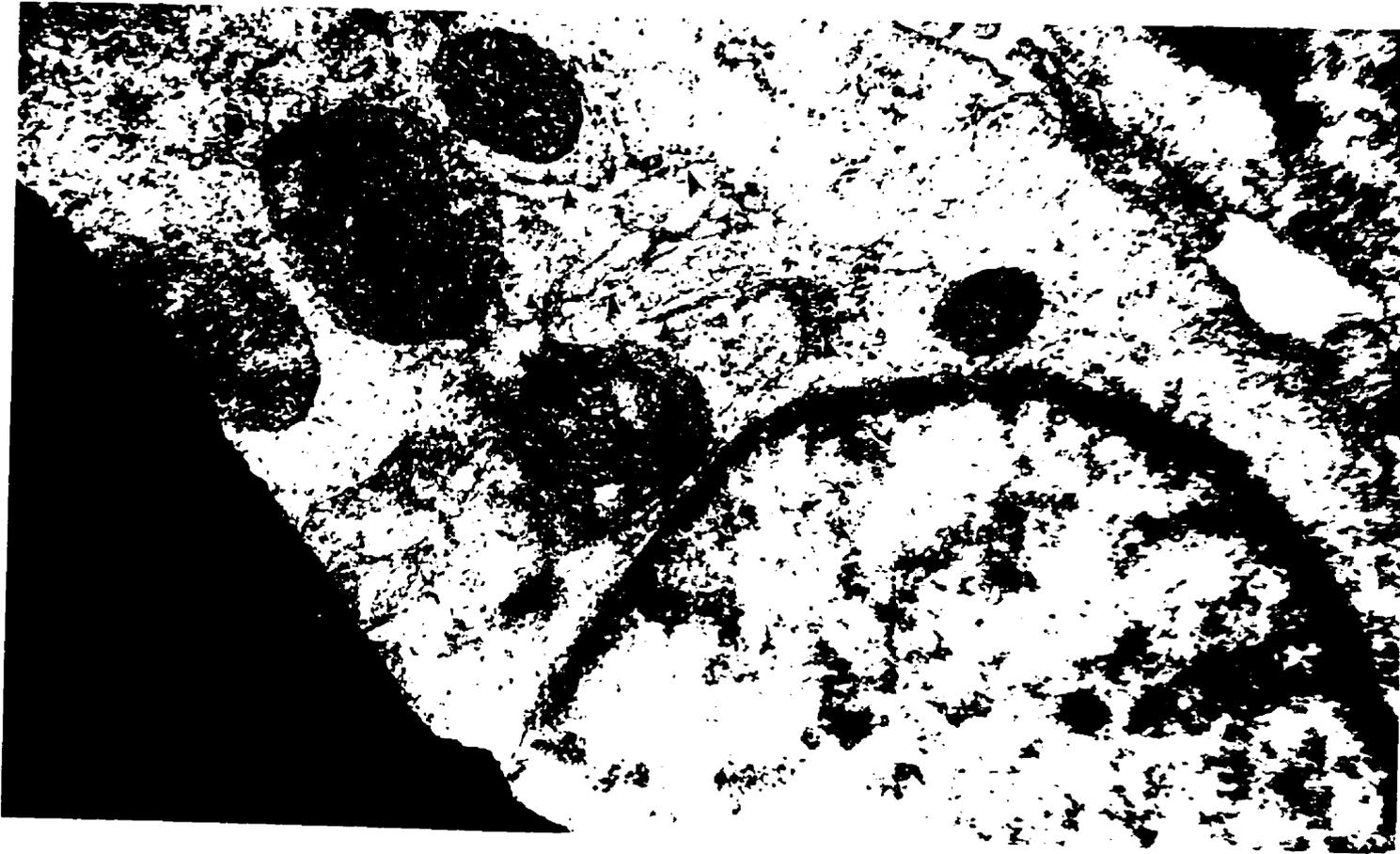
Figure 5.11c   Immunogold-VT1B subcellular targeting in XF-498 cells by Electron
Microscopy

Post-embedding immunoelectron microscopy of astrocytoma cell lines,
treated with VT1 for 1h at 37 C°. Fixation and embedding was followed by anti-
VT1B and gold labeled (10 nm) secondary antibody. Golgi labeling is shown by
arrows (mag. 96000X). Experimental details are presented in Chapter, 2. This
experiment was repeated two times with a similar pattern of results.
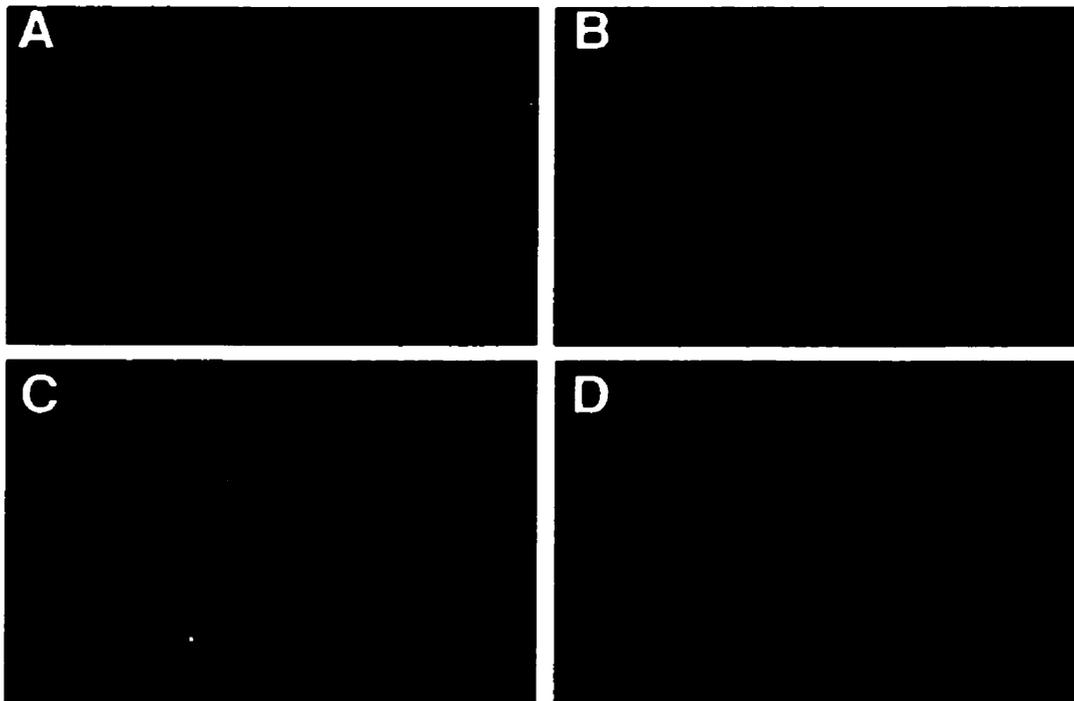
Figure 5.12a Double labeling of lysosomal marker with RITC-VT1B in astrocytoma cell lines

Lysosomal labeling of astrocytoma cells (SF-539) was performed by 24 hrs incubation with FITC-dextran (0.5 mg/ml), the marker for lysosomes. RITC-VT1B was added in the last 1h of the 2hrs chase and incubated at 37° C. RITC-VT1B localization is shown in panels A & C and FITC-dextran localization is demonstrated in panels B & D. Colocalization of the toxin and lysosomal marker was not observed in these cells.

Experimental details for cell preparation are as outlined in legend to Fig. 5.1.
These experiments were performed twice with a similar pattern of results.
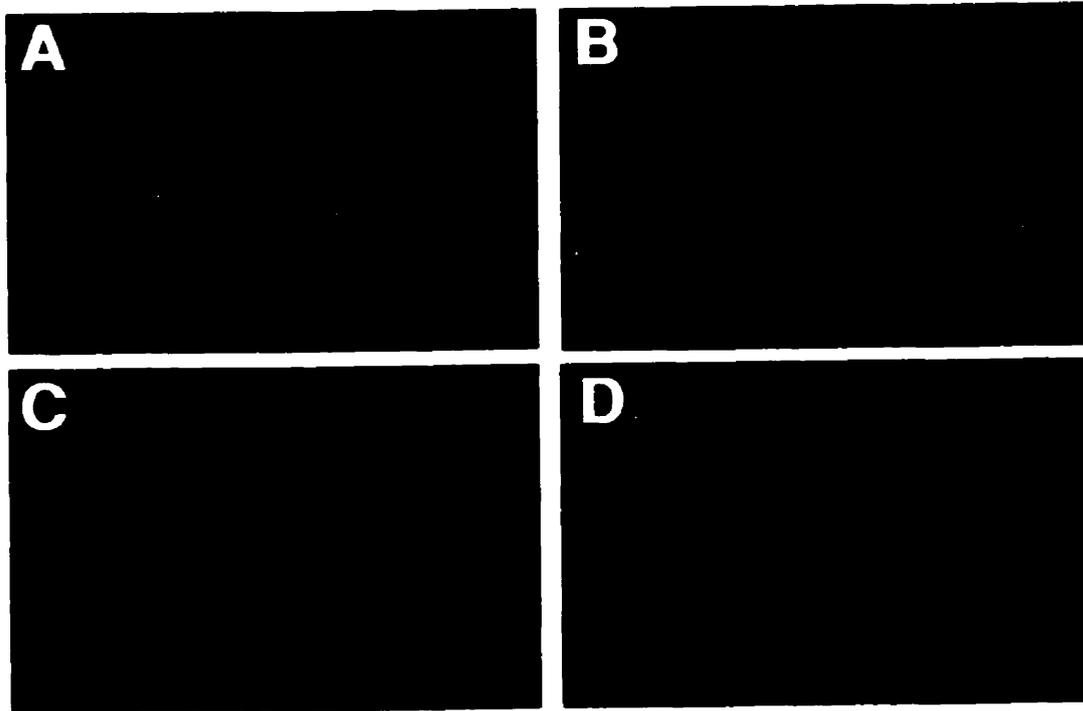
Figure 5.12b double labeling of lysosomal marker with RITC-VT1B in astrocytoma
cell lines

Lysosomal labeling of astrocytoma cells (XF+) was performed by 24 hrs incubation with FITC-dextran (0.5 mg/ml), a marker for lysosomes. RITC-VT1B was added in the last 1h of the 2hrs chase and incubated at 37° C. RITC-VT1B localization is depicted in panels A & C and FITC-dextran localization is demonstrated in panels B & D. Colocalization of the toxin and lysosomal marker was not observed in these cells.

Experimental details for cell preparation are as outlined in legend to Fig. 5.1.

These experiments were performed twice with a similar pattern of results.
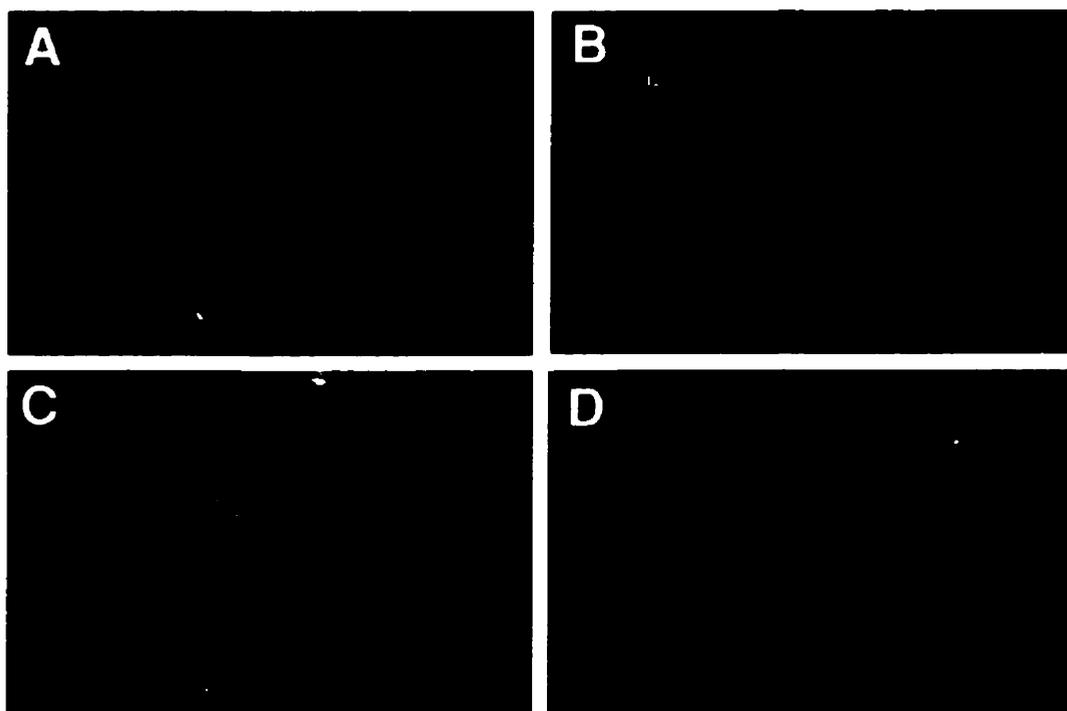
Figure 5.12c   **Double labeling of lysosomal marker with RITC-VT1B in astrocytoma cell lines**

Lysosomal labeling of astrocytoma cells (XF-498) was performed by 24 hrs incubation with FITC-dextran (0.5 mg/ml), a marker for lysosomes. RITC-VT1B was added in the last 1h of the 2hrs chase and incubated at 37° C. RITC-VT1B localization is demonstrated in panels B & D and FITC-dextran localization is shown in panels A & C. Colocalization of RITC-VT1B with FITC-dextran in XF-498 is demonstrated.

Experimental details for cell preparation are as outlined in legend to Fig. 5.1.

These experiments were performed two times with similar pattern of results.

Effect of Gb3 isoform expression on toxin subcellular targeting in other systems

FITC-VT1B internalization was investigated in SKOV3; its MDR variant, SKVLB cell; SKOV3 butyrate treated cells (SKOV3+), and Vero cells in vitro. In all of these systems the pattern of Gb3 isoform detected in SF-539 and/or in XF+ cells was observed. That is the elevated expression of Gb3 with shorter chain fatty acids targeted the toxin to the ER/nuclear envelope with a concomitant increase in cytotoxicity.

5.2.13 Ovarian tumor and MDR cell lines, SKOV3 & SKVLB

A) VT sensitivity Fig. 3.1A demonstrated significantly higher VT1 sensitivity of SKVLB compared to SKOV3.

B) Receptor isoform expression and fatty acids analysis of Gb3 The increased level of Gb3 isoforms with shorter fatty acid chains in SKVLB cells as compared to SKOV3 cells, is depicted in Fig. 3.2. To further confirm an increase in shorter chain fatty acids in Gb3 in SKVLB cells as compared to SKOV3 cells, fatty acid analysis was performed on a Gb3 sample from SKOV3 and SKVLB cells to determine the percentage of each fatty acid in Gb3. The results are presented in table 5.3. Significant differences exist in fatty acids 16:0 and 18:0 which are elevated in Gb3 from SKVLB cells and 22:0, 24:0, and 24:1 fatty acids that are significantly elevated in Gb3 from SKOV3 cells (Table 5.3).

C) Subcellular VT targeting Fig. 5.13 demonstrates the differential targeting of toxin in these two cells by fluorescence (Fig. 5.13a &b) and confocal microscopy (Fig. 5.13c). In SKVLB cells the toxin targets the nuclear membrane and the nucleus whereas in SKOV3 cells the majority of toxin is in a perinuclear location, suggestive of Golgi.
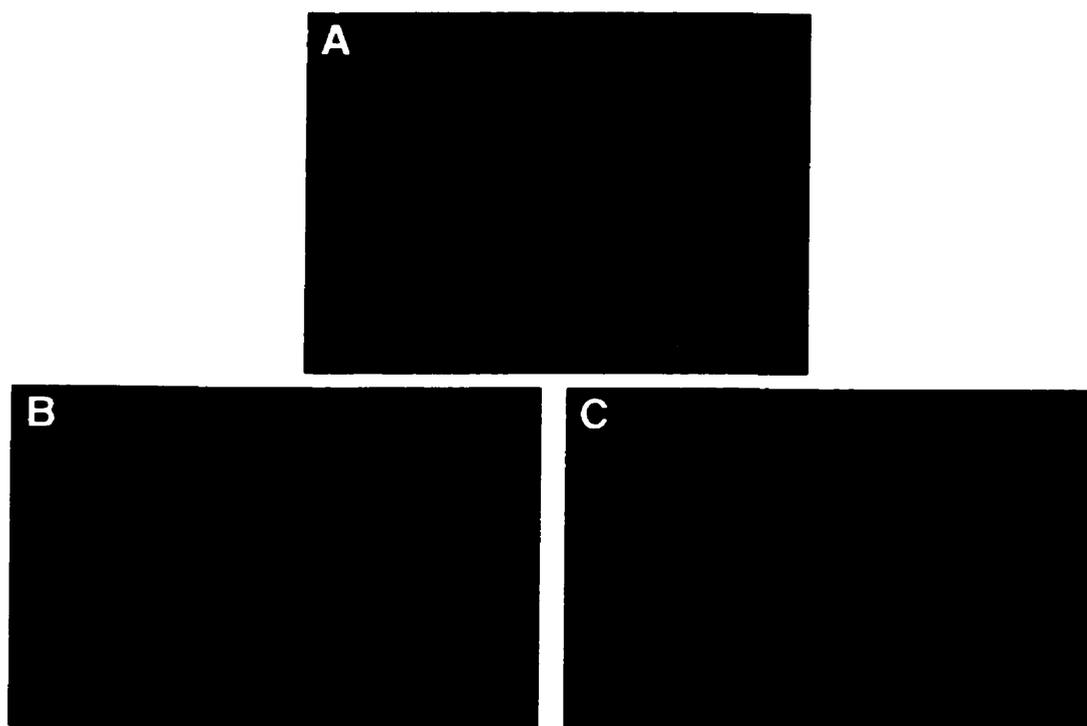
Figure 5.13a Study on FITC-VT1B subcellular targeting by Fluorescence Microscopy in SKOV3 cells

Ovarian tumor cells (SKOV3) grown on cover slips were incubated with FITC VT1B at 4°C(A) or 37°C(B,C) for 1 h and visualized under fluorescence microscopy. FITC-toxin is mainly targeted to a perinuclear location. Experimental details outlined in chapter 2.

This experiment was repeated four times with a similar pattern of results.
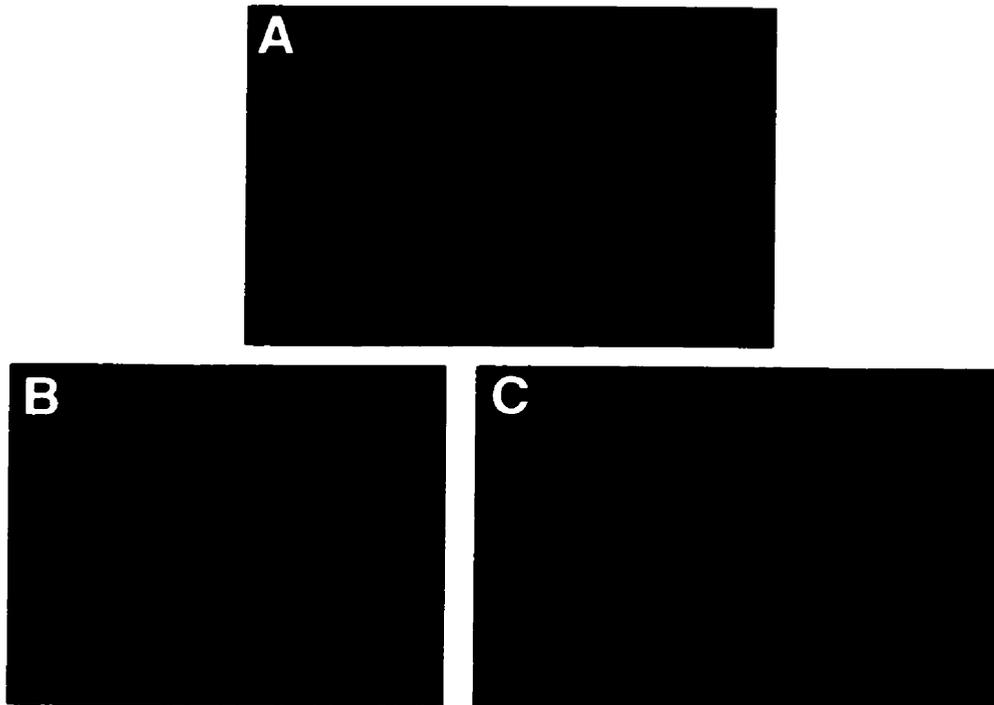
Figure 5.13b   Study on FITC-VT1B subcellular targeting by Fluorescence
              Microscopy in SKVLB cells

Ovarian MDR tumor cells (SKVLB) grown on cover slips were incubated
with FITC-VT1 or FITC VT1B at 4°C (A) or 37°C (B,C) for 1 h and visualized under
fluorescence microscopy. FITC-toxin is mainly targeted to the nuclear membrane.
Experimental details outlined in chapter 2.
This experiment was repeated four times with a similar pattern of results.
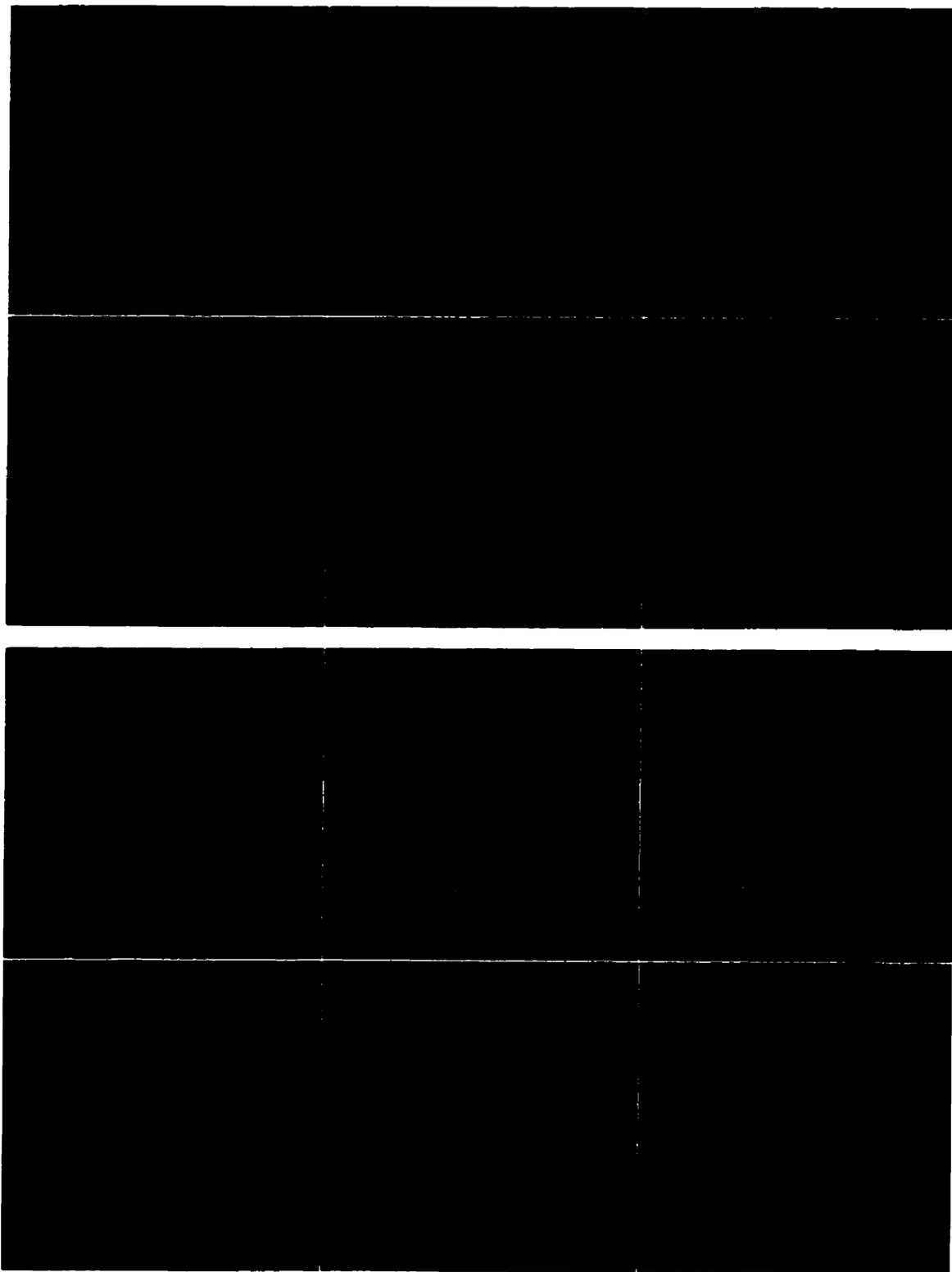
Figure 5.13c   Comparison of FITC-VT1B subcellular targeting in SKOV3 and
SKVLB cells by Confocal microscopy

Targeting of FITC-VT1B in ovarian carcinoma and MDR cells, studied by
confocal microscopy. x vs z (cross-sectional) scan (1-6) are illustrated in top panels,
for SKVLB cells where ER/nuclear targeting is shown; in bottom panels, for SKOV3
cells, where mainly perinuclear localization is demonstrated.

5.2.14 Ovarian tumor cells treated with butyrate

A) VT sensitivity. To investigate whether the effect of butyrate in cell sensitization and alteration in intracellular VT targeting from Golgi to ER/nuclear membrane and the correlation between toxin targeting and cell sensitivity could be observed in other systems, ovarian carcinoma cells were treated with 2mM butyrate for 24h and their sensitivity to VT1 was investigated. Fig. 5.14a shows the sensitizing effect of butyrate on SKOV3 cells with a CD50 of <0.05 ng/ml and over 98% cell death at 50 ng/ml observed in butyrate treated cells as compared to CD50 of about 50 ng/ml and about 40% cell death in untreated SKOV3 cells (more than 1000 fold increase in sensitivity to VT1).

B) Receptor isoform expression Fig.5.14b shows VT TLC overlay of Gb3 expression in SKOV3$^+$ cells. Elevated level of Gb3 level with higher intensity of lower band is suggestive of the elevated level of shorter fatty acid chain length in Gb3 isoform in SKOV3$^+$ (butyrate treated). Further fatty acid analysis is required to conclusively demonstrate the elevation of shorter chain fatty acids in Gb3 from SKOV3+ cells.

5.2.15 Vero cells treated with butyrate

To further explore the possibility of hypersensitizing other cells to VT1 by altering the targeting of VT1 from Golgi to ER/nuclear membrane, and the role of Gb3 fatty acid isoforms in this process in Vero cells (African Green Monkey's kidney cells) were tested. Vero cells, in which VT1 targeting of the Golgi was apparent (Fig. 5.15c), were treated with butyrate and its effect on receptor isoform expression, VT targeting inside the cell, thus the sensitivity to VT was examined.

A) VT sensitivity. Butyrate treatment causes a rapid response to VT cytotoxicity. Vero cells are sensitive to VT, but the cytotoxicity can not be detected in less than 48hrs. Vero cells which are routinely used to detect VT cytotoxicity, must be
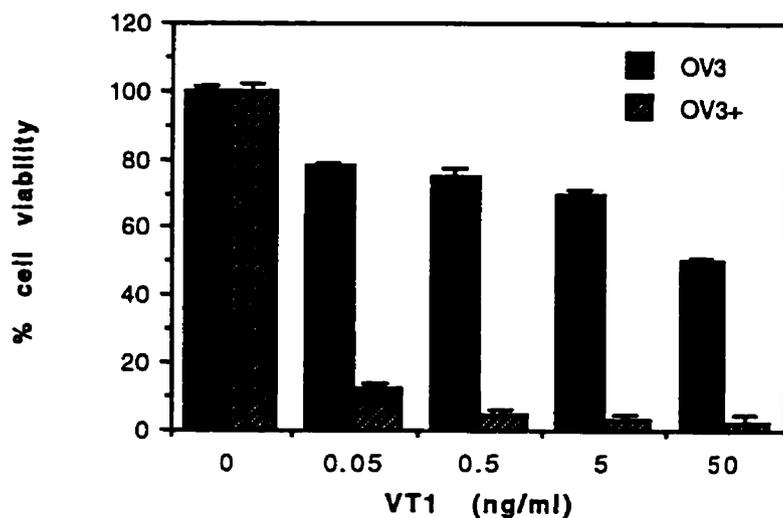
Figure 5.14a Study on VT1 cytotoxicity in ovarian tumor cells treated with butyrate

SKOV3⁺ (butyrate treated) cells were seeded in 96-well plates, experimental details are as outlined in legend to Fig. 5.2. Serial dilutions of VT1 was added to the cells with or without butyrate, and incubated for 3 days. SKOV3⁺ cells show elevated level of cytotoxicity to VT1 as compared to untreated cells (SKOV3). Each value represents the mean ± S.D. of triplicate wells.
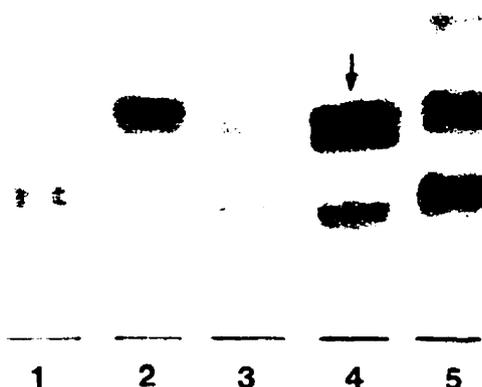
Figure 5.14b Study on receptor expression in ovarian tumor cells treated with butyrate

Glycolipids were extracted from SKOV3 and SKOV3+ cells as described in Chapter 2. An elevated level of Gb₃ was observed in butyrate treated SKOV3 cells (arrow). Gb₄ , as a negative control (1 ug, lane 1) and Gb₃, as a positive control (2 ug, lane 2), neutral glycolipids from untreated SKOV3 cells (lane 3), butyrate treated SKOV3 cells (lane 4), and kidney glycolipid as a standard (lane, 5).

FAME	Gb3 (SKVLB)	Gb3 (SKOV3)
12:0	1.33	1.54
14:0	3.24	5.74
16:0	38.34	26.71
16:1n7	3.85	1.25
18:0	34.37	18.21
18:1n9	12.84	7.69
18:1n7	3.72	
18:2n6	1.20	1.92
20:0	1.02	2.00
22:0	0.64	7.49
24:0	1.29	12.26
24:1n9	0.84	15.00
22:6n3	1.27	

Table 5.3   Analysis of Gb3 fatty acids purified from SKOV3 and SKVLB cells

FAME analysis of Gb3 isoforms from SKOV3, ovarian carcinoma and MDR variant cells, SKVLB, confirms the results depicted in Fig. 3.2. Level of short chain fatty acids show significant increase, accompanied by a decrease in long chain fatty acids in Gb3 from SKVLB cells as compared to Gb3 from SKOV3 cells.

incubated with VT for 72 hrs, however in Vero cells treated with butyrate 100% cell death was observed by 24 hrs incubation (Fig. 5.15a).

B) Receptor isoform expression. Shorter fatty acid chain expression of Gb_3 in Vero cells treated with butyrate is depicted in Fig. 5.15b.

C) Subcellular VT targeting. Increased nuclear targeting of toxin was observed in Vero+ cells and is demonstrated in Fig. 5.15c as compared to targeting of VT in untreated cells.

Collectively all the results presented are in support of the finding that subcellular targeting of VT is the key to sensitivity in these systems and the Gb_3 isoform with elevated short chain fatty acids is the determinant of the target in subcellular location of VT. Nuclear (membrane and/intranuclear) targeting is correlate of rapid induction of apoptosis and elevated sensitivity to VT, whereas lysosomal and/or Golgi targeting correlate lower sensitivity to VT.

Preliminary Results

In this part some preliminary results are presented that need further experimentation to lead to a conclusion.

5.2.16 Combinatorial effect of Gb_3 primary antibody and VT1 cytotoxicity in cancer human cells

In this part of the study two cell lines originally (before butyrate treatment), hypersensitive to VT (astrocytoma, SF-539 and SKVLB ovarian carcinoma cells) were chosen to investigate the effect of anti-Gb_3 primary antibody and VT1 together. In both cell lines, particularly SKVLB, the apoptotic effect of VT1 was accelerated in presence of primary antibody as detected by fluorescence microscopy (Fig. 5.16a & b). This may be suggestive of Gb_3 ligation being involved in this rapid response and also a separate epitope of Gb_3 being involved in anti-Gb_3 and VT binding. However, the anti-Gb_3 antibody alone as a control was not tested. Follow up on this preliminary results is necessary to reach a conclusion.
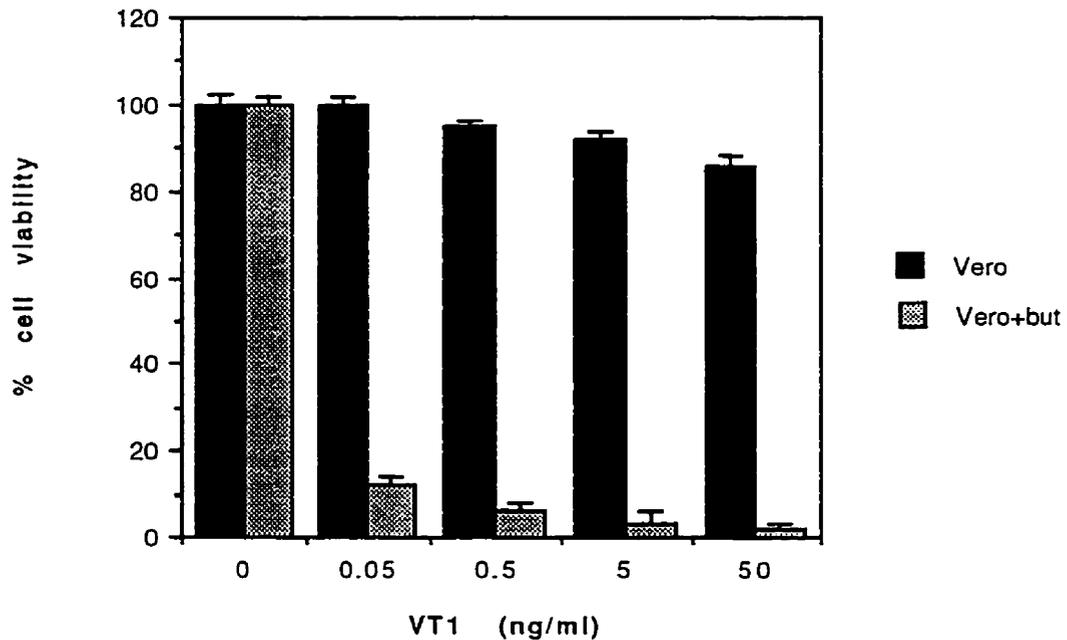
Figure 5.15a Study on VT1 cytotoxicity in butyrate treated vero cells

Vero and Vero⁺ (butyrate treated,2mM, 24hrs) cells were seeded and incubated with serial dilutions of VT1 for 24hrs. Experimental details are as outlined in the legend to Fig. 5.2 Vero⁺ cells show faster response to cytotoxicity of VT1.

Each value represents the mean ± S.D. of triplicate wells.

133
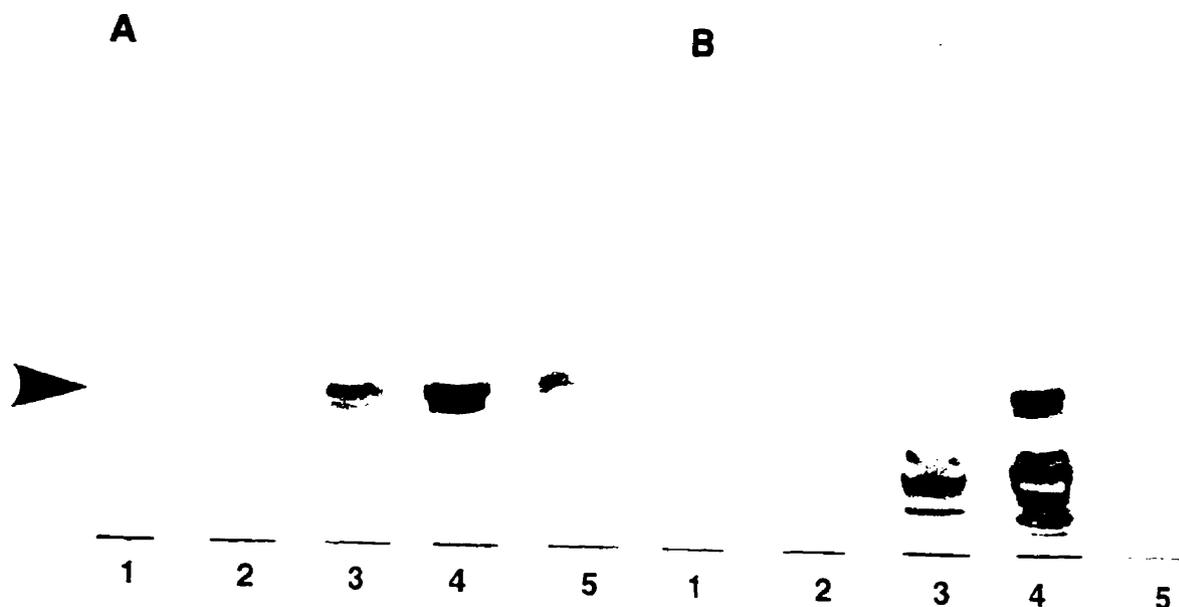
Figure 5.15b Study on Gb₃ expression in butyrate treated vero cells

Glycolipids were extracted from approximately equal numbers of Vero and Vero+ cells as described in Chapter 2. Elevated level of Gb3 was observed in butyrate treated Vero cells (arrow). VT1 TLC overlay (panel A) and orcinol stained (panel B). Gb4, negative control (1 ug, lane, 1), Gb3, positive control (1 ug, lane, 2), glycolipids from Vero (lane, 3), Vero + butyrate (lane, 4) cells, and kidney glycolipids as a standard (lane, 5) .
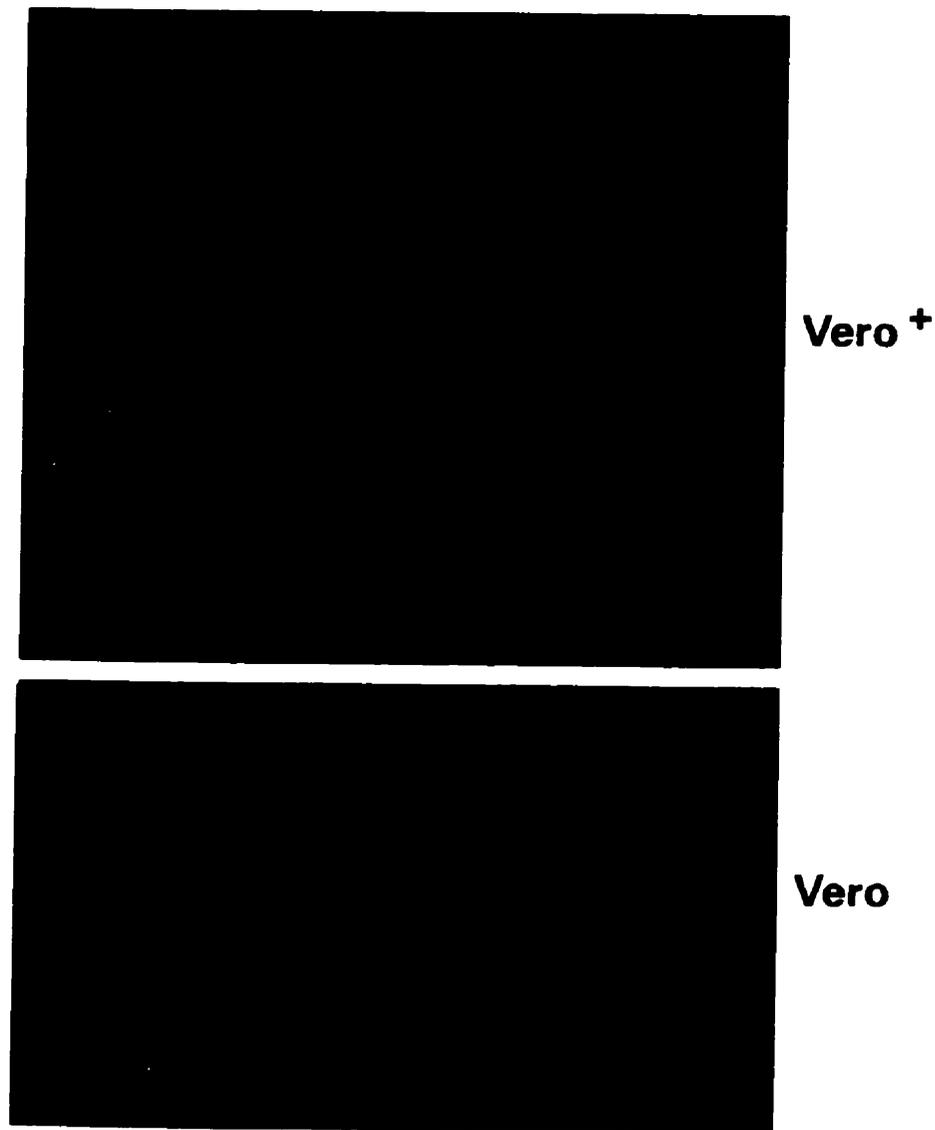
Vero [+]

Vero

Figure 5.15c Study on FITC-VT1B subcellular localization in vero cells treated
with butyrate

Pattern of FITC-VT1B targeting in butyrate treated Vero cells shows increased
diffusion and ER/nuclear envelope targeting of FITC-VT1B (top panel, Vero[+]) as
compared to untreated Vero cells (bottom panel, Vero) with significant Golgi
targeting of the toxin. Butyrate treatment altered the toxin targeting in these cells.
Experimental details are as outlined in chapter 2.
These experiments were repeated twice with a similar pattern of results.
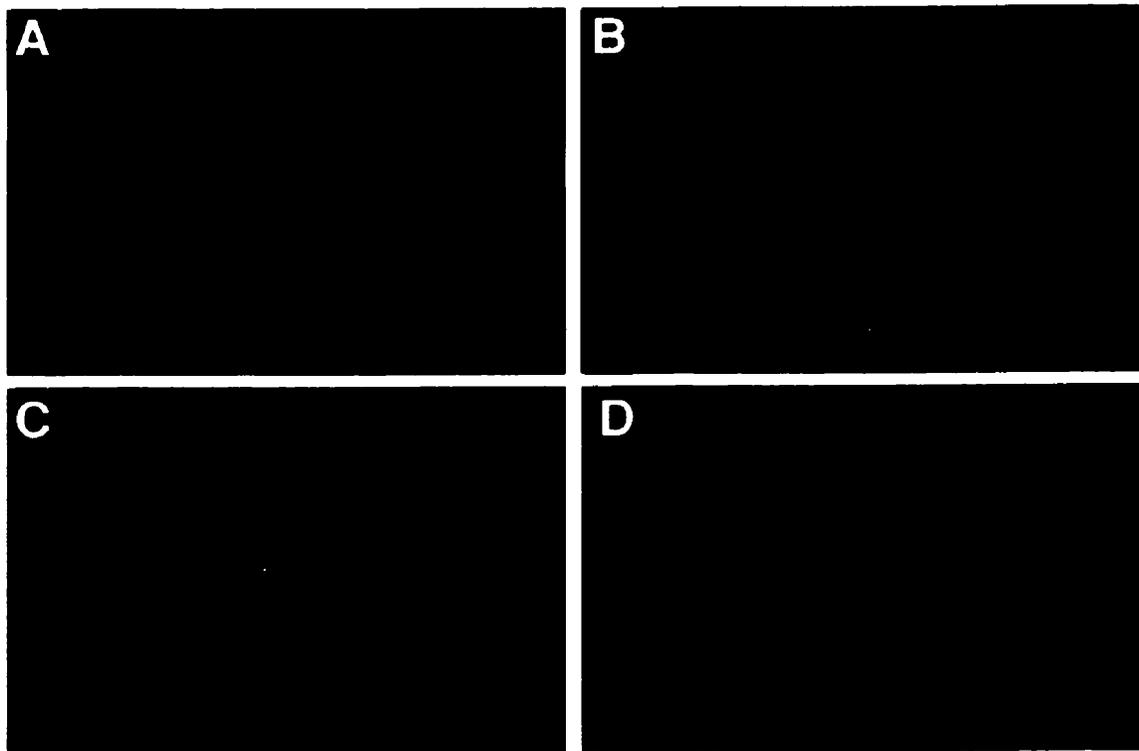
Figure 5.16a Additive effect of Gb₃ primary antibody to VT1 cytotoxicity in astrocytoma cells

SF-539 cells were treated with VT1 (10 ng/ml) and primary antibody to Gb₃ (1:20) at 37C° for different periods, A (1h), B, (2hrs), C, (3 hrs), stained with secondary anti-rat-FITC and D, (6 hrs), RITC-VT1. This combination with VT1 accelerated the cytotoxic effect of VT1 in SF-539 cells and apoptotic features (e.g. nuclear condensation and membrane blebbing) was detected by fluorescence microscopy (D).
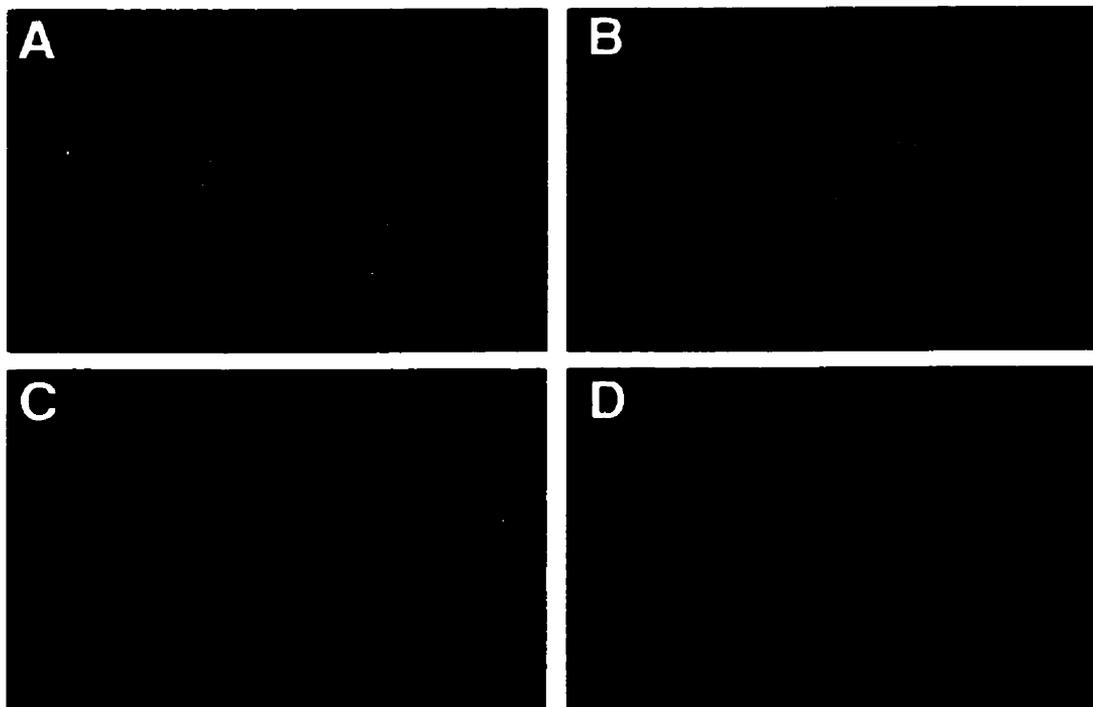
Figure 5.16b Additive effect of Gb3 primary antibody to VT1 cytotoxicity in MDR

ovarian carcinoma cells

SKVLB cells were treated with VT1 (10 ng/ml) and primary antibody to Gb3 (1:20) at 37C° for different periods, A (1h), B, (2hrs), C, (3 hrs), and D, (6 hrs), stained with secondary anti-rat-FITC. Apoptotic bodies are depicted in panel D.

5.2.17 F-actin rearrangement in astrocytoma cells in response to VT1 treatment

Phaloidin, a member of phallatoxins can bind F-actin at nanomolar concentrations and is water soluble, thus providing convenient probes for labeling F-actin in tissue sections and cell cultures (Iwig, et al., 1995). Phaloidin-RITC was used to investigate the effect of VT1 treatment on actin rearrangement in astrocytoma cells, SF-539. Prominant effect of VT1 treatment is demonstrated in Fig. 5.17b (C&D panels). However, this is a preliminary result and further study is necessary to reach a conclusion in this regard.
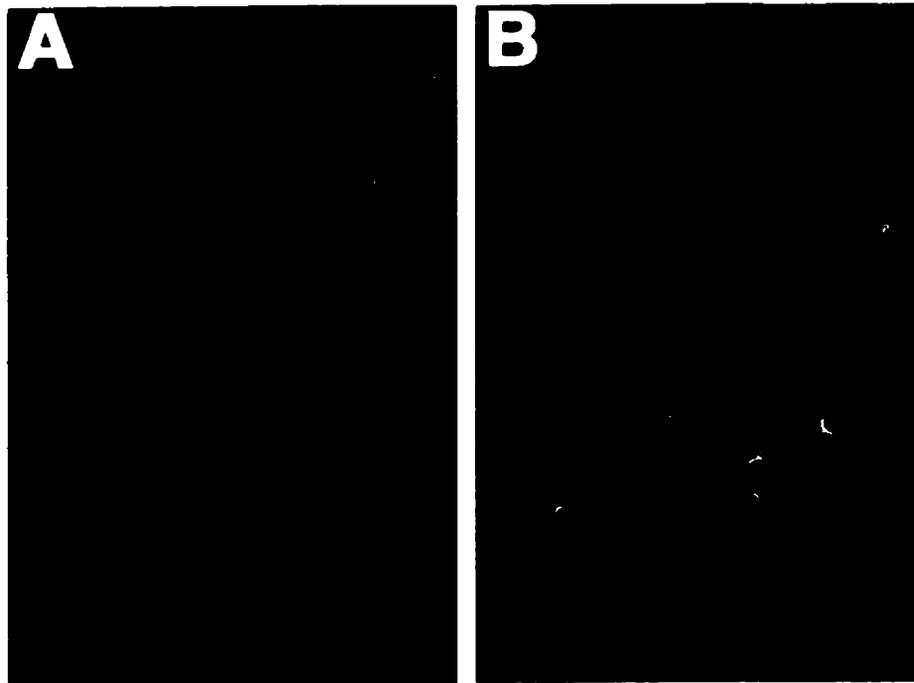
Figure 5.17a Double labeling of astrocytoma cells with Phaloidin-RITC and
VT1B-FITC

A   Phaloidin-RITC labeling of SF-539 cells
B   VT1B-FITC labeling of SF-539 cells
Actin filaments are uniformly distributed in the cells (A), and accumulation of
FITC-VT1B in nuclear membrane is clearly demonstrated (B).
Experimental details in Chapter, 2.
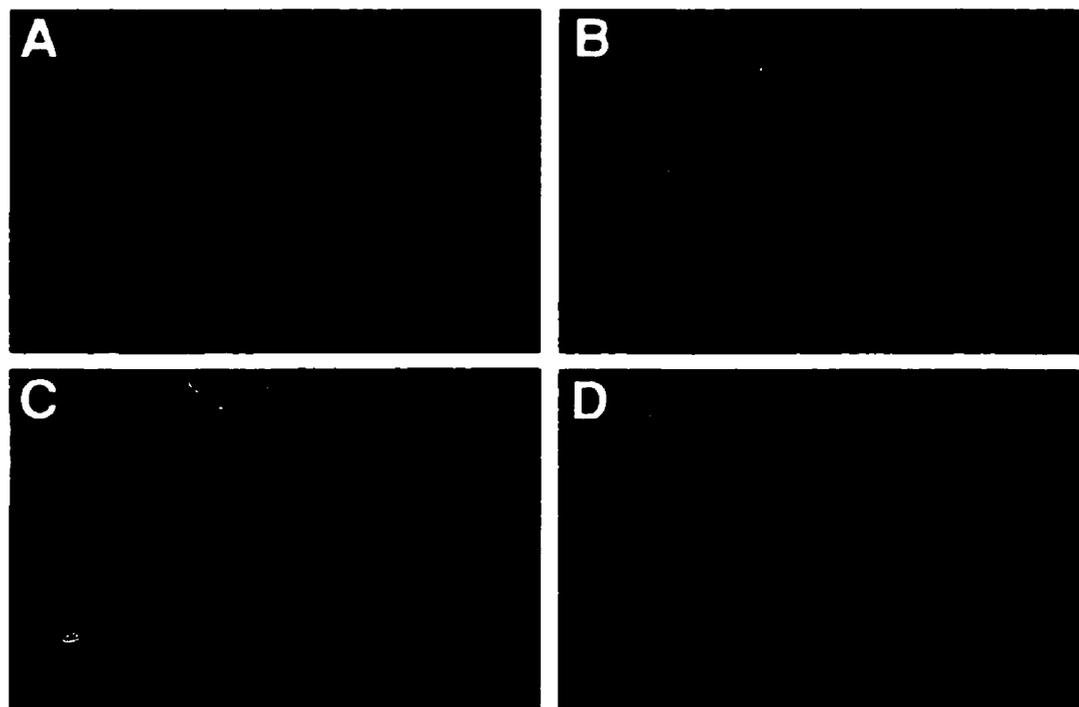
Figure 5.18b Phaloidin-RITC labeling of VT1 treated and untreated astrocytoma
cells

A&B Phaloidin-RITC localization in SF-539 not treated with VT1 is depicted in
panels A and B.

C&D Phaloidin-RITC localization in VT1 (10ng/ml) treated SF-539 cells is depicted
in panels C and D. F-actin rearrangement is significant.

5.3   DISCUSSION and SUMMARY

The differential sorting and subcellular localization of verotoxin following RME were studied in the following human Gb3 positive cell systems: astrocytoma cell lines with markedly different sensitivity to VT1, an human astrocytoma cell line with induced VT hypersensitivity by culturing in the presence of sodium butyrate, and multiple drug resistant mutants with increased sensitivity to VT as compared to parental human ovarian carcinoma cells. In all cases, an alteration in the intracellular routing of the receptor-bound internalized toxin from the Golgi/lysosomal pathway to that of retrograde transport to the nuclear envelope resulted in over a 5000 fold increase in cell sensitivity to VT. This change in intracellular routing may be due to sorting of Gb3 isoforms, since retrograde nuclear targeting was found to correlate with the synthesis of Gb3 containing elevated levels of shorter chain, and decreased levels of longer chain fatty acid species. Nuclear targeting was enhanced rather than reduced by Brefeldin A suggesting that retrograde transport of Gb3 to the nucleus is independent of Golgi function. Brefeldin A treatment induced nuclear toxin targeting in cells which undergo retrograde transport to the Golgi but not beyond. It has been shown that BFA inhibits the cytotoxicity of various toxins (Yoshida, 1991; Donta, 1993). In addition, a recent study has shown that cytotoxicity of VT1 was inhibited by BFA treatment in HeLa cells (Donta et al., 1995). However, in the present study an opposite effect of BFA on XF-498 cells was observed. In agreement with the present finding, it was shown that BFA treatment of HepG2 cells did not block retrograde transport from TGN (trans Golgi network) to ER (Strous, et al, 1993). In addition, BFA, as an inhibitor of coatomer binding, was shown to enhance retrograde transport while inhibiting anterograde transport (Lippincott, 1993). BFA treatment of HeLa cells was able to induce retrograde transport (Ivessa et al., 1995). Based on the results from the present studies, the existence of a direct vesicular traffic of Gb3

from the cell surface to the nuclear membrane or alternatively from the cell surface to an intermediate compartment of ER-Golgi is proposed.

Although the presence of Gb3 in a given cell is a good indicator of its sensitivity to VT cytotoxicity (Waddell, et al., 1988), the absolute concentration of Gb3 does not always indicate the relative sensitivity to VT (Jacewicz, et al., 1994). This was found for the human astrocytoma cell lines. Butyrate is a naturally occuring 4-carbon fatty acid which is found in some foods and is produced in the mammalian digestive tract by microbial fermentation of carbohydrates and endogenous substrates, such as mucus (Bergman, 1990). The principal short chain fatty acids with rapid absorption are acetate, propionate, and butyrate (Bergman, 1990). In this regard it will be interesting to investigate the effect of VTEC in production of these short chain fatty acids and their local and systemic effects on Gb3 expression, and the elevation of sensitivity to VT.

In addition to its multiple effect on mammalian cells from growth to gene expression, sodium butyrate has been shown to activate MAP kinase and as a result cell proliferation (Rivero et al., 1996). In a recent study butyrate enema therapy was shown to stimulate colonic repair by stimulating proliferation of epithelial cells of crypts (Kripke et al., 1989; Butzner et al., 1996). In the present study, butyric acid treatment of different cell lines elevated levels of Gb3 expression with a significant preferential increase in shorter fatty acids and the cells sensitivty to VT1 was significantly increased.

The fatty acid composition of the receptor (Gb3) may be important for its sorting to the nucleus. It has been shown that variation in the ceramide composition can affect sphingolipid sorting in the secretory pathway in epithelial cells (van der Bijl et al., 1996). This sorting process is lipid specific. Lipid transport routes and sorting are related to cell differentiation (van Genderen & van Meer,1995). Both retrograde transport and intoxication with Shiga toxin was shown to be induced by cAMP, supporting the idea that toxin sorting is regulated by

physiological signals and the fatty acid composition of Gb3 is involved in this signal transduction. Endocytosis of VT with its GSL receptor is a unique system which will provide a useful model to study both proteinaceous ligand and GSL fate in endocytic protein and lipid trafficking. VTs exclusively and specifically recognize Gb3 and therefore provide a method that allows the study of the flow of endogenous unlabeled glycolipid.

Taken together, these results suggest the existence of an optimum acyl chain length for the ability of Gb3 to target verotoxin to the nucleus and induce apoptosis. The ultra-sensitive SF-539 astrocytoma, XF+, SKOV3+, Vero+ cells and MDR ovarian cells show intracellular targeting of FITC-B subunit of VT1 to the nuclear membrane whereas the least sensitive XF-498 astrocytoma and SKOV3 ovarian carcinoma cells do not demonstrate such nuclear binding. Thus, intracellular targeting of the nuclear envelope by retrograde transport appears to determine cell hypersensitivity to verotoxin and sodium butyrate induces nuclear toxin targeting via globotriaosyl ceramide fatty acid isoform traffic.

(The results of this study are being prepared in a manuscript for J. Cell Biology.)

Chapter 6

Verotoxin Receptor Expression
in Ovarian Tumors and Metastases:
Implication for cancer therapy and
inhibition of angiogenesis

6.1 INTRODUCTION

The evidence presented thus far has shown the potential efficacy of VT1 as an anticancer agent in vitro. The elevation of a specific receptor in ovarian tumor and MDR cell lines was the path leading to investigation of the level of receptor expression in human ovarian tumor samples. Cancer of the ovary is the leading cause of death from neoplasia of the female reproductive tract and diagnosis at a late stage and resistance to chemotherapy rank among the most important therapeutic problems in its management (Barber, 1993). Approximately half of the patients presenting with the advanced-stage disease are resistant to conventional chemotherapy (Ozols and Young, 1994). Moreover, resistance to chemotherapy in these patients appears to be a complex and multifactorial problem (Perez and Hamilton, 1993). Since the introduction of the platinum chemotherapy agents twenty years ago, there has been little progress in the treatment of ovarian carcinoma. This is reflected in the fact that the survival rate in 1991 was only slightly better than in the past two decades (Barber, 1993). These statistics indicate the need for alternate treatment regimes. Since large scale screening for early detection of ovarian cancer is not financially feasible (Barber, 1993), the need for improved treatment becomes more vital.

This part of the localization study, was designed to investigate the histochemistry of VT binding and analysis of Gb_3 content of primary ovarian tumour tissue samples and metastases as a function of tumor type and clinical outcome.

(The results from this study will be presented in a paper)

6.2 RESULTS

6.2.1 VT1 receptor level in primary ovarian tumors and metastases

Gb_3 is essential for VT1 binding, internalization, and VT-induced cytotoxicity (Waddell, et al., 1990). The level of expression of Gb_3 specific isoforms in tumors

may provide a marker to index potential susceptibility to VT treatment. Gb₃ expression was investigated in different ovarian tumors , metastases and normal ovaries (Fig. 6.1 & Table 6.1). Altogether, 39 samples, including epithelial ovarian tumors and endometrioid cysts and 10 normal ovary controls, were selected for Gb₃ analysis in this study. The presence of Gb₃ was detected by VT1 TLC overlay and quantitated in comparison to a renal Gb₃ standard.

Normal ovaries. In the normal ovarian tissue samples, Gb₃ was completely absent in 2 of the 10 samples and was present at concentrations 0.0- 0.3 μg per 10 mg tissue in the other 8 samples (lanes, 1-10).

Serous tumors. Gb₃ was present in all five serous cyst-type adenomas (lanes 19-23), ranging in concentration from less than 0.5 μg/10mg tissue to >2.0 μg/10mg per tissue. The two cystadenoma samples (lanes, 19 &23: 2-3μg) had higher concentrations of Gb₃ than the cystadenofibroma samples (lanes, 20-22: 0.3-1μg). Gb₃ expression in the serous adenocarcinoma was more variable. The four serous papillary adenocarcinomas samples that were moderately differentiated (lanes 12 & 16-18) had barely detectable Gb₃ whereas those samples which were poorly differentiated (lanes 11 &15) had Gb₃ present in higher concentrations (0.5-1.0 μg per 10 mg tissue). The samples from moderately differentiated serous adenocarcinoma tumors (lanes, 13 &14) whose origin was uncertain (either ovarian or peritoneal) had a Gb₃ concentration of 3 μg per 10 mg tissue equivalent.

Endometrioid tumors. Gb₃ was present in concentrations ranging from 0.5 - 1.5 μg 10 mg tissue equivalent in the four endometrioid cyst samples and endometrioid carcinoma (lanes 24-28).

Mucinous tumors. Gb₃ was detected in 3/4 mucinous cystadenomas (lanes 29-32) at low concentrations of 0.1-0.3μg/10 mg tissue but increased in a mucinous cystadenocarcinoma (lane 33).

(The extraction of glycolipid from some of the above samples were performed by a summer student, Elizabeth Russel.)

Metastases . The level of Gb3 was examined in ovarian metastases to the colon, the small bowel and omentum, A tumor of unknown origin which had metastasized to the ovary was also studied. The expression of Gb3 was high in all the metastatic(drug resistant) tissues analyzed (even relative to primary ovarian tumor tissue) ranging from 3-8 μg per 10mg tissue (Fig. 6.2, lanes 5-10). Multiple drug resistant primary tumors showed higher Gb3 content relative to drug sensitive counterparts (Fig. 6.2 lanes 5-7,9,10 cf 8). In particular, the more slowly migrating isoforms of Gb3, likely containing shorter fatty acid chains within the lipid moiety, were elevated.

These data are summarized in table 6.1.

Table 6.1   Summary of the results in Fig. 6.1 and 6.2.

Tissue sample	Gb3 content(μg/10mg) ±SD	Number of samples
Normal ovary	0.1±0.1	10
Serous tumour	1.5±0.8	13
Mucinous tumour	0.18±0.17	5
Endometrioid tumour	1.2±0.7	5
Metastases	5.9±2.1	6
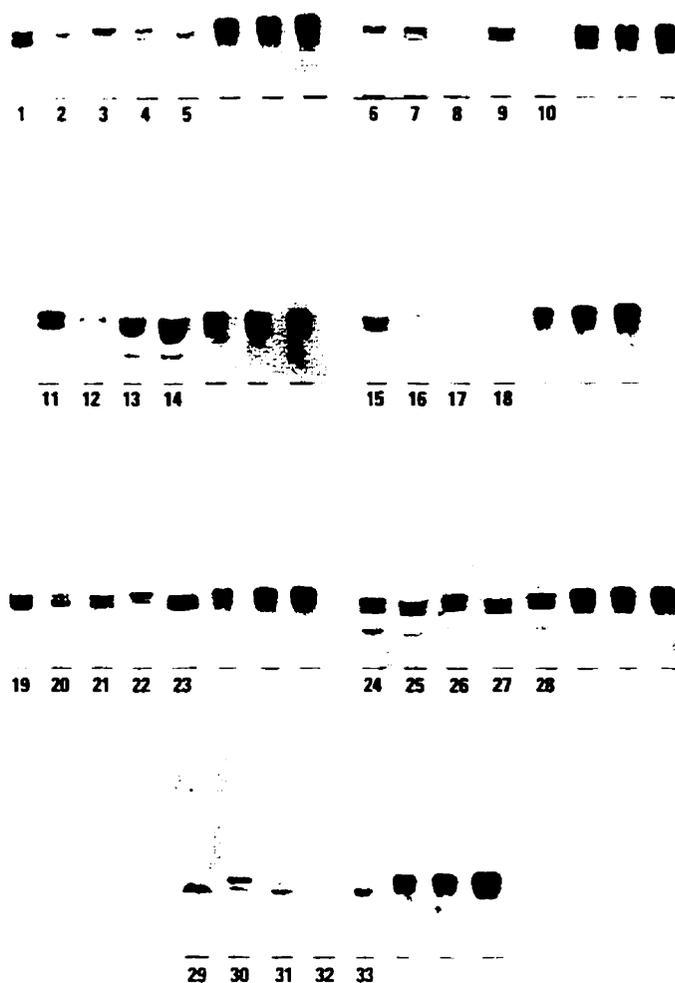
Figure 6.1   VT-1 TLC overlay of ovary and ovarian tumor glycolipids

'Normal' ovaries (lanes 1-10); Serous adenocarcinomas (lanes 11-18)-poorly (11 &15) and moderately differentiated serous papillary adenocarcinomas (12 & 16-18) and moderately differentiated serous adenocarcinoma (13 &14); Serous cyst adenomas (lanes 19 and 23) and cystadenofibromas (20-22); Endometriotic cysts (lanes 24-27) and an endometrioid adenocarcinoma (lane 28); Mucinous cystadenomas (lane 29-32) and a mucinous cystadenocarcinoma (lane 33).
The glycolipid samples (equivalent to 10 mg of tissue) were subjected to VT1 tlc overlay. Human renal Gb3 standards (1, 2, 3 µg) were run in the last three lanes of each plate.

6.2.2 FITC-VT1B overlay of ovarian tumor and metastatic frozen sections

Treatment of frozen ovarian or metastatic tumor sections with FITC-VT1B showed selective toxin binding to the tumor cells (Figs. 6.3-6.7). In addition, the lumen of blood vessels within and adjacent to the tumor mass showed extensive VT binding (Figs. 6.3 C,G, 6.5, 6.7 EF). In general, binding correlated with the levels of Gb3 extracted from the tissue. Poorly differentiated tumors generally expressed more Gb3 (Fig. 6.1 lanes 11,15) and showed marked FITC-VT1B binding (Fig.6.3 A,C, 6.7 A). Exceptions were noted however for differentiated tumors in which clinical outcome was unexpectedly poor, due to the rapid onset of a multidrug resistant phenotype (Fig. 6.2 lane 7, Fig. 6.3 I). In such MDR cases, extensive binding of the toxin to the differentiated tumor cells was seen (compare Fig. 6.3 E with I).

Little or no staining of VT1 B was seen in sections of normal ovary or normal bowel (Fig. 6. 4 & 6.8). Significant labelling of FITC-VT1B was observed in metastatic tissue (Figs 6.5, 6.6), correlating with high Gb3 content (Fig. 6.2). Ovarian metastases to the colon and small intestine were investigated. Extensive lumenal binding of invading blood vessels was observed (Fig. 6.5) and tumor cell foci showed selective binding while stromal cells remained VT unreactive (Fig. 6.6). Necrotic tissue was not stained (Fig.6.3 C). Significant binding of tumor foci and lumen of the blood vessels was observed in sections from poorly differentiated papillary serous tumor (Fig. 6.7A) and its metastasis to omentum (Fig. 6.7 C & D). Marked RITC-VT1 binding was observed (Fig. 6.9B) to the ascites cells from an ovarian carcinoma that were labeled with FITC-labeled epithelial marker (Fig.6.9A). These ascites cells expressed elevated level of Gb3 (data not shown).
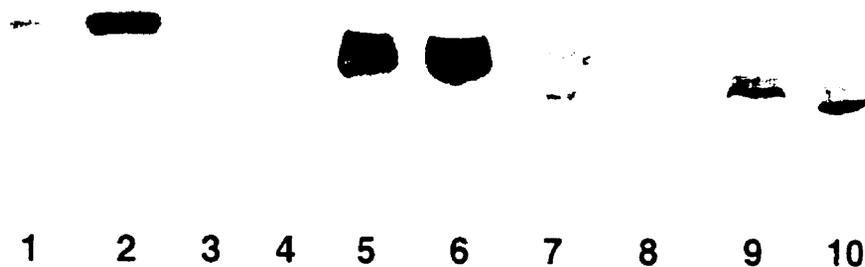
Figure 6.2 VT-1 TLC overlay of glycolipids from ovarian tumors and metastases

The neutral glycolipid fraction (10 mg tissue equivalents) from surgically removed metastatic ovarian tumors was separated by tlc and VT-1 toxin overlay was carried out. Lane 1: 0.5 μg Gb3 standard, lane 2: total human renal glycolipids, lane 3: 0.5 μg Gb4 standard, lane 4: normal ovary, lane 5: MDR small bowel metastasis, lane 6: MDR colon metastasis, lane 7: Differentiated papillary serous MDR ovarian tumor, lane 8: omentum metastasis (not MDR), lane 9: papillary serous MDR ovarian primary tumor, lane 10: MDR ascites tumor (from same patient as in lane 9).
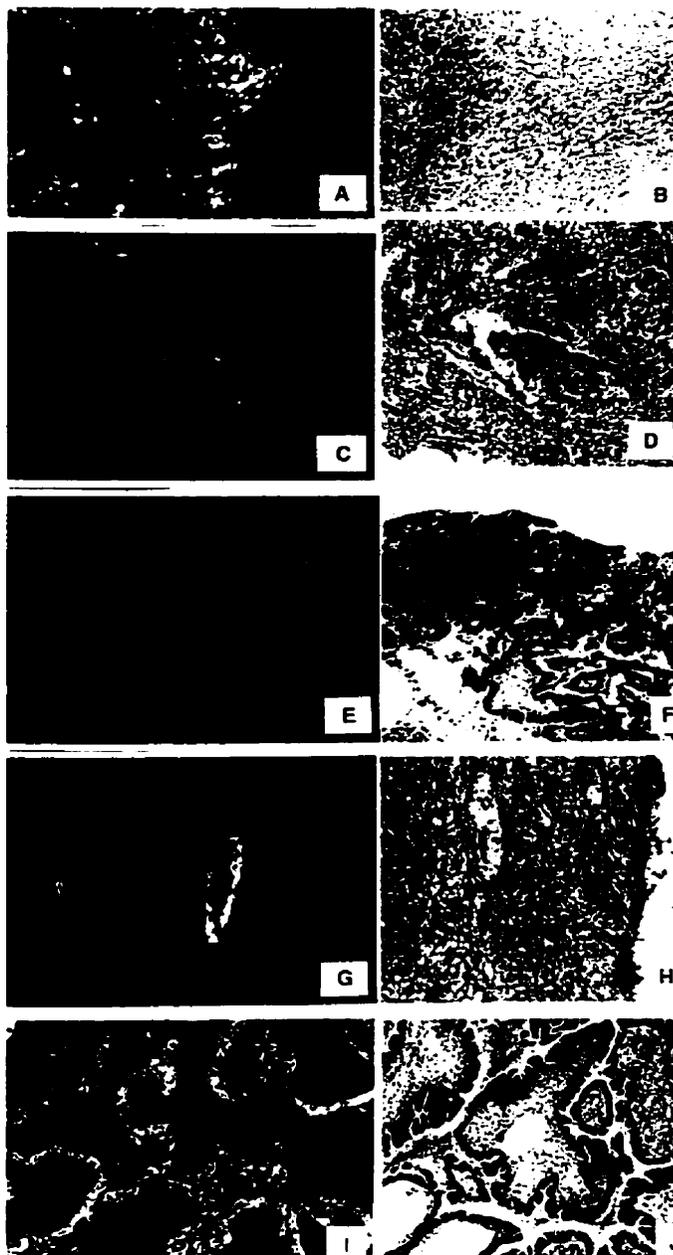
Figure 6.3   FITC-VT1B overlay of ovarian tumor cryosections.

A.   Poorly differentiated ovarian adenocarcinoma with associated desmoplastic stroma. Fluorescence demonstrates extensive toxin binding in the tumor but not in the stroma tissue (Mag. 50 X). Gb3 content is shown in Fig. 6.2 lane 7.
B.   Corresponding H&E (Mag. 50 X).

C.   Poorly differentiated ovarian adenocarcinoma, involving ovary and adjacent soft tissues. Fluoresence shows VT1 B staining of viable tumor cells (large arrow), extensive binding in blood vessels (arrows), but no binding to necrotic cells. (Mag. 35 X).

D.   Corresponding-H&E (Mag. 35X), arrowhead-longitudinally sectioned blood vessel, asterisks- necrotic cells.

E.   Ovarian adenocarcinoma (mucinous) well differentiated tumor. Fluorescence demonstrates only focal binding of VT1 B, as opposed to the diffuse binding in C (Mag. 35 X). Gb3 content is shown in Fig. 6.1 lane 31.

F.   Corresponding H&E (Mag. 350 X).

G.   Desmoplastic and inflamed stroma from same tumor as Fig. 3E. No tumor cells are present in this field. Fluorescence demonstrates VT1 B binding along the lumenal margin of blood vessels within the stroma. Stroma is VT unreactive. (Mag. 50 X).

H.   Corresponding H&E (Mag. 50 X)

I.   Differentiated papillary serous tumor which rapidly became multidrug resistant. Fluorescence demonstrates extensive VT 1B binding to the duct-like differentiated tumor. (Mag. 35 X). Gb3 content is shown in Fig. 6.2 lane 9.

J.   Corresponding H&E (Mag. 35 X)
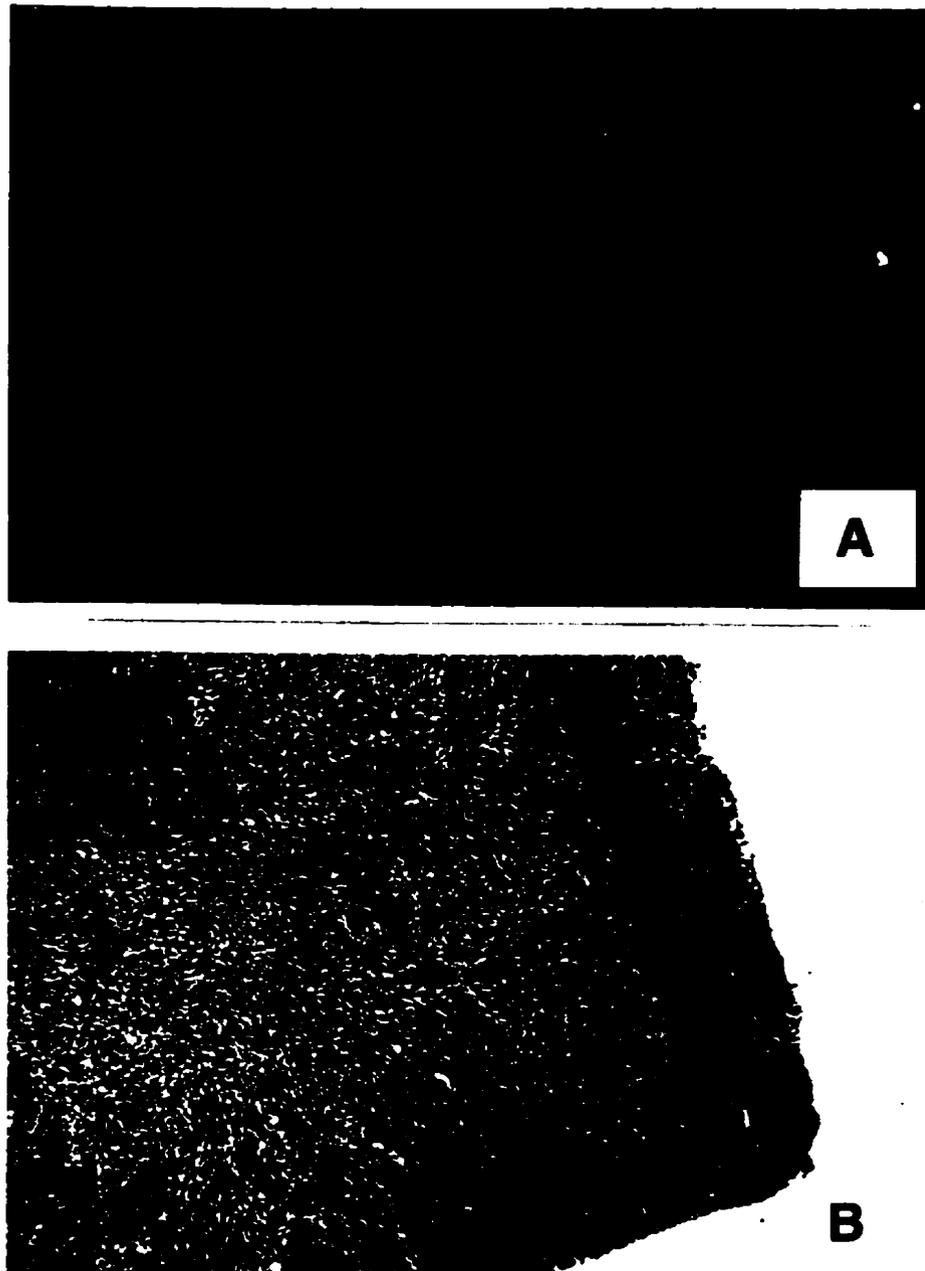
Figure 6.4   FITC-VT1B overlay of normal ovary

A.   FITC-VT1B treated normal ovarian section does not show detectable VT binding. (Mag. 75 X).

B.   Corresponding H&E (Mag. 75 X)
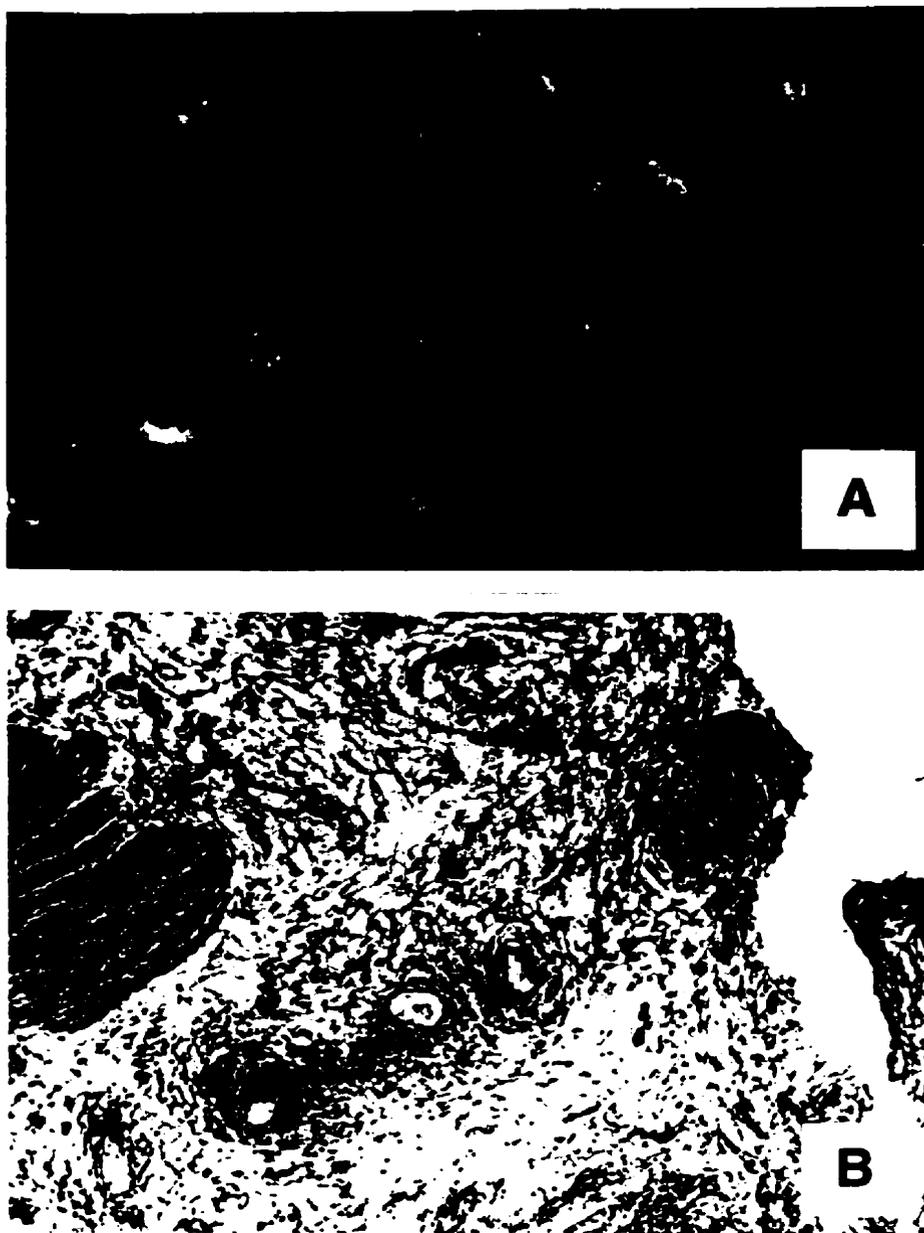
Figure 6.5   FITC-VT1B overlay of small bowel adjacent to metastasis from ovary

A.   Granulation tissue adjacent to metastasis of ovarian mucinous adenocarcinoma. Extensive fluorescent VT1 B binding to blood vessels. Small capillaries within the granular tissue are less intensely labelled. No binding in normal muscles (on the left side center) (Mag. 75 X). Gb_3 content is shown in Fig. 6.2 lane 6.

B.   Corresponding H&E (Mag. 75 X)
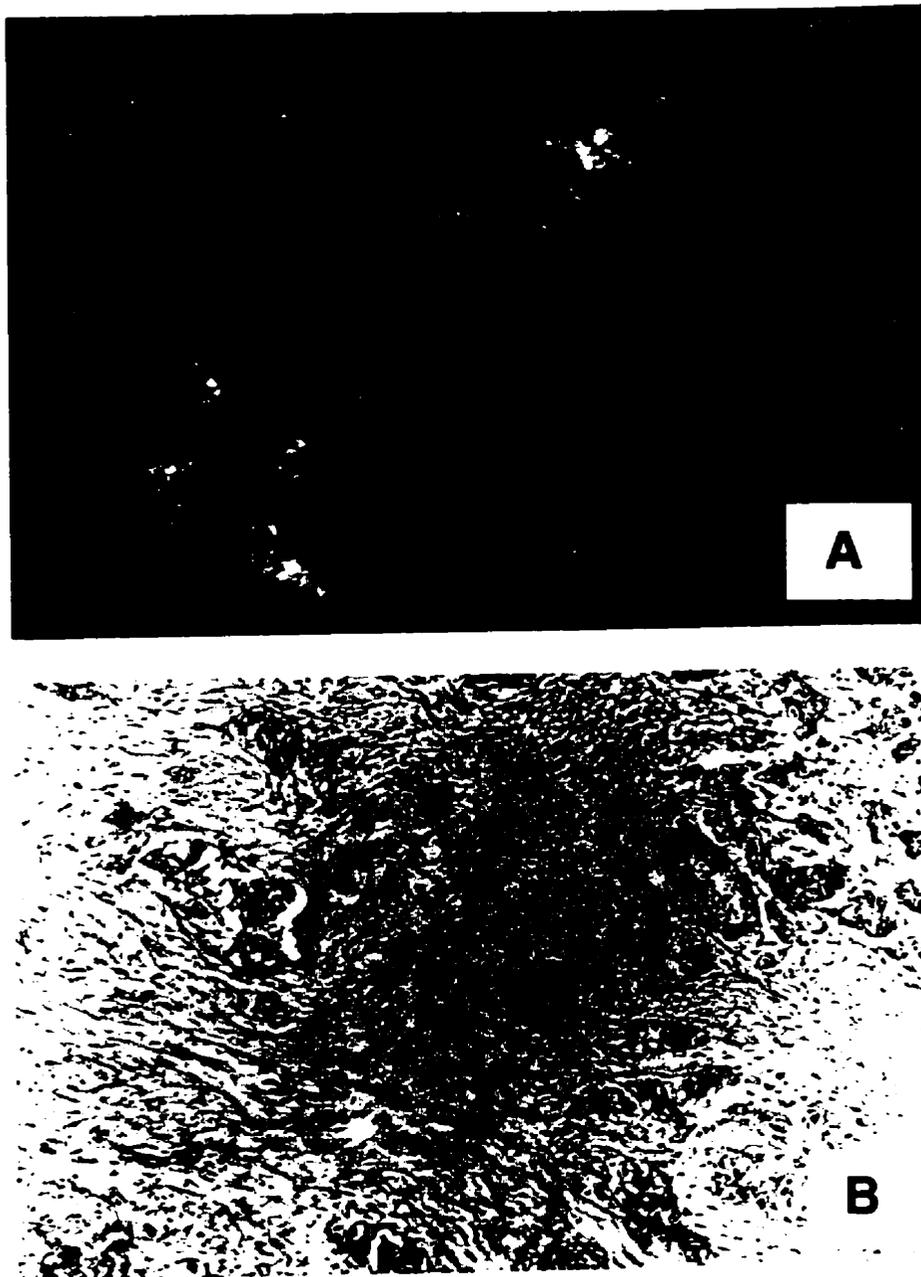
Figure 6.6 FITC-VT1B overlay of ovarian tumor metastasis to colon

A.   Poorly differentiated ovarian adenocarcinoma metastasized to the colon with associated desmoplastic stroma. Fluorescence demonstrates extensive VT binding in the tumor but not in the stroma tissue (Mag. 75 X). Gb3 content is shown in Fig. 6.2 lane 5.

B.   Corresponding H&E (Mag. 75 X)
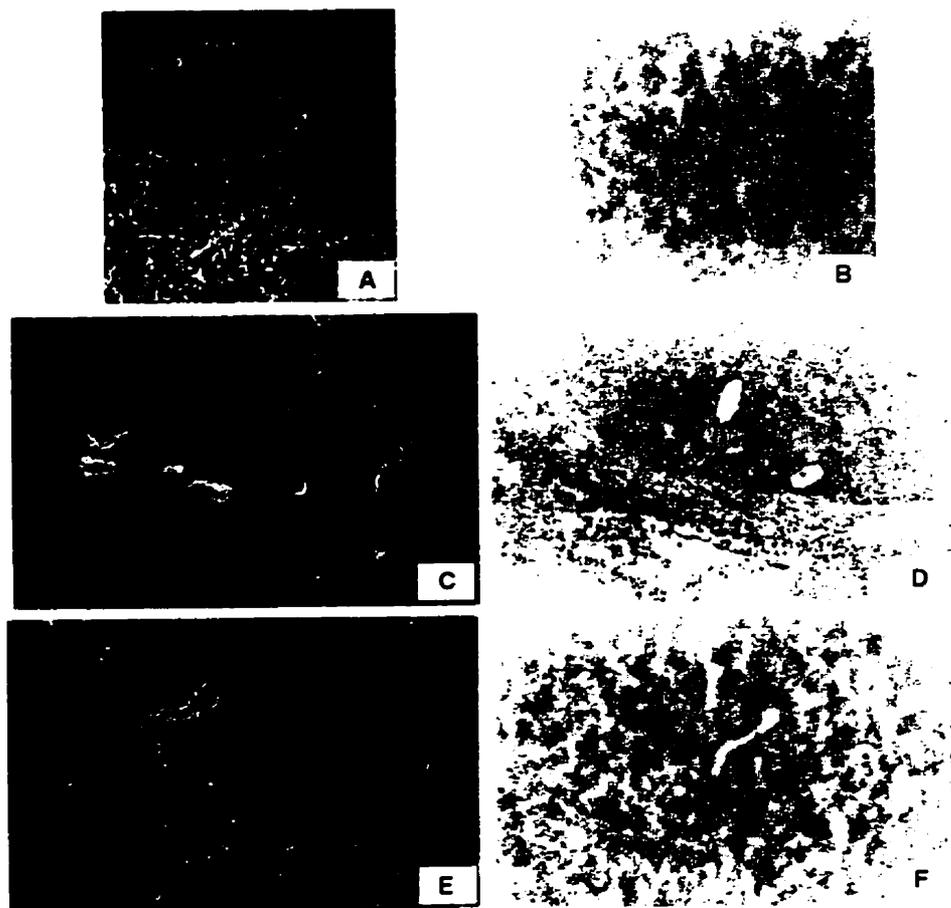
Figure 6.7   FITC-VT1B overlay of ovarian tumor and metastasis
                    cryosections.

A.   Poorly differentiated ovarian papillary serous tumor. Fluorescence
        demonstrates extensive VT 1B binding to tumor cells. (Mag. 35 X). Gb3
        content is shown in Fig. 6.2 lane 9.
B.   Corresponding H&E (Mag. 35 X)

C.   Omentum metastasis show significant localization of toxin to the lumen of
        the blood vessels.
B.   Corresponding H&E (Mag. 35 X)

E.   Blood vessel toxin localization, as well as tumor cells staining is depicted in
        this omentum metastasis section.
F.   Corresponding H&E (Mag. 35 X).
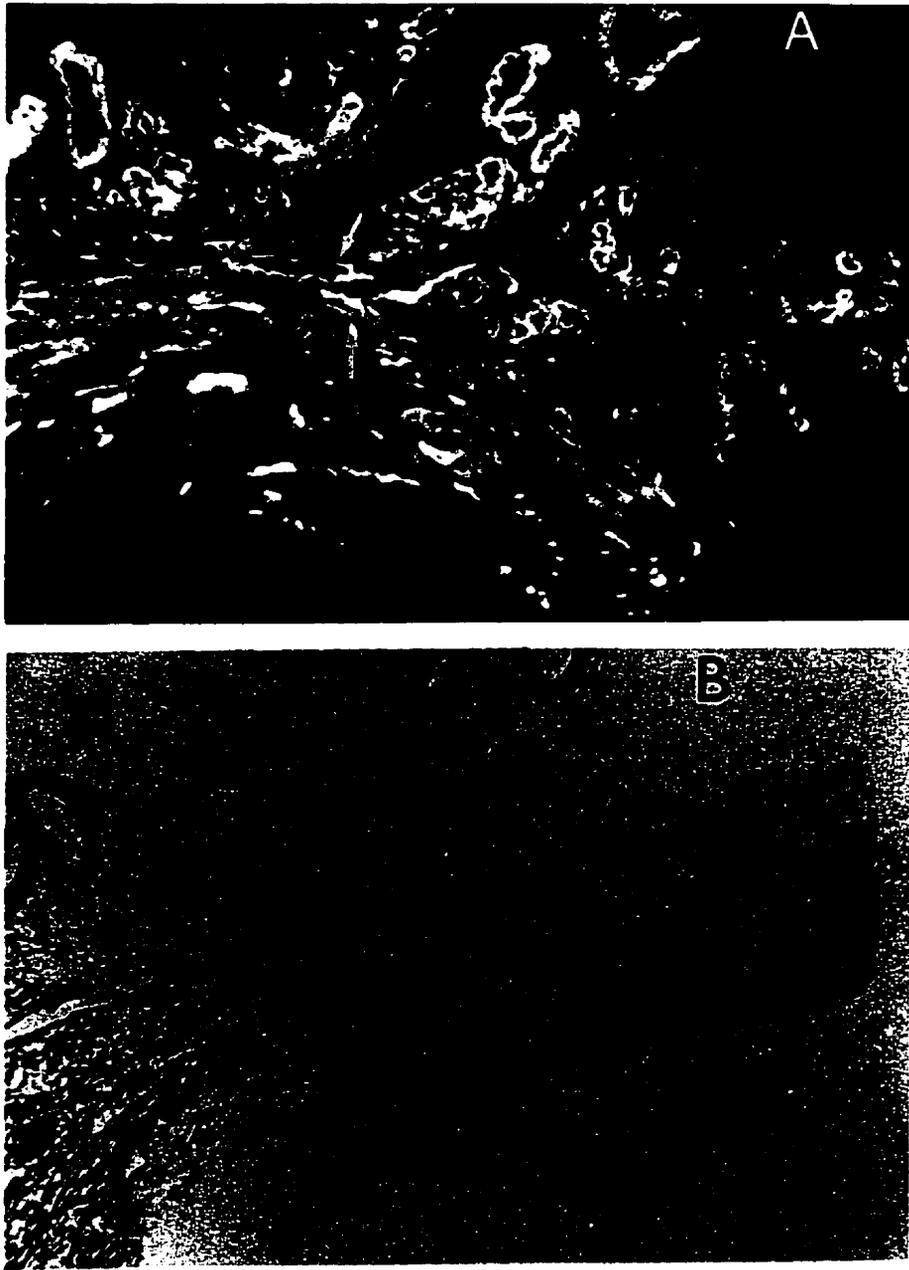
Figure 6.8   FITC-VT1B overlay of ovarian tumor cryosection

A.   Cryosection of papillary serous adenocarcinoma tumor sample. Significant labeling of FITC-VT1B is depicted in tumor foci and tumor vasculature. Lumenal staining of blood vessels is marked by arrows.

B.   Corresponding H & E (Mag. 35 X).
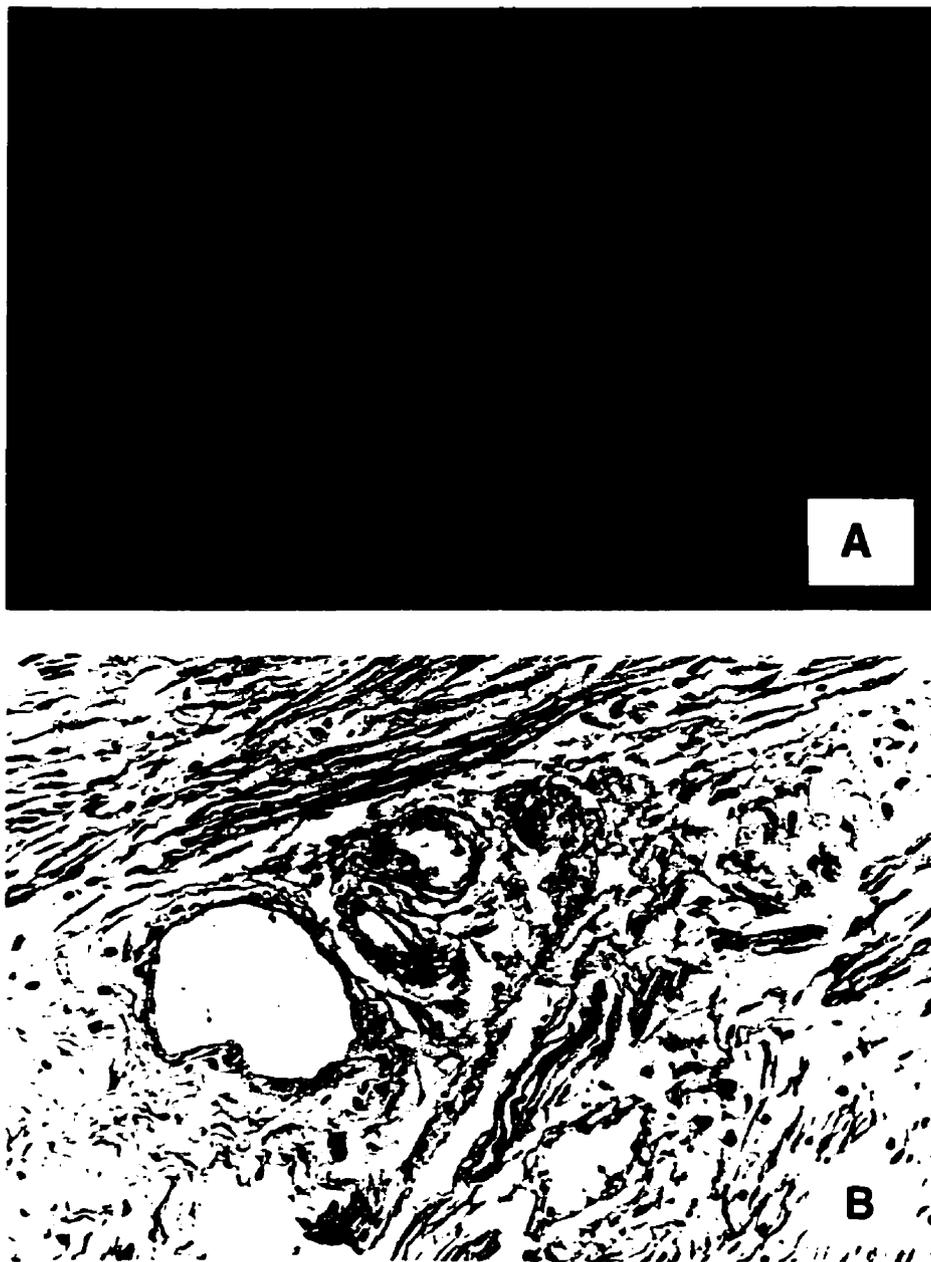
Figure 6.9   FITC-VT1B overlay of normal colon section

Adult colon tissue was obtained at autopsy and crysections stained with FITC-VT1 B. An area containing microvasculature is shown.
A.   FITC VT1 B staining (Mag. 250 X)
B.   Corresponding H&E.
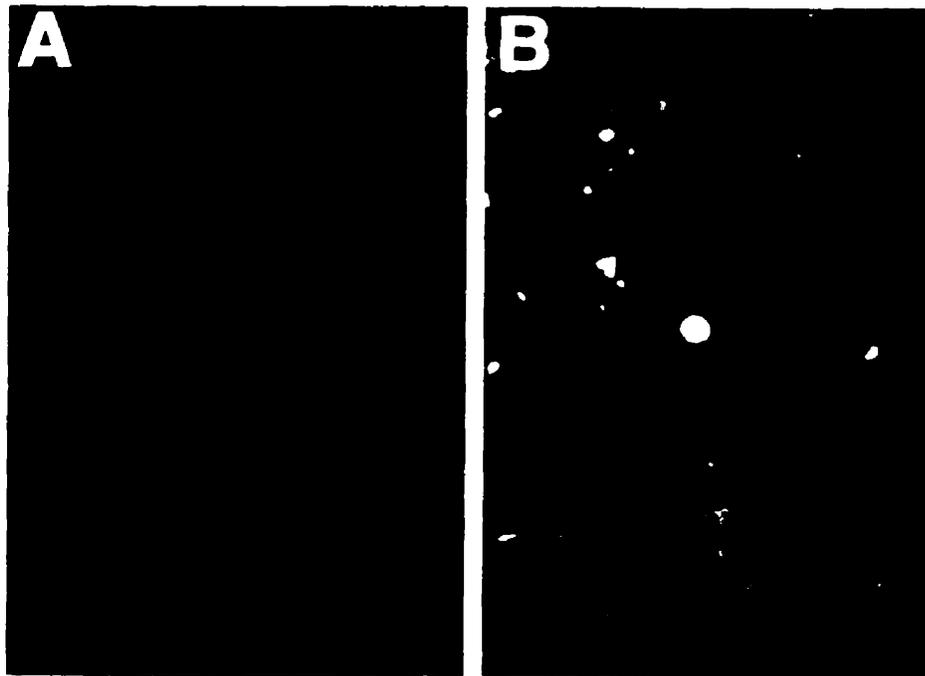
Figure 6.10   Double labeling of ascites cells from ovarian carcinoma to RITC-VT1

A.   RITC-VT1 was colocalized in tumor cells. Some nontumor cells are also
          labeled with RITC-VT
B.   Ovarian MDR tumor cells from ascities were labelled with epithelial
             cell marker-FITC to identify tumor cells

6.3   DISCUSSION and SUMMARY

While Gb3 was barely detectable in normal ovarian tissue samples, the amount of Gb3 in the tumor specimens was generally increased, suggesting that ovarian cancer cells may be more susceptible to verotoxin than their normal counterparts. There was marked variability of Gb3 content between different tumor types (endometrioid, mucinous, and serous- originating from surface epithelium of the ovary) suggesting differing susceptibility between neoplasms of different histopathology. Endometrioid tumors had consistently elevated Gb3 levels while serous tumors were more variable. For the serous papillary adenocarcinomas, Gb3 content was inversely related to the extent of differentiation. While the Gb3 content was lower for mucinous tumors, tissue section overlay also demonstrated more extensive VT binding to poorly differentiated, as opposed to differentiated, tumor sections. Estimation of Gb3 content by tlc overlay of the tissue lipid extract is only semiquantitative at best. Moreover, variation in the normal/tumor cell ratio in a tissue sample may render the analysis of total Gb3 less informative. Thus the VT tissue section overlay provides the most informative assay of Gb3 content. Differentiation is part of a mechanism for growth arrest and has great impact on prognosis of ovarian cancer. Poorly differentiated ovarian tumors are far more aggressive with worse prognosis than more differentiated tumors (Baak et al., 1986). The differentiated tumor which was multidrug resistant showed high Gb3 content (Fig. 6.2) and corresponding intense VT tissue staining (Fig. 6.3 G,H). This correlates with the previous observation that MDR ovarian cells lines are hypersensitive to VT and contain more Gb3 than the drug sensitive parental cell line (see Chapter 3) (Farkas-Himsley, et al., 1995) Ovarian tumors deemed to be drug resistant at surgery were analysed for Gb3 content by toxin overlay of the glycolipid extract (Fig. 6. 2 lanes 4-10). One of the samples analysed was found to have a less elevated Gb3 content than expected for MDR (Fig. 6.2 lane 8). On follow up, it was found that

this patient's aggressive tumor showed a surprisingly good response to chemotherapy and therefore was not MDR (MDR status of these tumors was clinically determined). Thus analysis of ovarian carcinoma Gb3 content may provide a method to predict the MDR phenotype (or response to treatment). In this regard, it is of interest to note that the level of glucosyl ceramide, a precursor of Gb3, has already been proposed as a marker for MDR (Lavie et al., 1996).

Gb3 expression was elevated in ovarian metastases (Fig. 6.2). VT1 B binding in sections from such tumors showed a clear discrimination between the metastatic tumor cell foci and the background normal stroma (Fig. 6.6). Blood vessels which vascularize the tumor were also highly VT reactive (Figs.6.3C,G, 6.5), 6.7. Both of these findings have significant clinical implications, since the development of drug resistance is the major obstacle in cancer treatment and angiogenesis, which is particularly important in ovarian cancers (Hollingsworth, et al., 1995), increases markedly in invasive tumors and metastases. The finding that blood vessels within the normal colon and ovary are not bound by VT suggests that Gb3 may be specifically up-regulated in blood vessels which vascularize tumors.

The fact that the microvasculature of the colon was not toxin reactive is also relevant in considering the role of verotoxin in the etiology of hemorrhagic colitis (Riley et al., 1983; Riley, 1985). While it is possible that there is heterogeneity in the expression of Gb3 within the GI microvasculature and between individuals, and receptor expression may vary regionally, this result may indicate that endothelial cells of the gastrointestinal tract require an additional signal to induce Gb3 synthesis, such as has been proposed is required for adult renal endothelial cell Gb3 synthesis and VT sensitivity (Lingwood, 1994). In contrast, following iv administration, verotoxin was found to bind effectively to a fraction of the gastrointestinal microvasculature of the rabbit (Richardson et al., 1992). A more extensive study of the binding of VT to different regions of the human GI tract will be required to resolve this question.

The present studies extend the earlier report (Chapters 3 and 4) that primary malignant ovarian tumor cell lines contained elevated levels of Gb3. Multi-drug resistant variants of ovarian tumor cell lines were markedly more sensitive to VT-cytotoxicity than the drug sensitive parental cell line and contained more Gb3 (Chapter 3; Farkas-Himsley, et al., 1995). This correlates with the present finding that those differentiated ovarian tumor samples which were atypically highly toxin reactive, were multidrug resistant in vivo. Gb3 expression and VT binding in differentiated ovarian tumors may thus provide a predictor of poor clinical outcome.

Since histological subtyping of ovarian carcinomas is of limited prognostic value (Baak, et al., 1986), Gb3 expression monitored by VT tissue section overlay could function as a marker of prognosis in ovarian tumors. Moreover, in this study the level of Gb3 expression was elevated in all of the metastatic samples, irrespective of the mechanism of drug resistance of the primary tumor.

Angiogenesis is essential for growth of both primary and secondary tumors. VT treatment may offer dual targeting of both the tumor and its vascular supply. Inhibition of angiogenesis provides a novel and more general approach for treating metastases by manipulation of the host microenvironment. Endothelial cells in tumor blood vessels divide rapidly, whereas those in normal tissues do not (Folkman, 1992). Extensive staining of blood vessels which vascularize the tumor was observed irrespective of the chemical Gb3 status of the tumor itself (Figs. 6.3.C G, 6.5, 6.7). Thus VT may have an antiangiogenic effect even for Gb3 negative tumors.

Genotoxic insults such as radiation and chemotherapy are known to induce apoptosis (Strasser, et al., 1994). In fact, apoptosis has been recognized as the major mechanism in the action of many chemotherapeutic agents (Barry, et al., 1990). However, the over expression of Bcl 2 renders the tumors resistant to the apoptotic activity of the anti-cancer drugs (Wang, et al., 1993). This is of considerable

therapeutic importance when tackling the thorny problem of drug resistance. Since some multidrug resistant tumor cell lines are oversensitive to VT, this pharmacological attack may prove to be of great importance in circumventing the problem of drug resistance. VT effectively induced apoptosis in drug resistance ovarian cell lines overexpressing P-glycoprotein (Chapter 3; Farkas-Himsley, et al., 1995).

The potential inhibition of tumor-induced angiogenesis, coupled with the increased levels of Gb_3 found in ovarian tumor cells and metastases, the most challanging aspect of cancer, indicate that VT treatment may offer a promising alternative for Gb_3 containing tumors.

The next step in this study was designed to investigate the anticancer effect of VT1 in tumors xenografted in nude mice, Chapter 7.

Chapter 7

VT treatment of astrocytoma xenografts in mice

7.1 Introduction

Based on the previous results of in *vitro* data, which showed rapid induction of apoptosis in astrocytoma cell lines, we have extended our studies to examine the effect of VT1 on astrocytoma cell growth *in vivo*.

In this chapter we demonstrate that VT1 therapy of human astrocytoma results in complete regression of tumor in nude mice by the induction of apoptosis *in vivo*. This is a major finding and indicates that VT1 may have the potential to be used as an anticancer agent for those tumor which express elevated level of receptor Gb3 isoform, amongst which are two of the most invasive cancers (ovarian and astrocytoma) both with poor prognosis.

7.2 RESULTS

7.2.1 Biodistribution of labeled VT1-B -subunit in Nude Mice.

To investigate the potential targeting of VT1 into the tumor tissue, VT1-B was labeled with sodium-iodide[131]. In a pilot experiment it was shown that radiolabeled verotoxin was able to target a human ovarian tumor sample engrafted into a nude mouse (Fig 7.1). After two hours, tissue samples were removed and the distribution of the radioactivity determined (Fig. 7.1). It was found that most of the radioactivity accumulated in the kidney (excreted). The labeling in lung could reflect the fact that human ovarian tumors metastasize to the lung in this animal model. The tissues to which significant toxin localization could be detected were the tumor itself (Fig. 7.2). Only trace amounts of label is located in brain, liver, and heart. The result from this study demonstrate the feasibility of using radiolabeled VT1 B-subunit to locate the tumor. This study demonstrates that, VT can selectively bind Gb3 expressing human tumors growing in mice.

7.2.2 Whole-body gamma-camera imaging

Fig. 7.2 shows a gamma-camera image of the mouse for which the data is presented in Fig. 7.1. Fig. 7.2A shows the image of the mouse with tumor and Fig. 7.2B shows the image of the same mouse after removal of the tumor. There is significant reduction of radiolabelled toxin due to the removal of the tumor, considering the small size of the tumor (30 mm^3) the accumulation of the radiolabeled toxin in tumor is significant. The high accumulation of ^{131}I-VT1 at the top of Fig 7.2 may represent the thyroid gland with 1000 times greater affinity for iodine than any other organ.

(This part of the experiment was performed in collaboration with DR. R. Reilly in division of nuclear medicine at Toronto Hospital.)
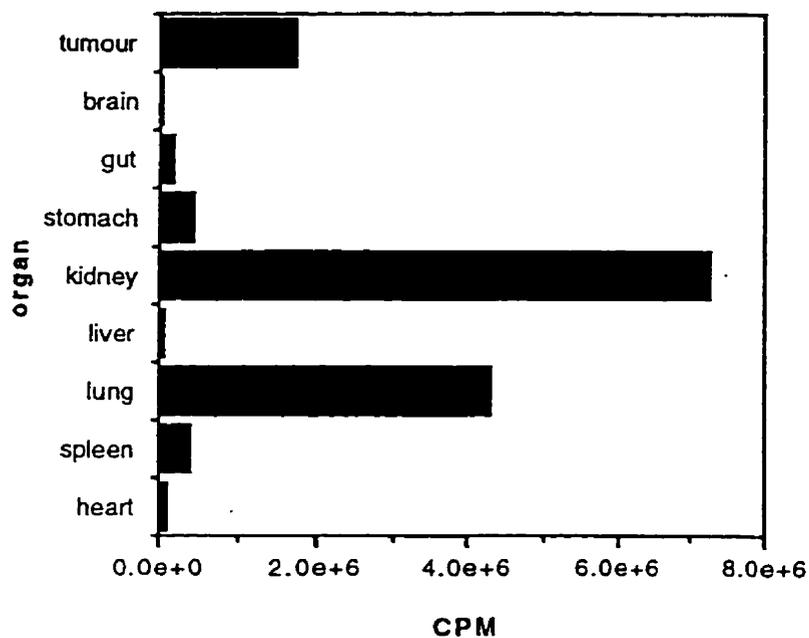
Figure 7.1   Biodistribution of ^{131}I-labeled VT1 B-subunit

Biodistribution of VT1 ^{131}IB-subunit administered i.p. in nude mouse bearing xenotransplants of human ovarian tumor. Considerable amount of labelling is targeted to primary tumor and trace amount of toxin was located in brain and heart where the potential VT1 side effect is considered.
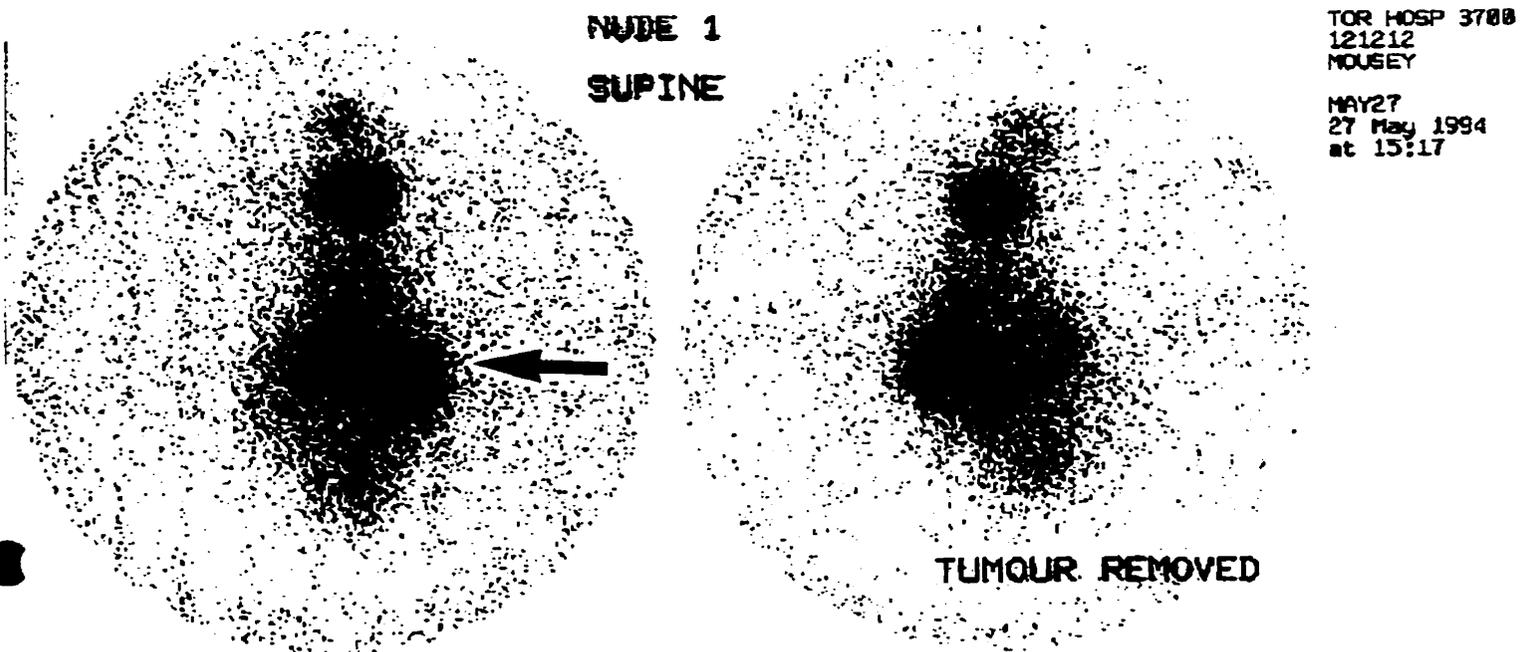
Figure 7.2   Whole-body gamma-camera imaging of a mouse xenografted with
            human ovarian tumor
    2 hrs after i.p. administration of ^{131}I-VT1 B-subunit.   Autoradiograph of
mouse with tumor (A) and after removal of tumor (B) is shown. Significant
labeling is concentrated at the tumor (arrow).

7.2.3 VT1 Induced Astrocytoma Tumor regression

Exponentially growing human astrocytoma (SF-539) cells were used to induce a s.c. tumor in nude mice. Mice with large tumors (> 50 mm2) received a single injection of 4 ug/kg or 8 µg/kg of VT1 intratumor (i.t.)which resulted in complete regression in 100% of the mice tested with no reccurrence of the tumor. Fig. 7.3a (A, B, C) and Fig. 7.3b (D, E, F, G) demonstrate the results of single i.t. injection of 2 different concentration of VT1 (8 µg/kg) and 4 µg/kg respectively) into a total of 7 nude mice bearing human astrocytoma tumors growing s.c. The tumor rapidly decreased in size and the mice were tumor free within 10-20 days. Mice remain tumor free for more than 40 days post VT1 treatment and were sacrified 50 days after VT1 treatment.

7.2.4 Injection of heat inactivated toxin in control mice

Tumors in control mice injected with a single dose of heat inactivated VT1 i.t. continued to grow and animal weight loss accompanied the tumor growth (see Fig. 7.4, A & B).

7.2.5 VT1 treatment of ovarian tumor in nude mice

Ovarian tumor, SKOV3 cells in vitro do not show a comparable level of sensitivity to VT1 as astrocytoma cells, SF-539. This difference in the level of sensitivity is reflected in in vivo study as well. Nude mice with ovarian tumor xenografts were not as sensitive to VT1. However at VT1 concentration of 8 µg/Kg there was considerable tumor regression (data not shown). Amazing regression of ovarian tumor was observed when VT1 at a concentration of 80 µg/Kg was injected into mice (i.t.) with ovarian tumors. In 20 hrs the tumor size regressed to 1/3 of the original size (data not shown), however the mice survived only 24 to 48 hrs post-injection of VT1.
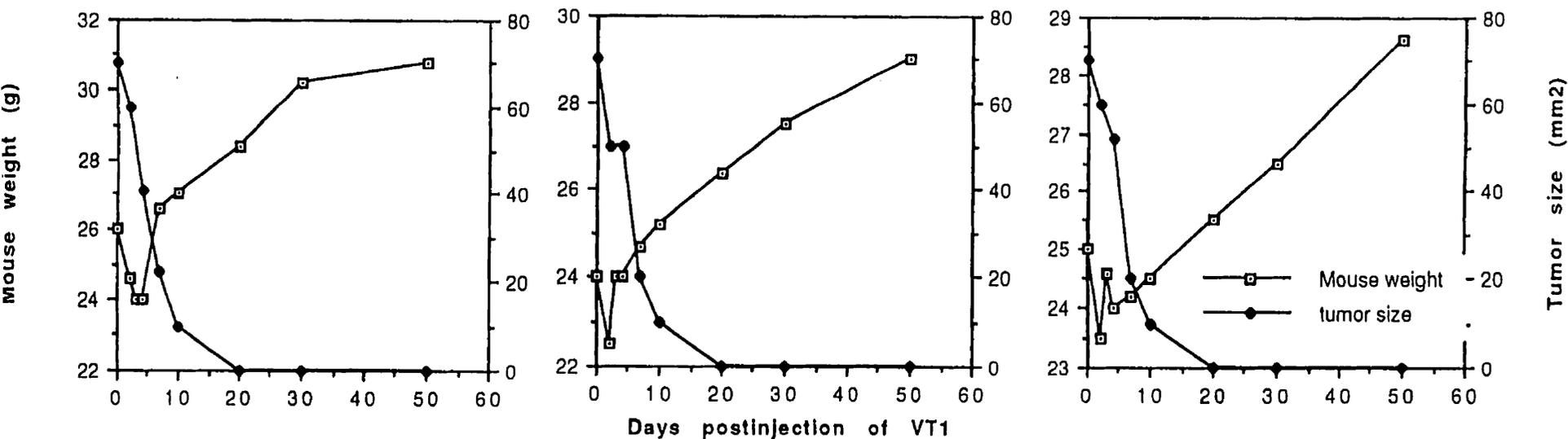
Figure 7.3a   VT1 treatment of human astrocytoma xenografted in nude mice

Three mice bearing xenotransplants of human astrocytoma (using SF-539 cell line) were injected with single dose of 8 ug/Kg (A, B, C) of VT1 i.t. This resulted in complete regression of tumor in 100% of mice with no recurrence after 50 days post-treatment with VT1.
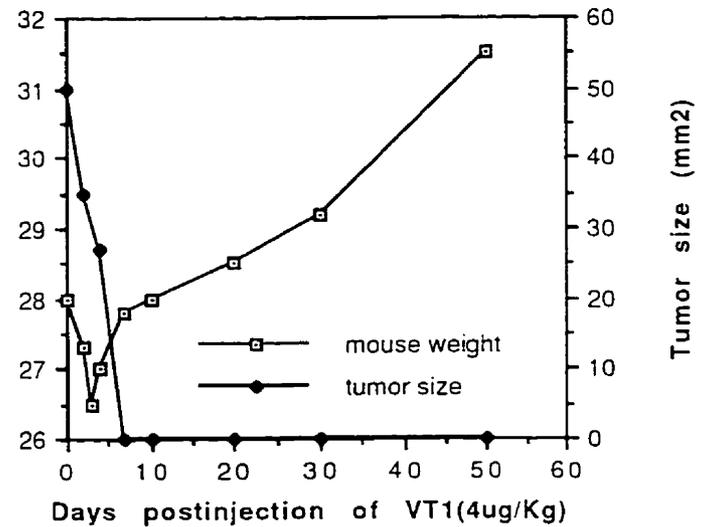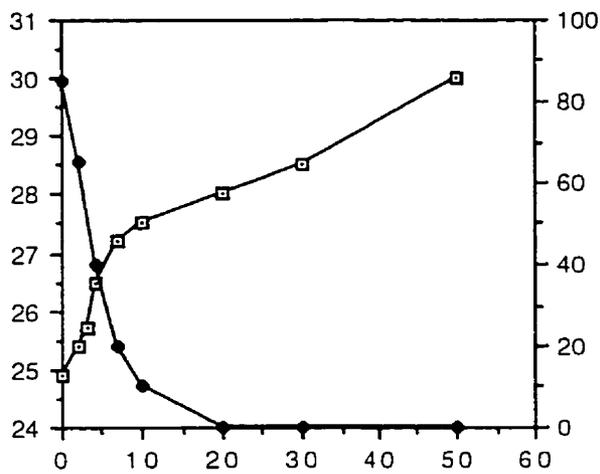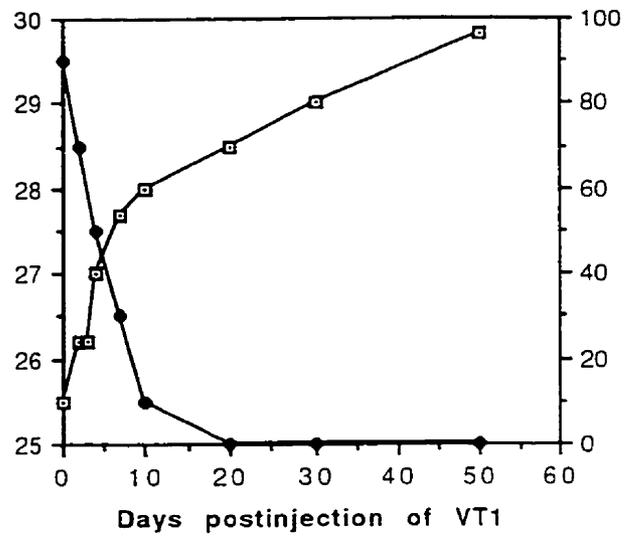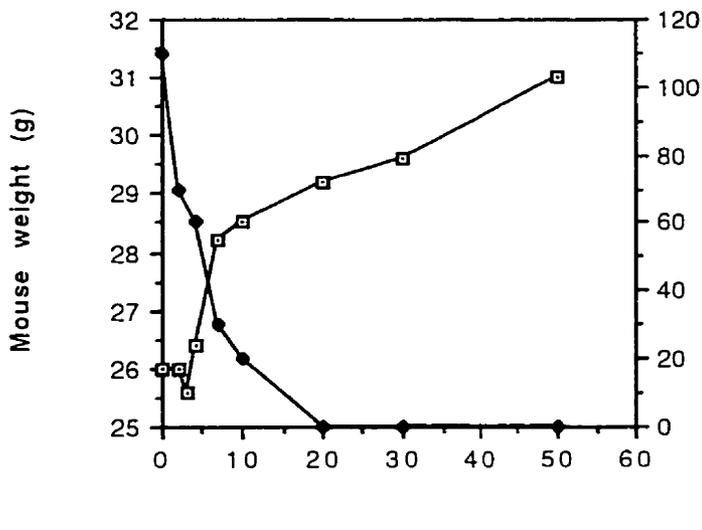
Figure 7.3b   VT1 treatment of human astrocytoma xenografted in nude mice

Four mice bearing xenotransplants of human astrocytoma (SF-539 cell line), were injected with single dose of 4 ug/KG (D,E, F, G) of VT1 i.t. This resulted in complete regression of tumor in 100% mice with no recurrence after 50 days post-treatment.
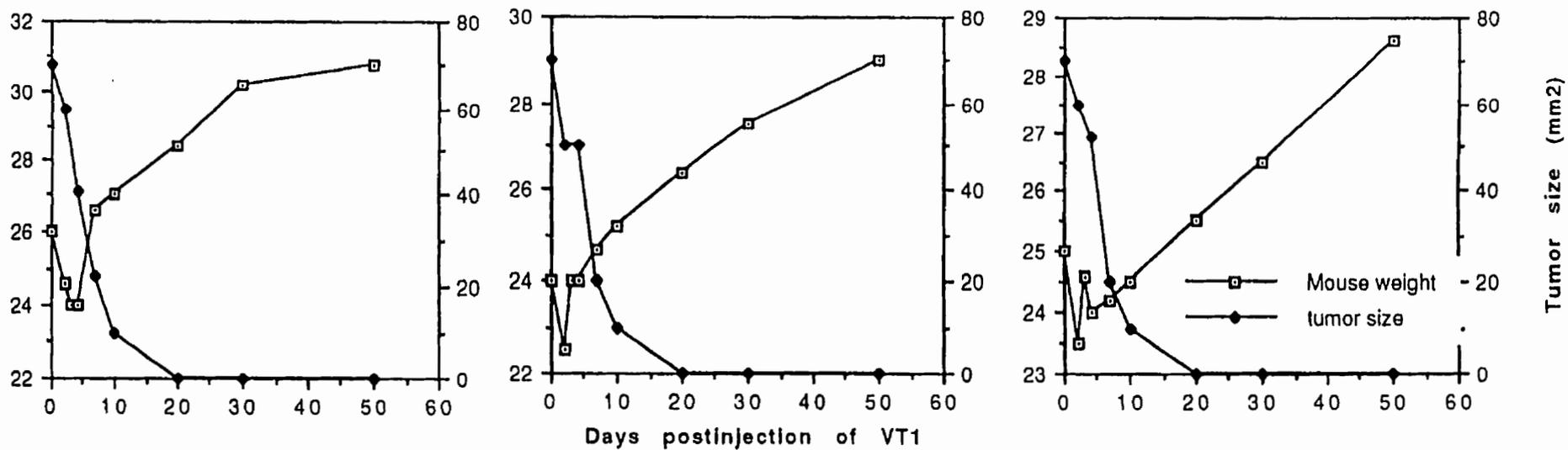
Figure 7.3a   VT1 treatment of human astrocytoma xenografted in nude mice

Three mice bearing xenotransplants of human astrocytoma (using SF-539 cell line) were injected with single dose of 8 ug/Kg (A, B, C) of VT1 i.t. This resulted in complete regression of tumor in 100% of mice with no recurrence after 50 days post-treatment with VT1.
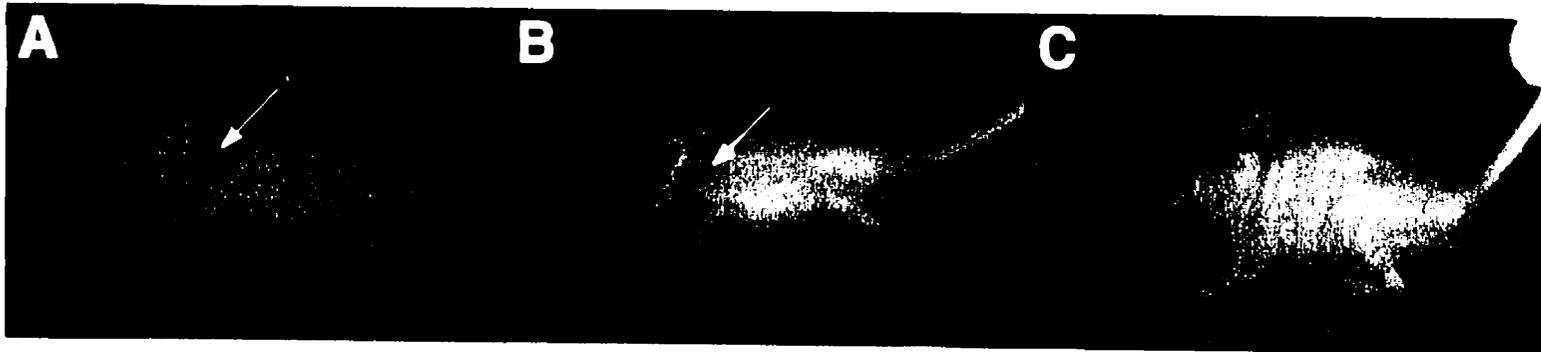
Figure 7.5a   Sample photographs of mice in this study

Fig 7. 5a (A-C) photographs of a nude mouse bearing astrocytoma tumor and treated with single injection of VT1. A, before injection of VT1, B, 3-days after treatment and C, 50 days post-treatment and tumor free.
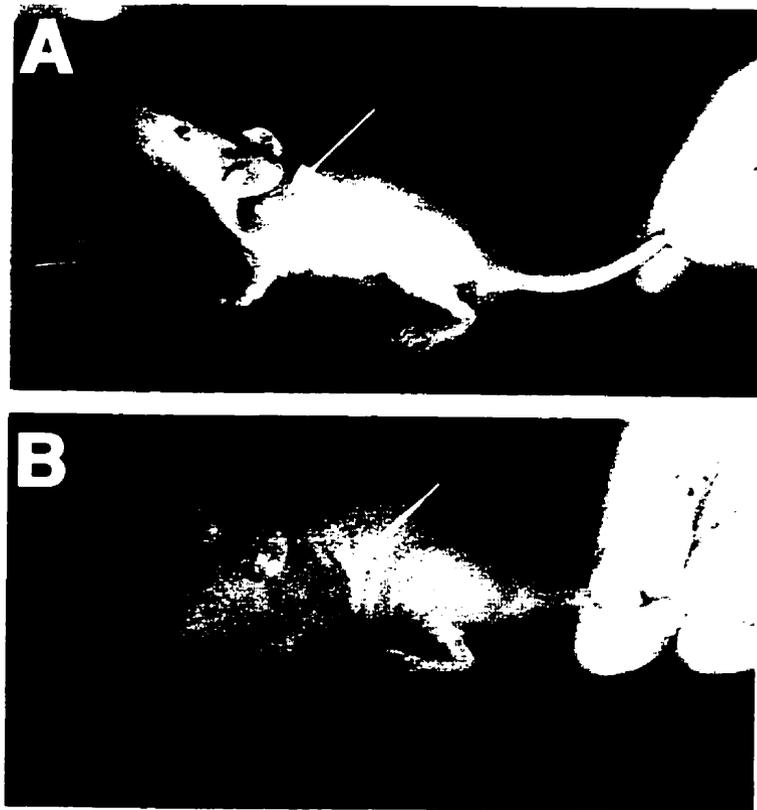
Figure 7.5b   Sample photographs of control mice in this study

Fig 7. 5b presents the sample photographs of control mice (A,B) with growing tumor and weight loss.

7.2.6 Mechanism of cell death in tumors treated with VT1

To investigate the mechanism of cell death in vivo in response to VT1 therapy, a method of detecting in situ apoptosis was employed. Fig. 7.6 shows the result of the TUNEL staining of paraffin embedded tumor sections (Gavrieli, et al., 1992. Histological examination and the TUNEL assay of tumor section (by nuclear staining) showed that the mechanism of action of VT1 in tumor regression was apoptosis. A high level of apoptosis particularly in the lumen of the blood vessels, reflects the rate of response to VT1 treatment. Apoptotic cells detected by the TUNEL assay are verified using in situ nick translation method (data not shown).

7.2.7 FITC-VT1B overlay of human brain tumor frozen sections

To investigate the ability of VT1 in targeting human astrocytoma tumor sample and thus the potential of VT1 in brain tumor therapy, frozen sections of different human brain tumor samples were tested. Treatment of frozen brain tumor sections with FITC-VT1B showed selective toxin binding to tumor cells (Fig 7.7). This selective binding of tumor cells can be used to detect VT1 sensitive tumors and specifically target the right candidates for VT1 treatment. The intensity of FITC-VT1B binding was proportional to the grade of the tumor. Low grade pediatric tumor did not demonstrate significant binding (data not shown), however the level of binding was elevated in adult, anaplastic astrocytoma (7.7a) and it was significantly elevated in glioblastoma multiforme (GBM) (Fig. 7.7b). In GBM sections tested, in addition to markedly increased overall binding, significant binding was observed in the blood vessels. Angiogenesis is the prominent feature of GBM (Burger, et al., 1991) which is targeted by the toxin. Thus in addition to induction of apoptosis (antimitogenic effect), VT1 may be capable of antiangiogenic effect.
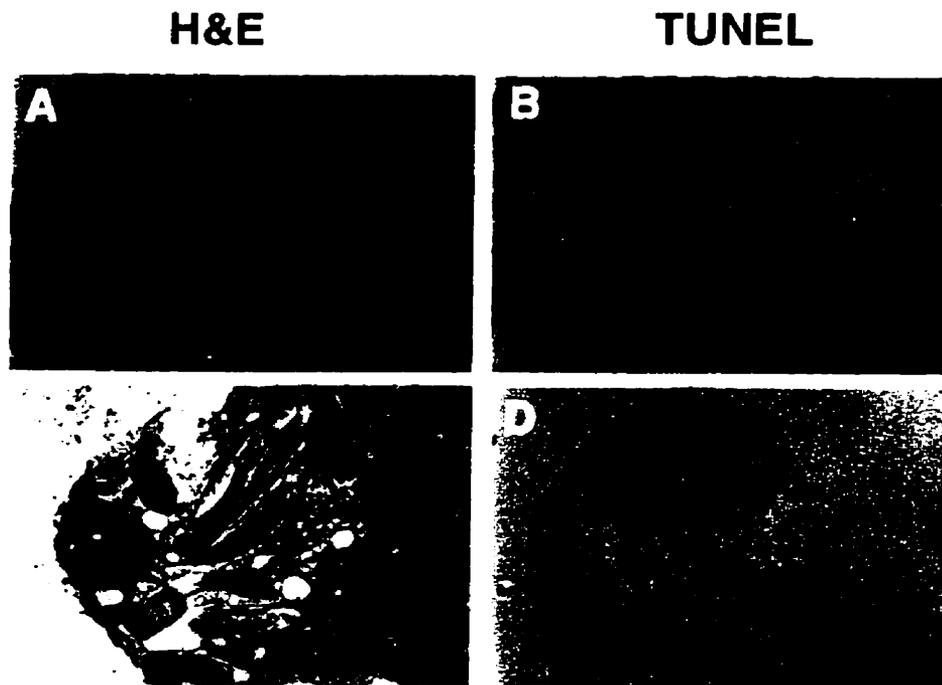
Figure 7.6a   TUNEL assay on mice astrocytoma xenograft section

Numerous stained nuclei of apoptotic cells in VT1 treated tumors (A,C) are present in paraffin embedded sections of astrocytoma xenograft in nude mice treated with single injection of 8 ug/Kg / mice after 24 hrs .

A&C   H&E assay demonstrating apoptotic nuclei.

B&D   TUNEL of the adjacent section of the same tumor to A and C respectively (Mag. 50x).
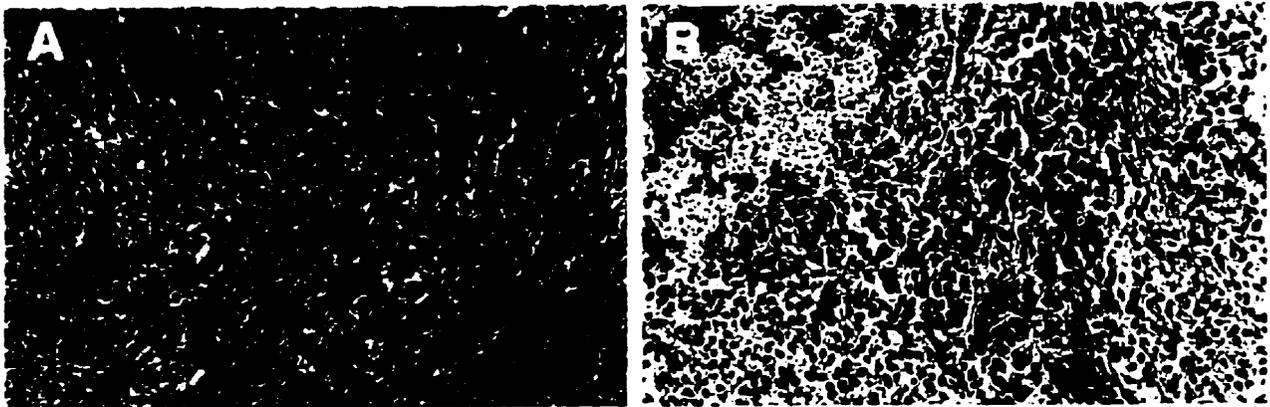
Figure 7.6b   TUNEL assay on mice ovarian tumor xenograft section

Numerous brown stained nuclei of apoptotic cells in VT1 treated tumors (A) are present in paraffin embedded sections of ovarian tumor xenograft in nude mice treated with single injection of 40 ug/Kg / mice after 24 hrs .

A   H&E assay demonstrating apoptotic nuclei.

B   TUNEL of the adjacent section of the same tumor (A) (Mag. 50x).
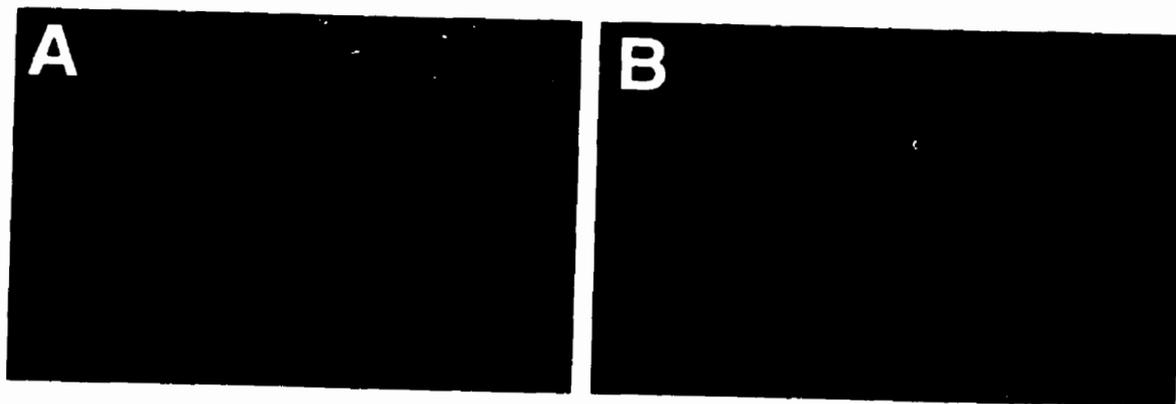
Figure 7.7a   FITC-VT1B overlay of human brain tumor cryosections

Anaplastic tumor
A   FITC-VT1B overlay of anaplastic brain tumor. Fluorescence shows specific
        binding in tumor cells.
B.   Corresponding H & E (Mag. 50x).
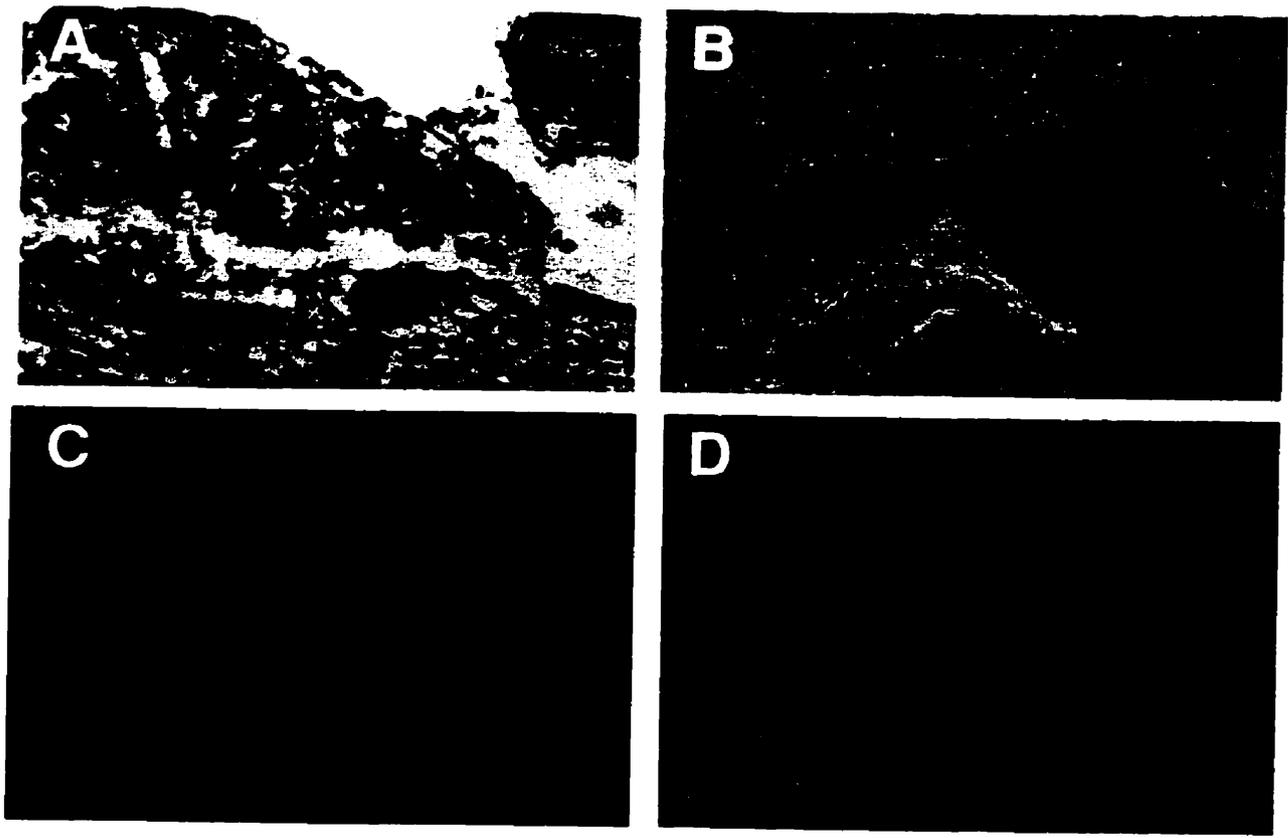
Figure 7.7b   FITC-VT1B overlay of human brain tumor cryosections

Glioblastoma multiforme
B &C   FITC-VT1B overlay of frozen sections of GBM tumor. Fluorescence
demonstrates extensive toxin binding in these cryosection and
significant binding in the blood vessels in tumor (A).
A&D   Corresponding H & E. (Mag. 75x)

7.3   DISCUSSION and SUMMARY

The hypothesis that despite its involvement in the etiology of human microvascular disease (Riley, et al., 1983; Karmali, 1989), verotoxin may provide a new approach to the clinical treatment of Gb_3 expressing neoplastic tissue was discussed earlier (Chapters, 1, and 6). A standard approach to the screening of potential antineoplastic regimes is to test the efficacy in tumour xenografts growing in nude mice (Davy, 1977; Danks, 1995).

The results presented clearly demonstrate that a single injection of VT1 into the tumor induces rapid apoptosis of human astrocytoma and complete regression of the tumor in vivo. We have also shown that the treatment of human astrocytoma cells in vitro will induce rapid apoptosis in these cells (Chapter, 4; Arab, et al., in press). Nuclear shrinkage and chromatin condensation, prominent features of apoptosis, were observed within 90 mins treatment of astrocytoma cells with VT1. To determine whether the ability of VT1 to induce apoptosis selectively in Gb_3 positive astrocytoma cells in vitro could be applied to *in vivo* studies and VT1 therapy, this study was extended to *in vivo* treatment of tumor in nude mice. Exponentially growing human astrocytoma cells (SF-539, which is the most sensitive cell line to VT1 cytotoxicity in vitro), was used to induce a s.c. tumor in nude mice. Mice with a large tumor (at least > 50 mm^2) received a single injection of 4 ug/kg or 8 ug/kg (1/10 and 1/5 of LD50 for normal mice (Richardson, et al., 1992)) of VT1 i.t. which resulted in complete regression in 100% of the tumors. Mice remained tumor free for more than 40 days post VT1 treatment and they were sacrified 50 days after VT1 treatment.

Interestingly, ovarian tumor, SKOV3 cells in vitro show a reduced level of sensitivity to VT1 compared to astrocytoma cells, SF-539. This level of sensitivity is reflected in in vivo study as well and SKOV3 tumors in mice were less responsive as compared to SF-539 tumor. However, amazing regression of ovarian tumor was

observed when VT1 at concentration of 80 ug/Kg was injected i.t. into the mice with ovarian tumor of 154 mm^2 size. In 20 hrs the tumor size regressed to 1/3 of the original size but the mice survived only 24 to 48 hrs post-injection of VT1. Two major questions remained unanswered: 1) the optimal dose of VT1 for ovarian tumor which is able to safely induce tumor regression and 2) the possibility that the release of a sudden and high level of cytokines from rapid regression of ovarian tumor induced the death of the animals. Currently a new soluble form of Gb3 (receptor for VT1) is being developed which may allow safe administration of a high dose of VT1 to a patient with a tumor sensitive to VT1.

Ligation of Gb3 has been shown to induce apoptosis (Mangeney, et al., 1993; Inward, et al., 1995; Arab, et al., in press). These findings are consistent with a role for Gb3 in growth control. Selective induction of apoptosis in tumor cells is the strategy for developing new therapy in cancer (Kerr, 1994). Such selectivity is achieved in VT1 therapy since VT only enters cells which express significant levels of receptor (Gb3) (Arab, et al., submitted).

Astrocytomas, arising from astrocytes, constitute the majority of all primary brain tumors and are the most common gliomas (over 80%, a proportion that increases with age) (Laws, et al., 1984). Angiogenesis is a prominent feature of aggressive astrocytomas and is an essential feature of the histological diagnosis and grading of these tumors (Burger, et al., 1991). Despite the advent of promising adjuvant therapies and drugs which have impacted positively on patient survival in other tumor types in recent times (Kays and Laws, 1995), no such promising therapy has yet been found for the patient with a malignant astrocytoma. Moreover, median survival for patients with glioblastoma multiforme, the most malignant form of astrocytoma, is approximately 12 months (Muller, et al., 1977; Laws, et al., 1984; McCormack, et al., 1992). In this context, it is imperative that new therapeutic strategies be explored for malignant astrocytomas.

An antineoplastic effect of α2-interferon was shown for certain tumors (Colamonici et al., 1994) and recently, the α2-interferon receptor, which shares amino acid similarity to the VTB subunit (Maloney and Lingwood, 1993) has been found to have anti-oncogenic properties (Sexl et al., 1994). Interestingly, chronic daily administration of a low dose of interferon-α has been shown to induce complete regression of life threatening hemangiomas in infants (Ezekowitz et al., 1992). Moreover, inhibition of angiogenesis, provides a novel and more general approach for treating cancers and metastasis by manipulation of the host microenvironment (Fidler, 1990). Angiogenesis is a prominent feature of aggressive astrocytoma. Tumor vasculature has been found to be targeted by FITC-VT1B in this study (Chapter, 6; Arab, et al., submitted). Endothelial cells in tumor blood vessels divide rapidly, whereas those in normal tissues do not (Folkman, 1992). The division of endothelial cells is induced by a variety of angiogenic factors. Treating neoplasms by targeting both the tumor cells (chemotherapy) and the organ environment (angiogenesis inhibitor) has been shown to produce additive or synergistic effects in mice bearing tumors (Teicher, et al, 1992). VT1 may be capable of targeting both the cancer and inducing apoptosis in the blood vessels thereby inhibiting angiogenesis. Further experimentation with labeling of the endothelial cells in the lumen of the blood vessels with a specific marker is required to confirm this speculation.

Histological examination and TUNEL assay of tumor sections showed that the mechanism of action of VT1 in tumor regression is apoptosis. A considerable level of apoptosis was observed in the blood vessels in tumor sections from these mice (Fig., 7.6a). In addition, the blood vessels within human frozen brain tumor samples were extensively stained with FITC-VT1B or FITC-VT1 (Fig. 7.7a &b). This antiangiogenic effect of VT1 by itself is significant in VT1 therapy of astrocytoma. It may be feasible to use VT therapy even in the absence of Gb_3 in tumor.

It has been demonstrated that FITC-VT1 or -VT1B is capable of selectively targeting the metastatic foci in human tumor samples and selectively targets the lumen of the blood vessels in the tumor mass (not in normal tissues) (Chapter, 6); (Arab, Russel et al., submitted). Treatment of frozen brain tumor sections with FITC-VT1B or FITC-VT1 showed selective toxin binding to the tumor cells (Fig.7.7) and extensive binding was demonstrated in the samples of high grade brain tumors (glioblastoma multiforme). The selective binding of tumor and blood vessels can be used to detect tumors sensitive to VT1 and specifically target the right candidate for VT1 treatment. In addition, I^{131}-labeled VT1B was able to specifically target the tumor in vivo (Fig. 7.1 & 7.2). These methods have the potential of being used in the clinical detection of tumor or possible metastases.

The results of this study have important implications for future approaches to the therapy of human highly malignant astrocytoma tumors for which there is no effective therapy as yet.

(The result from in vivo study of verotoxin is being prepared in a manuscript for Proc. Natl. Acad. Sci. USA.)

Chapter 8

Summary

and

General Discussion

8.1 Role of the Gb₃ fatty acid content in retrograde transport and signal transduction

8.1.1 Effect on retrograde transport

Although the different members of the verotoxin family all bind specifically to Gb₃, the specific cytotoxicity of each toxin is different (Head, et al., 1988). Surprisingly, this difference in specific cytotoxicity is a function of the B receptor binding and not the A cytotoxic subunit (Head, et al., 1991). Thus the glycolipid binding subunit determines not only the specificity but the specific activity of the verotoxins. Studies indicate that decreasing the chain length of the surrounding phospholipid results in a change of the relative binding selectivity between different Gb₃ fatty acid isoforms (Arab and Lingwood, 1996). Heterogeneity of the lipid component of GSLs plays an important role in VT-mediated cytotoxicity and VT1 binding capacity of Gb₃ is a function of the fatty acid chain length (Pellizzari, et al., 1992; Kiarash, et al., 1994). It was suggested that the differences in fatty acid content could change the conformation of the carbohydrate moiety (Nyholm, et al., 1996).

Human astrocytoma tumor cell lines (Chapter 4) are highly sensitive to VT (Arab, et al., in press) but this sensitivity varies significantly for cell lines of apparently equivalent Gb₃ content. This difference in sensitivity correlates with differences in fatty acid chain length of the Gb₃ and differences in intracellular retrograde transport of toxin. Growth of the least sensitive astrocytoma cell line (XF-498) in butyrate was found to result in >5000 fold increase in VT sensitivity concommitent with the ability to internalize FITC labelled VT1 B subunit from the cell surface to the nuclear membrane (Chapter 5; Arab and Lingwood in preparation). The results in Chapter 5 show that the change in toxin routing following butyrate treatment is accompanied by a change in fatty acid composition of Gb₃, such that a slower migrating Gb₃ band (likely containing a shorter fatty acid

chain) is detected on thin layer chromatography (Arab and Lingwood in preparation). This species was absent in the least sensitive astrocytoma (XF-498), present in the most sensitive (SF-539), and markedly elevated for the XF$^+$ cells grown in butyrate. Fatty acid analysis of this Gb3 species showed, as expected, that it contained an higher percentage of shorter chain fatty acids. The level of C16:0 was increased from 5% to 25% and the level of C24:0 reduced from 39% to 9% as compared to Gb3 from XF-498 cells (Table 5.1).

In another system, ovarian tumor cell lines, it was shown that MDR variants of the cell line showed a significant increase in sensitivity to VT in vitro as compared with the parental cell line (Chapter, 3; Farkas-Himsley, et al., 1995). and express high levels of the P-glycoprotein (Bradley, Naik et al., 1989). Using fluorescent toxin B subunit, it was demonstrated that the MDR phenotype resulted in the targeting of the toxin to the ER/nuclear membrane whereas the parental tumor cell line targeted the toxin, for the most part, to vesicles in a non-nuclear location. Similarly on analysis of the Gb3 content of the MDR resistant variants, a lower band of Gb3 was detected on TLC overlay, similar to that detected following butyrate treatment of astrocytoma cells. Fatty acid analysis confirmed the elevated level of short chain fatty acid in Gb3 in both systems (Table 5.1 & 5.3). Thus induction of the MDR phenotype behaves in a similar manner to addition of exogenous butyrate, to switch on the nuclear retrograde transport pathway for Gb3, coincident with a change in the fatty acid component of the Gb3 fraction and increased VT1 sensitivity.

The finding that MDR cells are hypersensitive to verotoxin, indicates the potential of using verotoxin as an adjunct to classical tumor chemotherapy. Moreover the suggestion that this hypersensitivity can be induced by means other than induction of the classical MDR phenotype, ie, by butyrate treatment, suggests that tumor cells in vivo may well be liable to be induced to become more VT sensitive. In this regard, butyrate has been approved for human administration

(Chen et al., 1994). In conclusion, VT identifies an intracellular routing (signal transduction pathway) dependent on Gb3 fatty acid content which is activated in MDR and butyrate treated cells to markedly increase tumor cell VT sensitivity. This retrograde transport targets VT to the nuclear membrane for rapid induction of apoptosis (Chapter, 5; Arab and Lingwood in preparation).

Based on these findings a model has been proposed (Lingwood, 1996) to explain this intracellular sorting of Gb3 for retrograde transport according to fatty acid content (Figure 8. 1).

The hypothesis here is that the change in Gb3 fatty acid content resulted in an altered conformation of the carbohydrate. This would then require some form of 'sorting protein' which, for example targets the shorter chain fatty acid Gb3 to the Golgi-ER intermediate compartment and then to the nucleus or directly from the plasma membrane to the nucleus. It can be postulated that P-glycoprotein may act as a sorter protein, since P-glycoprotein has been shown to bind and translocate glucosylceramide with shorter fatty acid chain length (Helvoort, et al 1996). The shorter fatty acid chain length of Gb3 would determine the localization and thus the function of protein ligand (VT in this case), resembling the role of fatty acid in localization and function of G-proteins (Wedegaertner, et al., 1995).

The results described here show that the retrograde transport of VT1 is cell type specific and may reflect the difference in fatty acid composition of Gb3. This is in agreement with the finding that the morphology and the mechanism of functioning of the endosomal system depend, to the large extent, on the cell type (van Meer, 1993; van der Bijl et al, 1996). Based on our results we propose that these two types of astrocytoma cell lines might have different endocytotic trafficking pathways. In XF-498 , the less sensitive cells to VT1 treatment, the majority of Gb3 bound VT1 is sorted from the peripheral early endosomes, to the lysosomes and the Golgi apparatus. However, in sensitive SF-539 cells VT1 is directed to the nucleus where it apparently exerts an apoptotic signal to start the
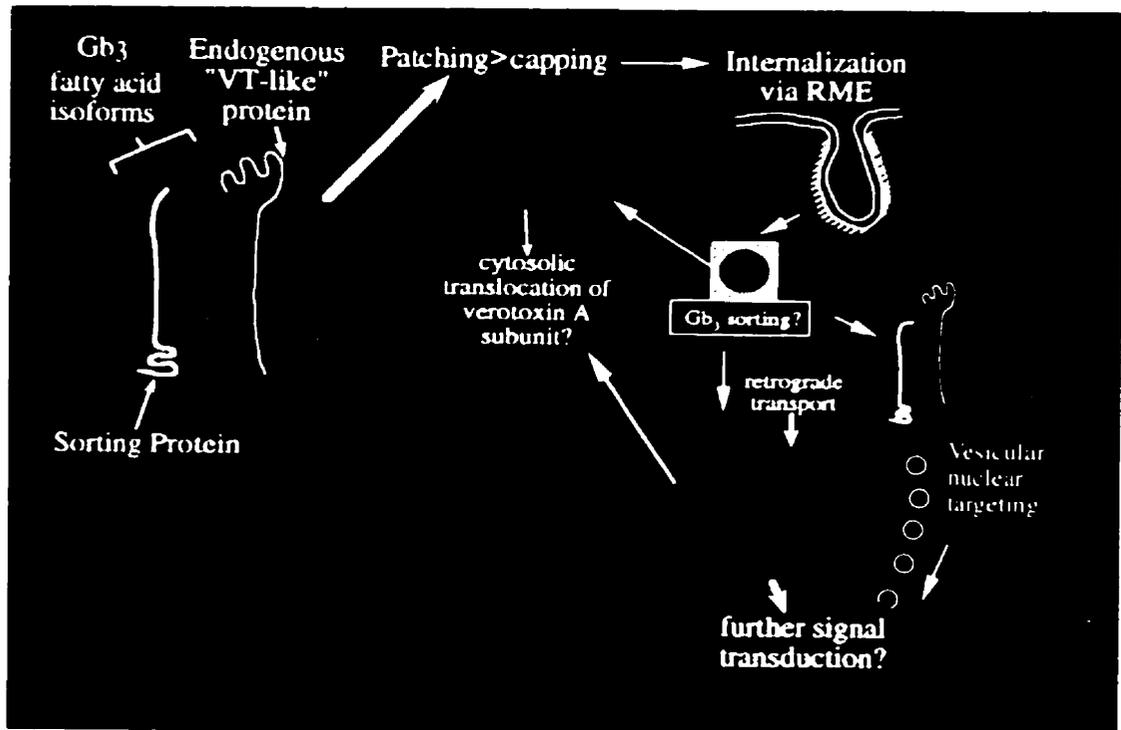
Figure 8.1: Model of retrograde transport of Gb3 and binding ligands

programmed cell death pathway. This level of signal transduction for evoking apoptosis is beyond the point of no return. This could be the reason for the inability of bcl-2 to prevent apoptosis induced by VT1 (personal communication, J. Wiels). Bcl-2 oncoprotein is an inner membrane protein of mitochondria that blocks apoptosis (Oltvai and Korsmeyer, 1994).

8.1.2 Effect on signal transduction

Both the α_2-interferon receptor subunit and the extracellular domain of CD19 show sequence homology with verotoxin B subunit (Maloney and Lingwood, 1993) and therefore they may contain a Gb_3 binding site (Lingwood and Yiu, 1992) ; (Ghislain, et al., 1994). Moreover, in addition to α2-interferon antineoplastic activity for certain tumors (Colamonici, et al., 1994; Sexl, Wagner et al., 1994), these studies show that its receptor, even in the absence of α2-interferon, has a distinct antineoplastic activity. A ligand independent interferon receptor antineoplastic effect could be related to its Gb_3 binding activity. A defect in Gb_3 content of B cells was shown to compromise both α_2-interferon and CD19-ligation dependent signal transduction pathways (Ghislain, et al., 1994; Maloney et al., 1994).

Internalization of Gb_3 by verotoxin is inhibited in the presence of monodansyl cadaverine (Khine and Lingwood, 1994). Prevention of internalization protects cells against VT induced cytopathology. Thus the retrograde transport pathway is involved in the mechanism by which VT holotoxin or B subunit induces apoptosis (Mangeney, et al., 1993; Inward, et al., 1995; Arab, et al., in press).

There is increasing evidence for the role of fatty acid protein acylation in signal transduction (Casey, 1995; Wedegaertner, et al., 1995; Helvoort, 1996). Conjugation of various proteins to fatty acid species of only minimal difference in chain length has been shown to result in targeting of such proteins to the different

membranes within the cell (Robbins et al., 1995), suggesting that fatty acid acylation may be a more generalized mechanism for subcellular membrane targeting and signal transduction.

It was shown that the suppressive effect of shed gangliosides on immune responses in vitro is dependent not only on the carbohydrate but also the fatty acid chain length (Ladisch et al., 1994; 1995).

8.1.2a. Effect on the role of Gb3 as proliferation signal

Based on the following findings from this and other studies collectively, it can be postulated that Gb3 is a proliferating and/or survival signal and different isoforms of Gb3 are the determinant of its function in this signal transduction pathway.

While the normal function of Gb3 is not clearly understood, Gb3 has been implicated in the modification of cell growth parameters. Cell binding of the VT1-B subunit alone can induce apoptosis (Mangeney, et al., 1993; Arab, et al., in press; Chapter, 4) in B cells and Gb3 (CD77) containing B cells are prone to apoptosis during B-cell differentiation (Mangeney, et al., 1991). Sensitivity to VT1 is a function of cell cycle with the cells at the G1/S boundary, (where Gb3 isoforms with shorter fatty acid chains are prominent), being particularly sensitive (Obrig, et al., 1988; Pudymaitis and Lingwood, 1992). Moreover, human renal endothelial cells in quiescent phase in intact tissue do not express Gb3 (Lingwood, 1994), however upon growth in culture these cells show elevated level of Gb3 expression and rapid growth (Obrig, et al., 1988).

In this study it was demonstrated that the multidrug resistant ovarian tumor cells show elevated level of Gb3 and are significantly more sensitive to VT1 that the parental cell line. Intensive binding of FITC-VTB was observed in high grade brain tumor, glioblastoma multiforme.In this regard accumulation of glucosylceramide was recently reported in multidrug-resistant cancer cells (Lavie, et al., 1996) and

MDR1 P-glycoprotein was identified as a translocase for glucosylceramide, a precursor of Gb3 (Helvoort, et al., 1996). Moreover, tamoxifen treatment significantly inhibited formation of glucosylceramide and lactosylceramide (precursors of Gb3) in human breast cancer cells (Cabot et al., 1996) demonstrating the involvement of these GSLs in pathogenesis of MDR in breast cancer. Since inhibition of the synthesis of these GSLs reversed the MDR in these cells.

Astrocytoma cells (XF^+), treated with butyrate function the same way in internalizing and targeting VT to the nucleus as ovarian MDR cells (SKVLB). Both systems have been shown to express elevated level of specific Gb3 isoforms with shorter fatty acid chain length. These isoforms of Gb3 may be responsible for MDR properties of SKVLB cells. In other words, XF+ may represent an MDR variant of the parental cell. However, additional experimentation is required to confirm this speculation. In this regards, stable differentiation of a human colon adenocarcinoma cell line by sodium butyrate was shown to be associated with multidrug resistance (Ho, 1994). Based on preliminary results that there is homology between P-glycoprotein sequence and VT1B (data not shown), it is possible that MDR1 P-glycoprotein can also translocate the Gb3 to the outer leaflet of plasma membrane where the signal transducing function of Gb3 for cell proliferation or survival is mediated. However, further experimentation is required to confirm this possibility.

It is interesting to note that, a 66-years old woman with p genotype (Chapter, 1), undergoing surgery to remove a gastric carcinoma was tested with an small amount (25 ml) of an inappropriate P blood group transfusion to test the reaction (Levine et al., 1951; Kannagi, et al., 1982). Because of the reaction due to incompatible blood group, the tumor was only partially removed. The patient's anti P blood group glycolipid titre (anti pk, P and P1) was dramatically increased (Kannagi, et al., 1982) and likely as a result of such antibodies, no recurrence of the tumor occurred and the patient lived 22 more years with no further signs of cancer.

Her serum carried antibody to p antigen. The mechanism of anti-Gb3 and VT1 tumor targeting may share a common basis. In the present studies, preliminary result of anti-Gb3 in combination with VT1 increased the rate of induction of apoptosis in SKVLB and SF-539 cells (Chapter, 5; Fig. 5.17).

Therefore, Gb3 may provide the signal for cell proliferation or survival and any ligand of Gb3 may be capable of antineoplastic effect. The role of glycolipids in development of neoplasia and cell proliferation needs more investigation.

8.2 Targeting of Verotoxin in human primary tumors in vitro

As reported in chapter 5 and 7, verotoxin is exquisitely specific in binding to ovarian and brain tumor cells. Tissue sections overlaid with FITC-VT1B show extensive VT binding to tumor cells and blood vessels within the tumor mass. Both primary and secondary ovarian tumor and metastasis sections indicated VT targeting of tumor tissue. Tumor foci were stained but stromal tissue was consistently negative. The level of Gb3 expression was a predictor of ovarian MDR tumors and potentialy can be standardized for diagnosis of such tumors. In the ovarian carcinomas and glioblastoma multiforme, the lumen of blood vessels within the tumor mass were also heavily labelled by VT (Chapter, 5 & 7; Farkas-Himsley, et al., 1995; Arab, et al., submitted) indicating that VT is able to bind to the activated endothelial cells which vascularize the growing tumor. As it has been shown in culture (Obrig, et al., 1993), activated endothelial cells can be extremely toxin sensitive. Thus it is likely that verotoxin will prove antiangiogenic, in addition to being a direct antineoplastic agent, which is relevant to ovarian cancer and brain tumor (GBM) with significant level of angiogenesis (Hollingsworth, et al., 1995). Moreover, recently interferon was used for treatment of life threatening hemangiomas in infants to act as an antiangiogentic agent (Ezekowitz, et al., 1992). Metastases from the ovarian tumors were also found to be highly toxin reactive and only the tumor cell foci within the metastases were labelled (Arab, et al.,

submitted). The expression of Gb3 was significantly elevated in ovarian metastatic samples irrespective of the Gb3 status of the parental tumor. This is in agreement with studies indicating Gb3 was a marker of metastatic potential (Mannori, Cecconi et al., 1990).

Glial tumors accounts for about half of all intracranial tumors and malignant gliomas are the most common malignant brain tumors, essentially considered incurable (Kays and Laws, 1995). The infiltrative growth pattern of these tumors precludes curative neurosurgery, and such tumors fail to respond to irradiation, chemotherapy, or immunotherapy (McCormack, et al., 1992). Malignancy has been recognized to result not only from enhanced cell proliferation, but from decreased apoptosis (Muller, et al., 1977). In the present study, the rapid induction of apoptosis was observed in astrocytoma cell line (Chapter, 4). Toxin localization studies in human primary brain tumor samples demonstrated extensive binding of FITC-VT1B, proportional to tumor grade, with GBM showing maximal binding (Chapter, 7). Significant binding was observed in the lumen of the blood vessels in GBM, which may indicate the potential of antiangiogenic effect of VT (Chapter, 7). VT1 treatment of astrocytoma xenografts in mice resulted in complete regression of the tumor in 100% of mice tested (Chapter, 7).

8.3 Anti-angiogenic and Anti-mitogenic effect of verotoxin

In conclusion, the process of cancer metastasis is highly selective and is regulated by a number of different mechanisms (Fidler, 1990). The outcome of metastasis depends on the properties of both tumor cells and host factors. Therapy of cancer metastases can therefore be targeted against tumor cells or host homeostatic factors that facilitate this process (Fidler, 1990). The major barriers to the treatment of metastasis are the biological heterogeneity of cancer cells and the rapid emergence of tumor cells with resistance to most conventional anticancer agents (Fidler, 1985). Inhibition of angiogenesis, however, provides a novel and

more general approach for treating metastases by manipulation of the host microenvironment. Endothelial cells in tumor blood vessels divide rapidly, whereas those in normal tissues do not (Folkman, 1992). The division of endothelial cells is induced by a variety of angiogenic factors. Treating neoplasms by targeting both the tumor cells (chemotherapy) and the organ environment (angiogenesis inhibition) has been shown to produce synergistic effects in mice bearing tumors (Teicher, 1992). Interestingly, VT1 is potentially capable of both targeting the cancer cells and inducing apoptosis in blood vessels and inhibiting angiogenesis. Potential use of VT1 as an antiangiogenic agent goes far beyond neoplasia and may be used in non-neoplastic conditions as well (eg. atherosclerosis, proliferative retinopathies, etc.).

Moreover, synthesis of a soluble form of free globotriaose oligosaccharide which is capable of inhibiting VT/receptor binding is under way (Dr. Mylvaganam, unpublished results). Thus such competitive inhibitors could potentially be administered into the systemic circulation to prevent possible distal side effects following injection of verotoxin directly into a tumor site. This would be particularly effective for astrocytomas in the brain since these are, in general, protected from the systemic circulation and it might well be possible to inject the toxin directly into the brain lesion with minimal escape of the toxin into the systemic circulation. Injection of VT1 at 10 times LD50 into mouse brain did not induce any lethal effect (Personal communication, Dr. Moscarello).

In conclusion, Verotoxin is a potent, Bcl-2 independent, rapid inducer of apoptosis in several human tumors and toxin sensitivity is a function of cell growth which induces a new intracellular traffic pathway. This pathway is most marked in multiple drug resistance, sensitized tumor cell (butyrate treated), and in activated endothelial cells which vascularize the tumor. Verotoxin may provide a new potential therapeutic modality for several human neoplasia for which little optimism in clinical treatment currently exists.

Future work

A novel finding of the present study demonstrates that the intracellular routing of verotoxin from the cell surface to the nuclear membrane by retrograde transport is an important signal transduction pathway which is related to its cytotoxicity. The shorter fatty acid chain length of the VT receptor Gb3 was found to play the determinant role in VT routing and thus its cytotoxicity. For future work, one direction worthy of serious consideration is finding the exact role and routing of these Gb3 isoforms. In this regard it would be interesting and informative to investigate the role of caveolae in verotoxin transport. Caveolae are glycolipid-enriched structures found on the surface of many mammalian cells and are involved in protein sorting (Brown, 1992; Patron, 1996). The emerging picture of caveolar structure is of a lipid-based microdomain made up of caveolin and specific lipids, with a crucial role for cholesterol. The basic caveolin-lipid structure may act as a scaffold with which other components, such as trimeric G proteins, receptor, and receptor bound toxin, can associate in a regulated fashion. This could act to concentrate specific molecules in a discrete region of the plasma membrane for a number of different functions, including internalization and signal transduction in the case of VT. In a recent study it was shown that caveolin cycles constitutively between the plasma membrane and ER/Golgi intermediate compartment (ERGIC). It was proposed that caveolin may shuttle fatty acids and cholesterol between plasma membrane and the ER/Golgi (Conrad, et al., 1995). In addition, since it was shown that verotoxin is not internalized only through clathrin coated pits in Vero cells (Grinstein unpublished observation), and based on the present finding that verotoxin was seen in the ER/nuclear membrane, the involvement of caveolae in routing of VT is therefore worthy of investigation. In this regard it will be fairly simple to identify first if caveolae are involved in translocation of VT by double immunofluorescence labeling of the cells with fluorescence-labeled VT and

fluorescence-labeled antibody to caveolin which is commercially available (m Ab 2234, Glentech, Inc.). Second, to follow the path of caveolae containing VT (time course study), with both fluorescence and immunogold electron microscopy using a colocalization study with specific markers such as ERGIC-53 (specific ERGIC marker), β-COP marker for both ERGIC and Golgi. Dissection of the pathway into distinct, individual steps is possible by using specific markers and placing a roadblock at each signature location within the pathway. In this way different perturbants can be used in the same pathway to confirm the findings. To investigate the involvement of caveolae in VT internalization and routing and confirm the finding by a different approach, biochemical analysis of subcellular fractions may be employed. Briefly, cells treated with labelled-VT (different time course) can be fractionated on a Percoll gradient which should yield three distinct fractions (Smart, et al., 1995); cytosol, highly purified plasma membrane, and intracellular membranes (Golgi, ER, lysosomes, endosomes, mitochondria, etc.). An aliquot of each fraction can then be analyzed by immunoblotting with anti-caveolin. It is also possible to purify caveolae from cells by a detergent-free method (Smart, et al., 1995) to follow VT internalization and transport.

Moreover, there are other issues that are worth following up for future work which include:

• To screen for all other tumor cell lines sensitive to VT in order to find if there is any correlation between tumor cell line sensitivity and sensitivity of representative tumors to VT.

• to determine the molecular events in induction of apoptosis by VT:

P53, P^{Rb}, CKI contribution, and further investigation of bcl-2.

• to investigate the role of lipid second messenger in induction of apoptosis by VT

ceramide, sphingosine, sphingosine-P level before and after VT treatment.

Preliminary results have shown that the nuclear membrane contains Gb$_3$ (data not shown). In this regard, further experimentation is required to decipher

the interactions and contribution of the Gb3 isoforms. Several studies can be performed to further address the question of fatty acid chain length in verotoxin sorting and signaling inside the cell and to determine if VT possesses a nuclear localization signal (NLS) that was not yet identified. Some of the proposed experiments are as follow:

• To explore the possibility of VT containing an unidentified NLS as of yet, through mutation and deletion analysis.

•Determining the isoforms of Gb3 in nuclear membrane before and after VT holotoxin or VTB and before or after butyrate treatment.

These results can determine whether there is a selection of fatty acid isoforms for the retrograde transport to the Golgi/RER, nuclear membrane and what is the effect of butyrate treatment on nuclear Gb3.

Same study can be performed for subcellular organelles and their contribution in this signaling process.

•Reconstitution of Gb3 specific homologues in Gb3 negative cells and following the sorting and signaling in each system.

These results will clearly assist the investigator to determine the optimal chain length of Gb3 in the process of induction of apoptosis by VT.

• Saturate the above cells with the anti-Gb3 and examine the binding , internalization, sorting and signaling of VT/VTB in these cells.

These results will increase our understanding of subpopulation of Gb3 which are involved in VT or anti-Gb3 binding. Do VT and anti-Gb3 bind to the same epitopes of Gb3? Does the differential binding to the epitopes of Gb3 also affect the routing and signaling? What is the contribution of the sugar moiety of Gb3 in addition to binding to ligands with respect to different epitopes being involved?

• To compare the Gb3 fatty acid content of tumors and determine the Gb3 fatty acid profiles.

The results should be useful in order to identify tumors sensitive to VT.

• To compare Gb3 expression in two clinical patterns of cancer metastases: I) a primary tumor is removed, but within a few months metastases appear.

II) metastases disappear after removal of primary tumor.

The results from this investigation would determine the Gb3 contribution in above clinical patterns of metastases and can be expanded to other patterns as well.

References

Abe A. Inokuchi J. Jimbo M. Shimeno H. Nagamatsu A. Shayman JA.Shukla GS. Radin NS. (1992). Improved inhibitors of glucosylceramide synthase. J. Biochemistry. 111:191-6.

Anderson, K. M., T. M. Seed, J. Peng, A. Jajeh, J. Meng and J. E. Harris (1994). "Morphologic changes of apoptosis induced in human chronic myelogenous leukemia "blast" cells by SC41661A (Searle), a selective inhibitor of 5-lipoxygenase." Scanning Microscopy 8(3): 675-686.

Antony, C., C. Cibert, G. Geraud, A. Santa Maria, B. Maro, V. Mayau and B. Goud (1992). "The small GTP-binding protein rab6p is distributed from medial Golgi to the trans-Golgi network as determined by a confocal microscopic approach." J Cell Sci 103: 785-796.

Arab, S. and C. A. Lingwood (1996). "Influence of phospholipid chain length on verotoxin/globotriaosyl ceramide binding in model membranes: comparison of a surface bilayer film and liposomes." Glycoconj J 13: 159-166.

Arab, S., E. Russel, W. Chapman, B. Rosen and C. Lingwood (submitted). "Overexpression of Verotoxin receptor glycolipid in ovarian tumours and their metastases." Cancer.

Arab, S., M. Murakami, P. Dirks, B. Boyd, S. Hubbard, C. Lingwood and J. Rutka (in press). "Verotoxins inhibit the growth of and induce apotosis in human astrocytoma cells." Neuropathol Exp Neurol.

Arab, S. and Lingwood, C. "Intracellular targeting of the nuclear envelope by retrograde transport determines cell hypersensitivity to verotoxin: Sodium butyrate induces nuclear toxin targeting via globotriaosyl ceramide fatty acid isoform traffic." (manuscript in preparation).

Arab, S., Rutka J·, and Lingwood, C. "Verotoxin treatment induces apoptosis and the complete, rapid, long term elimination of human astrocytoma xenografts in nude mice."(manuscript in preparation).

Azuma, N., J. O'Brien, H. Moser and Y. Kishimoto (1994). Stimulation of acid ceramidase activity by saposin D. Arch. Biochem. Biophys. 311: 354-357.

Baak, J. P. A., E. C. M. Wisse-Brekelmans, F. A. Langley, A. Taterman and J. F. M. Delemarre (1986). "Morphometric data to FIGO stage and histological type and grade for prognosis of ovarian tumours." J Clin Pathol 39: 1340-1346.

Banfield, D. K., M. J. Lewis, C. Rabouille, G. Warren and H. R. Pelham (1994). "Localization of Sed5, a putative vesicle targeting molecule, to the cis-Golgi network involves both its transmembrane and cytoplasmic domains." J Cell Biol 127: 357-371.

Barber, H. R. K. (1993). Ovarian Carcinoma. Etiology, Diagnosis and Treatment. New York.

Barinaga, M. (1995). "Shedding light on blindness." Science 267: 452-453.

Barrett, T. J., M. E. Potter and N. A. Strockbine (1990). "Evidence for participation of the macrophage in Shiga-like toxin II-induced lethality in mice." Microbial Pathog 9: 95-103.

Barry, M., C. A. Behink and A. Eastman (1990). "Activation of programmed cell death (apoptosis) by cisplatin, other anticancer drugs, toxins and hyperthermia." Biochem Pharma 40: 2353-2362.

Beck, W. T. (1983). "Vinca alkaloids-resistant phenotype in cultured leukemic lymphoblasts." Cancer Treat Res 67: 875-882.

Beck, W. T. (1987). "The cell biology of multidrug resistance." Biochem Pharmacol 36: 2879-2887.

Bergman, E. (1990). "Energy contribution of volatile fatty acids from the gastrointestinal tract in various species." Physiol. Rev. 70: 567-590.

Bigner, S., Mark, J., Bigner, D. (1990). Cytogenetic of human brain tumors. Can. Genetics Cytogenet. 47: 141-154.

Bitzan, M., S. Richardson, C. Huang, B. Boyd, M. Petric and M. Karmali (1994). "Evidence that Verotoxins (Shiga-like toxins) from Escherichia coli bind to P blood group antigens of human erythrocytes in vito." Infect Immun 62: 3337-3347.

Boggs, J. M. (1987). "Lipid intermolecular hydrogen bonding: influence on structural organization and membrane function." Biochim Biophy Acta 906(353-404).

Boring, C. C., Squires,T. S. , Tong, T. and Montgomery, S (1994). "Cancer Statistics." CA 44: 7-26.

Bosch, I. and J. Croop (1996). "P-glycoprotein multidrug resistance and cancer." Biochem Biophys Acta 1288: F37-F54.

Bouck, N. (1990). "Tumor angiogenesis: the role of oncogenes and tumor suppressor genes." Cancer Cells 2: 179-185.

Boulanger, J., M. Huesca, S. Arab and C. A. Lingwood (1994). "Universal method for the facile production of glycolipid/lipid matrices for the affinity purification of binding ligands." Anal Biochem 217: 1-6.

Boyd, B. and C. A. Lingwood (1989). "Verotoxin receptor glycolipid in human renal tissue." Nephron 51: 207-210.

Boyd, B., G. Tyrrell, M. Maloney, C. Gyles, J. Brunton and C. Lingwood (1993). "Alteration of the glycolipid binding specificity of the pig edema toxin from globotetraosyl to globotriaosyl ceramide alters in vivo tissue targetting and results in a VT1-like disease in pigs." J Exp Med 177: 1745-1753.

Bradley, G., M. Naik and V. Ling (1989). "P-glycoprotein expression in multidrug-resistant human ovarian carcinoma cell lines." Canc Res 49: 2790-2796.

Bremer, E. (1994). Glycosphingolipids as effectors of growth and differentiation. Curr. Topics in Membrane, Academic Press, 40: 387-411.

Brown, Y. and Green, M. (1991). Molecular and cellular mechanism of receptor mediated endocytosis. DNA Cell Biol. 10:399-409.

Burczak, J.D., Soltysiak, R.M., and Sweeley, C.C. (1984). " Regulation of membrane-bound enzymes of glycosphingolipid biosynthesis. " J. Lipid Res. 25: 1541-1547.

Bruzik, K. S. (1988). "Conformation of the polar head group pf sphingomyelin and its analgoues." Biochem Biophys Acta 938: 315-321.

Buckner, J., L. Brown, J. Kugler, T. Cascino, J. Krook, J. Mailliard, C. Kardinal, L. Tschetter, J. O'Fallon and B. Scheithauer (1995). "Phase II evaluation of recombinant interferon alpha and BCNU in recurrent glioma." J Neurosurg 82: 430-435.

Burger, KN., van der Bijl, P., van Meer, G. (1996). Topology of sphingolipid galactosyltransferasese in ER and Golgi: transbilayer movement of monohexosyl sphingolipids is required for higher glycosphingolipid biosynthesis. J. Cell. Biol. 133: 15-28.

Burger, P. C., B. W. Scheithauer and F. S. Vogel (1991). Surgical pathology of the nervous system and its coverings. Brain Tumors. 4: 193-437.

Butzner, J., R. Parmar, C. Bell and V. Dala (1996). "Butyrate enema therapy stimulates mucosal repair in experimental colitis in the rat." Gut 38: 568-573.

Cabot, M., A. Giuliano, A. Volner and T. Han (1996). "Tamoxifen retards glycosphingolipid metabolism in human cancer cells." FEBS Lett 394: 129-131.

Caputto R. (1992). Endogenous inhibition of glycosyltransferases of gangliosides: possible biochemical and morphogenetic functions. [Review]. Biochemical Society Transactions. 20:705-10.

Calderwood, S. B., F. Auclair, A. Donohue-Rolfe, G. T. Keusch and J. J. Mekalanos (1987). "Nucleotide sequence of the Shiga-like toxin genes of Escherchia coli." Proc Natl Acad Sci 84: 4364-4368.

Carlsen, S. A., J. E. Till and V. Ling (1976). "Modulation of membrane drug permeability in Chinese hamster ovary cells." Biochem Biophys Acta 455: 900-912.

Casey, P. (1995). "Protein lipidation in cell signaling." Science 268: 221-.

Cerame, MA., Guthikonda, M., Kohli, CM. (1985). Extraneural metastasis in gliosarcoma: A case report and review of the literature. Neruo. Surge. 17: 413-418.

Chen, Z.-X. and T. R. Breitman (1994). "Tributyrin: A prodrug of butyric acid for potential clinical application in differentiation therapy." Cancer Res 54: 3494-3499.

Christopher-Hennings, J., J. A. Willgohs, D. H. Francis, U. A. K. Raman, R. A. Moxley and D. J. Hurley (1993). "Immunocompromise in gnotobiotic pigs induced by verotoxin-producing Escherichia coli (0111:NM)." Infect Immun 61(6): 2304-2308.

Clark, A. R., C. A. Purdie, D. J. Harrison, R. G. Morris, C. C. Bird, M. L. Hooper and A. H. Wyllie (1993). "Thymocyte apoptosis induced by P53-dependent and independent pathways." Nature 362: 849-852.

Cohen, A., G. E. Hannigan, B. R. G. Williams and C. A. Lingwood (1987). "Roles of globotriosyl- and galabiosylceramide in verotoxin binding and high affinity interferon receptor." J. Biol. Chem. 262(35): 17088-17099.

Cohen, A., V. Madrid-Marina, Z. Estrov, M. Freedman, C. A. Lingwood and H.-M. Dosch (1990). "Expression of glycolipid receptors to Shiga-like toxin on human B lymphocytes: a mechanism for the failure of long-lived antibody response to dysenteric disease." Int. Immunol. 2: 1-8.

Colamonici, O., B. Porterfied, P. Domanski, R. Handa, S. Flex, C. Samuel, R. Pine and M. Diaz (1994). "Ligand-independent anti-oncogenic activity of ther alpha subunit of the type 1 interferon receptor." J Biol Chem 269.

Cosson, P. and F. Letourneur (1994). "Coatomer interaction with di-lysine endoplasmic reticulum retention motifs." Science 263: 1629-1631.

Costello, R. and Delmaestro, R. (1990). "Human cerebral endothelium; isolation and charcterization of cells derived from microvessels of non-neoplastic and malignant glial tissue." J. Neuro-oncol 8: 231-243.

Cramer, D. W. and W. R. Welch (1983). "Determination of ovarian cancer risk. II. Inferences regarding pathogenesis." J Natl Cancer Inst 71: 717-721.

Crook, S. J., J. M. Boggs, A. I. Vistnes and K. M. Koshy (1986). "Factors affecting surface expression of glycolipids influence of lipid environment and ceramide composition on antibody recognition of cerebroside sulfate in liposomes." Biochem 25: 7488-7494.

Curatolo, W. (1987). "Glycolipid function." Biochimica et Biophysica Acta 906: 137-160.

Danks, M. K., C. A. Schmidt, M. C. Cirtain, D. Parker Suttle and W. T. Beck (1988). "Altered catalytic activity of DNA cleavage by topoisomerase II from human leukemic cells selected for resistance to VM-26." Biochem 27: 8861-8869.

DeGrandis, S., H. Law, J. Brunton, C. Gyles and C. A. Lingwood (1989). "Globotetraosyl ceramide is recognized by the pig edema disease toxin." J. Biol. Chem. 264: 12520-12525.

Donohue-Rolfe, A., N. A. Kelley, M. Bennish and G. T. Keusch (1986). "Enzyme-linked immunosorbent assay for Shigella toxin." J. Clin. Microbiol. 24(1): 65-68.

Drago, J., Murphy, M., Carole, S.M., Harvey, R.P. and Barlett, P. (1991). Fibroblast growth factor-mediated proliferation of central nervous system precursors depends on the endogenous production of insulin-like growth factor I. Proc. Natl. Sci. Acad. USA. 88: 2199-2203.

Dressler, K. A., S. Mathais and R. N. Kolesnick (1992). "Tumor necrosis factor-alpha activates the sphingomyelin signal transduction pathway in a cell-free system"Science 255: 1715-1718.

Duverger, E. P.-M., C Mayer, R Roche, A Monsingny, M (1995). "Nuclear import of glycoconjugates is distinct from the classical NLS pathway." J. Cell Sci. 108: 1325-1332.

Eggens, I., B. Fenderson, T. Toyokuni, B. Dean, M. Stroud and S.-I. Hamomori (1989). "Specific interaction between Le^x and Le^x determinants." J Biol Chem 264(16): 9476-9484.

Endo, Y., K. Tsurugi, T. Yutsudo, Y. Takeda, K. Ogasawara and K. Igarashi (1988). "Site of the action of a vero toxin (VT2) from Escherichia coli O157:H7 and a Shiga toxin on eukaryotic ribosomes." Eur J Biochem 171: 45-50.

Escriba, P. V., A. V. Ferrer-Montiel, J. A. Ferragut and J. M. Gonzales-Ros (1990). "Role of membrane lipids in the interaction of daunomycin with plasma membranes from tumor cells: implications in drug-resistance phenomena." Biochem 29: 7275-7282.

Espand, R. F., R. M. Espand, R. S. Gupta and E. J. Cargoe (1991). "Reversal of intrinsic multi-drug resistance in Chinese hamster ovary cells by amiloride analogues." Br J Cancer 63: 247-251.

Ezekowitz, R. A., J. B. Mulliken and J. Folkman (1992). "Interferon α2a therapy for life-threatening hemangiomas of infancy." N Engl J Med 326: 1456-1463.

Falcieri, E., P. Goggi, L. Zamai and M. Vitale (1994). "Ultrastructural features of apoptosis." Scan Microscopy 8: 653-666.

Farkas-Himsley, H. (1976). "Bacteriocins as growth inhibitors of neoplastic and tumorigenic cells." IRCS Med Sci 4: 291-295.

Farkas-Himsley, H. and C. E. Musclow (1980). "Bacteriocins, effect on mammalian cells: mode of analyzed by flow cytometry and cell sorting." Cell & Mol Biol 26: 597-603.

Farkas-Himsley, H. and H. Yu (1985). "Purified colicin as cytotoxic agent of neoplasia: comparative study with crude colicin." Cytobios 42: 193-207.

Farkas-Himsley, H., B. Rosen, R. Hill, S. Arab and C. A. Lingwood (1995). "Bacterial colicin active against tumour cells in vitro and in vivo is verotoxin 1." Proc Natl Acad Sci 92: 6996-7000.

Farkas-Himsley, H., R. Cheung and W. A. F. Tompkins (1975). "Selective antitumor agents: Bacteriocins from gram-negative bacteria." IRCS Med Sci 3: 148.

Farker, P. and Speck, JC. (1978). Protein and cell membrane iodinations with a sparingly soluble chloroamide, 1,3,4,6-tetrachloro-3-alpha, 6-alpha-diphenyl-glycoooluril. Biochem. Biophys. Res. Commun. 80: 849-857.

Fenderson, B. A., E. M. Eddy and S.-I. Hakomori (1990). "Glycoconjugate expression during embryogenesis and its biological significance." BioEssays 12(4): 173-179.

Fidler, I. J. (1990). "Department of Cell Biology, University of Texas M.D. Anderson Cancer". Cancer Res 50: 6130-6138.

Folch, J., M. Lees and G. H. Sloane-Stanley (1957). "A simple method for the isolation and purification of total lipids from animal tissues." J Biol Chem 226: 497-521.

Folkman, J. (1992). "The role of angiogenesis in tumor growth." Semin Cancer Biol 3: 65-71.

Folkman, J. and Y. Shing (1992). "Angiogenesis." J Biol Chem 267(16): 10931-10934.

Folkman, T. (1995). "Angiogenesis in cancer,vascular, rhumatoid and other disease." Nature, Med 1: 27-31.

Fraser, M., Chernaia, M., Kozlov, Y., and James, M. (1994). "Crystal structure of the holotoxin from Shigella dysenteriae at 2.5A resolution". Struct. Biol. 1: 59-64.

Friches, E., M. K. Danks, C. A. Schmidt and W. T. Beck (1991). "Decreased DNA topoisomerase II in daunorubicon resistant Eherlich ascites tumor cells." Cancer Res 51: 4213-4218.

Fu, X.-Y. (1992). "A transcription factor with SH2 and SH3 domains is directly activated by an interferon α-induced cytoplasmic protein tyrosine kinase(s)." Cell 70: 323-335.

Gahmberg, C. G. and S. Hakomori (1973). "External labeling of cell surface galactose and galactosamine in glycolipid and glycoprotein of human erythrocytes." J Biol Chem 248(12): 4311-4317.

Gannon, V. P. J. and C. L. Gyles (1990). "Characteristics of the Shiga-like toxin produced by Escherichia coli associated with porcine edema disease." Vet. Microbiol.(24): 89-100.

Garred, O., E. Dubinina, P. K. Holm, S. Olsnes, B. van Deurs, J. V. Kozlov and K. Sandvig (1995). "Role of processing and intracellular transport for optimal toxicity of Shiga toxin and toxin mutants." Exp Cell Res 218(1): 39-49.

Gartner, S., E. Connzelmann and K. Sandhoff (1983). J. Biol. Chem. 258: 12378-12385.

Ghislain, J., C. A. Lingwood and E. N. Fish (1994). "Evidence for glycosphingolipid modification of the type 1 IFN receptor." J Immunol 153: 3655-3663.

Ghislain, J., C. Lingwood, M. Maloney, L. Penn and E. Fish (1992). "Association between the IFN alpha receptor and the membrane glycolipid globotriaosyl ceramide." J Interfer Res 12: s114.

Gillard, B. K., L. T. Thurman, R. G. Harrell, Y. Capentanaki, M. Saito, R. K. Yu and D. M. Marcus (1994). "Biosynthesis of glycosphingolipids is reduced in the abseance of a vimentin; intermediate filament network." J Cell Science 107: 3545-3555.

Gregory, C. D., T. Tursz, C. F. Edwards, C. Tetaud, M. Talbot, B. Caillou, A. B. Rickinson and M. Lipinski (1987). "Identification of a subset of normal B cells with a Burkitt's lymphoma (BL)-like phenotype." J. Immunol. 139(1): 313-318.

Gregory, G. D., C. F. Edwards, A. Milner, J. Wiels, M. Lipinski, M. Rowe, T. Tursz and A. B. Rickinson (1988). "Isolation of a normal B cell subset with a Burkitt-like phenotype and transformation in vitro with Epstein-Barr virus." Int J Cancer 42: 213-220.

Griffin, P. W. and R. V. Tauxe (1991). "The epidemiology of infections caused by Escherichia coli 0157:H7, other enterohemorrhagic E. coli, and the associated hemolytic uremic syndrome." Epidem Rev 13: 60-98.

Griffiths, G. and K. Simons (1986). "The trans Golgi network: sorting at the exit site of the Golgi complex." Science 234: 438-434.

Griffiths, G., M. Ericsson, J. Krijnse-Locker, T. Nilsson, B. Goud, H. D. Soling, B. L. Tang, S. H. Wong and W. Hong (1994). "Localization of the Lys, Asp, Glu, Leu tetrapeptide receptor to the Golgi complex and the intermediate compartment in mammalian cells." J Cell Biol 127: 1557-1574.

Hakomori, S. (1986). "Glycosphingolipids." Scientific American 254(5): 40-53.

Hakomori, S. and R. Kannagi (1983). "Glycosphingolipids as tumor associated and differentiation markers." J. Natl. Can. Inst 71: 231-251.

Hakomori, S.-I. (1981). "Glycosphingolipid in cellular interaction, differentiations, and oncogenesis." Ann Rev Biochem 150: 733-764.

Hakomori, S.-I. (1984). "Philip Levine Award Lecture: Blood group antigens and their modifications as human cancer antigens." Am J Clin Path 82(6): 635-647.

Hakomori, S.-I. (1990). "Bifunctional role of glycosphingolipids." J. Biol. Chem. 265(31): 18713-18716.

Hakomori, S.-I. (1993). "Structure and function of sphingoglycolipids in transmembrane signaling and cell-cell intractions. Biochemical society transactions. 21: 583-595.

Hannun, Y. A. (1994). "The sphingomylin cycle and the second messenger function of ceramide." J Biol Chem 269(5): 3125-3128.

Hannun, Y. A. and B. R.M. (1987). "Lysosphingolipids inhibit protein kinase C: Implications for the sphingolipidoses." Science 235: 670-674.

Hannun, Y. A. and C. M. Linardic (1993). "Sphingolipid breakdown products: anti-prolifeartive and tumor-suppressor lipids." Biochem Biophys Acta 1154: 223-236.

Hannun, Y. A. and R. M. Bell (1989). "Functions of sphingolipids and sphingolipid breakdown products in cellular regulation."Science 243: 500-507.

Harri, E.W. Loeffler, H.P., Sigg, H., Stahelin, H., and H. Tamm. (1963). "Uber die isolierung neuer stoffwechselproduckte aus penicilillium brefeldianum." DODGE. Helv. Chim. Acta 46: 1235-1243.

Harel, R., and Futerman, A.H. (1993). Inhibition of sphingolipid synthesis affects axonal outgrowth in cultured hippocampal neurons. J. Biol. Chem. 268: 14476-14481. 1993.

Harris, S. L. and M. G. Waters (1996). "Localization of a yeast early Golgi mannosyltransferase Och1p, involves retrograde transport." J Cell Biol 132: 985-998.

Hartwell, L. and M. B. Kastan (1994). "Cell cycle control and cancer." Science 266: 1821-1828.

Hatva, E. A., P. Kaipinen, J. Mentula, A. Jaskelainen, M. Paetau, Haltia and K. Alitalio (1995). "Expression of endothelial cell-specific receptor tyrosine kinase and growth factors in human brain tumors." Am J Pathol 146: 368-378.

Head, S. C., M. A. Karmali, M. E. Roscoe, M. Petric, N. A. Strockbine and I. K. Wachsmuth (1988). "Serological Differences Between Verocytotoxin 2 and Shiga-like Toxin II." Lancet i: 751.

Head, S., K. Ramotar and C. A. Lingwood (1990). "Modification of the glycolipid-binding specificity of verocytotoxin Polymyxin B and other cyclic amphipathic peptides." Infect Immun 58: 1532-1537.

Head, S., M. Karmali and C. A. Lingwood (1991). "Preparation of VT1 and VT2 hybrid toxins from their purified dissociated subunits: Evidence for B subunit modulation of A subunit function." J Biol Chem 266(6): 3617-3621.

Helvoort, A. Smith, A., Sprong, H., Fritzsche, I, Schinkel, A., Borst, P., and van Meer, G. (1996). "MDR1 P-glycoprotein is a lipid translocase of broad specificity, while MDR3 P-glycoprotein specifically translocates phosphatidylecholine." Cell 87:507-517.

Helvoort, A. and van Meer, G. (1995). "Intracellular lipid heterogeneity caused by topology of synthesis and specificity in transport. Example: sphingolipids."FEBS Lett 369: 18-21.

Higgins, C. F. and M. M. Gottesman (1992). "Comparison of two stage epidermal carcinogenesis initiated by 7,12-dimethylbenz(a)antracene or N-Methyl-N'-nitro-N-nitrosoguanidine in newborn and adult SENCAR and BALB/c mice." Cancer Res 41: 770-773.

Hill, R. P. and H. Farkas-Himsley (1991). "Further studies of the action of a partially purified bacteriocin against a murine fibrosarcoma." Cancer Res 51: 1359-1365.

Hiraiwa, M., Kishimoto, Y. (1996). Saposins and their precursor, prosaposin: Multifunctional glycoprotein. Trend Glycosci. Glycotech. 8: 341-356.

Hoe, M. H., P. Slusarewicz, T. Misteli, R. Watson and G. Warren (1995). "Evidence for recycling of the resident medial/trans Golgi enzyme, N-acetylglucosaminyltransferase I, in ldlD cells." J Biol Chem 270: 25057-25063.

Hollingsworth, H. C., E. C. Kohn, S. M. Steinberg, M. L. Rothenberg and M. J. Merino (1995). "Tumor angiogenesis in advanced stage ovarian carcinoma." Am J Pathol 147(1): 33-41.

Hudson, T. H. and D. M. Neville Jr (1987). "Temporal separation of protein toxin translocation from processing events." J Biol Chem 262(34): 16484-16494.

Hunter, T. and J. Pines (1994). "Cyclins and cancer II: cyclin D and CDK inhibitors come of age." Cell 79: 573-582.

Hyde, S. C., P. Emsley, M. J. Hartshorn, J. Mimmach, U. Gileadi, S. R. Pearce, M. P. Gallagher, D. R. Gill, R. E. Hubbard and C. F. Higgins (1990). "Structural

model of ATP binding proteins associated with cystic fibrosis, multi drug resistance and bacterial transport." Nature 346: 362-365.

Igarashi, K., T. Ogasawara, K. Ito, T. Yutsudo and Y. Takeda (1987). "Inhibition of elongation factor 1-dependent aminoacyl-tRNA binding to ribosomes by Shiga-like toxin 1 (VT1) from Escherichia coli O157:H7 and by Shiga toxin." FEMS Microbiol. Letts. 44: 91-94.

Inokuchi, J.-I., M. Jimbo, K. Momosaki, H. Shimeno, A. Nagamatsu and N. S. Radin (1990). "Inhibition of experimental metastasis of murine Lewis lung carcinoma by an inhibitor of glucosylceramide synthase and its possible mechanism of action." Cancer Res. 50: 6731-6737.

Inward, C. D., J. Williams, I. Chant, J. Crocker, D. V. Milford, P. E. Rose and C. M. Taylor (1995). "Verocytotoxin-1 induces apoptosis in vero cells." J Infect 30: 213-218.

Ito, H., A. Terai, H. Kurazono, Y. Takeda and M. Nishibuchi (1990). "Cloning and nucleotide sequencing of Vero toxin 2 variant genes from Escherichia coli 091:H21 isolated from a patient with the hemolytic uremic syndrome." Microbial Patho 8: 47-60.

Ivessa, NE., De Lemoos-Chiarandinnni, C., Gravotta, D., Sabatini, DD., Kreibich, G. (1995). The brefeldin A-induced retrograde transport from the Gogi apparatus to the ER depends on calcium sequestered to intracellular stores. J. Biol. Chem. 270: 25960-7.

Iwig, M., Czeslick, E., Muller, A., Gruner, M., Spindler, M. and Glaesser, D. (1995). Growth regulation by cell shape alteration and organization of the cytoskeleton. Europ. J. Cell Biol. 67: 145-157.

Jacewicz, M. S., M. Mobassaleh, S. K. Gross, K. A. Balasubramanian, P. F. Daniel, S. Raghavan, R. H. McCluer and G. T. Keusch (1994). "Pathogenesis of Shigella diarrhea: XVII. A mammalian cell membrane glycolipid, Gb_3, is required but not sufficient to confer sensitivity to Shiga toxin." J Infect Dis 169:538-546.

Jackson, M. P., R. J. Neill, A. D. O'Brien, R. K. Holmes and J. W. Newland (1987). "Nucleotide sequence analysis and comparison of the structural gene for Shiga-like toxin I and Shiga-like toxin II encoded by bacteriophages from Escherichia coli 933J." FEMS Microbiol. Lett. 44: 109-114.

Jans, D. (1994). "Nuclear signaling pathways for polypeptide ligands and their membrane receptors?" FASEB 8(11): 841-847.

Jarvis, W. D., R. N. Kolesnick, F. A. Fornari, R. S. Traylor, D. A. Gewirtz and S. Grant (1994). "Induction of apoptotic DNA damage and cell death by activation of the sphingomyelin pathway." Proc Natl Acad Sci, USA 91: 73-77.

Jarvis, W. D., S. Grant and R. N. Kolesnick (1996). "Ceramide and the induction of apoptosis." Clin Canc Res 2: 1-6.

Ji, L., Zhang, G., Yamagata, T. (1995). The hydrolysis of cell surface glycosphingolipids by endoglycoceramidase reduces epidermal growth factor receptor phosphorylation in A431 cells. Glycobiology. 5: 343-350.

Johnson, G. and Hollborow, E. (1989). Immunochemistry. Handbook of immunology Weir, Herzenberg . 1:284.

Juliano, R. L. and V. Ling (1976). "A surface glycoprotein modulating drug permeability in chinese hamster ovary cell mutants." Biochem Biophys Acta 455: 152-162.

Kanfer, J. and S. Hakomori (1983). Sphingolipid Biochemistry. New York, Plenum Press.

Kannagi, R., E. Nudelman and S. Hakomori (1982). "Possible role of ceramide in defining structure and function of membrane glycolipids." Proc. Natl. Acad. Sci. U.S.A. 79: 3470-3474.

Kannagi, R., R. Stroup, N. A. Cochran, D. L. Urdal, W. W. Young Jr. and S.-I. Hakomori, . (1983). "Factors affecting expression of glycolipid tumor antigens: Influence of ceramide composition and coexisting glycolipid on the antigenicity of gangliotriaosylceramide in murine lymphoma cells." Cancer Res 43: 4997-5005,.

Kaplan, B. (1994). Clinical and pathophysiological aspects of hemolytic uremic syndrome. Recent Advances in Verocytotoxin-Producing Excherichia Coli Infections. M. A. Karmali and A. G. Goglio, Elsevier Science B.V.: 301-304.

Karlsson, K.-A. and N. Stromberg (1987). "Overlay and solid-phase analysis of glycolipid receptors for bacteria and viruses." Methods Enzymol. 138: 220-232.

Karmali, M. A. (1989). "Infection by Verocytotoxin-producing Escherichia coli." Clin. Microbiol. Rev. 2: 15-38.

Karmali, M. A., M. Petric, C. Lim, P. C. Fleming, G. S. Arbus and H. Lior (1985). "The association between hemolytic uremic syndrome and infection by Verotoxin-producing Escherichia coli." J. Infect. Dis. 151: 775-782.

Karmali, M. A., M. Petric, M. Winkler, M. Bielaszewska, J. Brunton, N. van de Kar, T. Morooka, G. B. Nair, S. E. Richardson and G. S. Arbus (1994). "Enzyme-linked immunosorbent assay for detection of immunoglobulin G antibodies to Escherichia coli vero cytotoxin1." J Clin Microbiol 32(6): 1457-1463.

Kaufmann, S. H. (1989). "Induction of endonuleolytic DNA cleavage in human myelogenous leukemia cells by etoposide, camtothectin, and other cytotoxicanticancer drugs: a cautionary note." Cancer Res 49: 5870-5878.

Kaye, A.H. and Laws, E.R. (1995). Brain tumors. Churchill livingstone. New York, USA.

Kaye, S. A., C. B. Louise, B. Boyd, C. A. Lingwood and T. G. Obrig (1993). "Shiga toxin-associated hemolytic uremic syndrome: Interleukin-1ß enhancement of Shiga toxin cytotoxicity toward human vascular endothelial cells in vitro." Infect Immun 61(9): 3886-3891.

Keizer, H. G. and H. Joenja (1989). "Increased cytosolic pH in multi-drug resistant human tumor cells: effect of veramamil." J Natl Cancer Inst 81: 706-709.

Kerr, J. and S. Searle (1972). "Shrinkage necrosis: a distinct mode of cell death." J Pathol 105: 13-18.

Kerr, J. F. R., C. M. Winterford and B. V. Harmond (1993). "Apoptosis. Its significancae in cancer and cancer therapy." Cancer 73: 2013-2025.

Keusch, G., M. Jacewicz, M. Levine, R. Hornick and S. Kochwa (1976). "Pathogenesis of Shigella diarrhea. Serum anticytotoxic antibody response produced by toxigenic and nontoxigenic Shigella dysenteriae." J. Clin. Invest. 57: 194-202.

Khine, A. A. and C. A. Lingwood (1994). "Capping and receptor mediated endocytosis of cell bound verotoxin(Shiga-like toxin) 1; Chemical identification of an amino acid in the B subunit necessary forefficient receptor glycolipid binding and cellular internalization." J Cell Physiol 161: 319-332.

Khine, A. A. and C. A. Lingwood (submitted). "CD77 Dependent Retrograde Transport of CD19 to the Nuclear Membrane: Functional Relationship between CD77 and CD19 during Germinal Center B-cell Apoptosis." .

Kiarash, A., B. Boyd and C. A. Lingwood (1994). "Glycosphingolipid receptor function is modified by fatty acid content: Verotoxin 1 and Verotoxin 2c preferentially recognize different globotriaosyl ceramide fatty acid homologues." J Biol Chem 269(15): 11138-11146.

Kim, J. H., A. A. Khine, C. A. Lingwood, W. Furuya, M. F. Manolson and S. Grinstein (1996). "Dynamic measurement of the pH of the Golgi complex in living cells using retrograde transport of the verotoxin receptor." J Cell Biol 134: 1387-1396.

Kok, J. and D. Hoekstra (1994). Glycosphingolipid trafficking in the endocytic pathway., Acadamic press.

Kok, J., K. Hoekstra, S. Eskelinen and D. Hoekstra (1992). "Recycling pathways of glucosylceramide in BHK cells: Distinct involvment of early and late endosomes." J. Cell Sci. 103: 1139-1152.

Kolesnick, R. N. (1991). "Sphingomyelin and derivatives as cellular signals." Prog Lipid Res 30(1): 1-38.

Konowalchuk, J., J. I. Speirs and S. Stavric (1977). "Vero response to a cytotoxin of Escherichia coli." Infect. Immun. 18: 775-779.

Kornfeld, S. and I. Mellman (1989). "The biogenesis of lysosomes." Annu. Rev. Cell Biol. 5: 483-525.

Krenik, K. D., G. M. Kephart, K. P. Offord, S. L. Dunnette and G. J. Gleich (1989). "Comparison of antifading agents used in immunofluorescence." J Immunol Methods 117(1): 91-97.

Kripke, S., A. Fox, J. Berman, R. Settle and J. Rombeay (1989). "Stimulation of intestinal mucosal growth with intracolonic infusion of short chain fatty acids." JPEN 13: 109-116.

Ladisch, S., A. Hasegawa, R. Li and M. Kiso (1995). "Immunosuppressive activity of chemically synthesized gangliosides." Biochem 34(4): 1197-1202.

Ladisch, S., R. Li and E. Olson (1994). "Ceramide structure preadicts tumor ganglioside immunosuppressive activity." Proc Natl Acad Sci, USA 91: 1974-1978.

Lavie, Y., H. Cao, S. L. Bursten, A. E. Giuliano and M. C. Cabot (1996). "Accumulation of glucosylceramides in multidrug-resistant cancer cells." J Biol Chem 271(32): 19530-19536.

Laws, E. J., W. F. Taylor, M. B. Clifton and H. Okazaki (1984). "Neurosurgical management of low grade astrocytomas of the cerebral hemisphers." J Neurosurg 61: 665-673.

Lee, MC., Lee, WS., Huhang, SW. (1994). The biological role of ganglioside in neuronal differentioation--effects of GM1 ganglioside on human neuroblastoma SH-SY5Y cells. J. of Korean Med. Sci. 9: 170-187.

Lee, W., J. Klock and B. Macher (1981). "Neutral glycospingolipids in hairy cell leukemia." Biochem 20: 6505-6508.

Letourneeur, F. G., E.C. Hennecke, S. Demolliere, C. Duden, R. Emr, S.D. Riezman, H. Cosson, P. (1994). "Coatomer is essential for retrieval of dilysine-tagged proteins to the endoplasmic reticulum." Cell 79: 1199-1207.

Levine, P., o. Bobbit, R. Waller and A. Kuhmichel (1951). "Isoimmunization by a new blood factor in tumor cells." Proc Soc Exp Biol Med 77: 403-405.

Lewis, M. J. and H. R. Pelham (1990). "A human homologue of the yeast HDEL receptor." Nature 348: 162-163.

Lewis, M. J. and H. R. Pelham (1992). "Ligand-induced redistribution of a human KEDEL receptor from the Golgi complex to the endoplasmic reticulum." Cell 68: 353-364.

Lewis, M. J. and H. R. Pelham (1996). "SNARE-mediated retrograde traffic from the Golgi complex to the endoplasmic reticulum." Cell 85: 205-215.

Li, S., S. Sonnino, G. Tettamanti and Y. Li (1988). Accumulation of globotriaosylceramide in a case of leiomyosarcoma. J. Biol. Chem. 263: 6588-6591.

Lieberman, E., E. Heuser, G. Donnel, B. Landing and G. Hammond (1966). "Hemolytic Uremic Syndrome. Clinical and pathological considerations." N. Eng J Med 275: 227-236.

Lindberg, A., Brown, J., Strömberg, N. Westling-Ryd, M. Schultz, J. and K-A Karlsson. (1987). Identification of the carbohydrate receptor for Shiga toxin produced by Shigella dysenteriae type 1. J. Biol. Chem. 262:1779-1785.

Lindgren, S. W., J. E. Samuel, C. K. Schmitt and A. D. O'Brien (1994). "The specific activities of Shiga-like toxin type II (SLT-II) and SLT-II-related toxins of enterohemorrhagic Escherichia coli differ when measured by vero cell cytotoxicity but not by mouse lethality." Infect Immun 62(2): 623-631.

Ling, V. and L. H. Thompson (1973). "Reduced permeability in Chinese hamster ovary cells as a mechanism of resistance to cholchicine." J Cell Physiol 83: 103-116.

Linggood, M. A. and J. M. Thompson (1987). "Verotoxin production among porcine strains of Escherchia coli and its association with edema disease." J Med Microbiol 24: 359-362.

Lingwood, C. A. (1979). "Action of galactose oxidase on galactolipids." Can J Biochem 57: 1138-43.

Lingwood, C. A. (1994). "Verotoxin-binding in human renal sections." Nephron 66(1): 21-28.

Lingwood, C. A. (1996). "Aglycone Modulation of Glycolipid Receptor Function." Glycoconj J 13: 495-503.

Lingwood, C. A. and S. C. K. Yiu (1992). "Glycolipid modification of α-interferon binding: Sequence similarity between α-interferon receptor and the verotoxin (Shiga-like toxin) B-subunit." Biochem. J. 283(1): 25-26.

Lingwood, C. A., H. Law, S. Richardson, M. Petric, J. L. Brunton, S. DeGrandis and M. Karmali (1987). "Glycolipid binding of purified and recombinant Escherichia coli-produced verotoxin in vitro." J Biol Chem 262(18): 8834-8839.

Liotta, L. A., P. S. STeeg and W. G. Stevenson (1991). "Cancer metastasis and angiogenesis: an imbalance of positive and negative regulation." Cell 64: 327-336.

Lippincott-Schwartz, J. (1993). Membrane cycling between the ER and Golgi apparatus and its role in biosynthetic transport. Sub-Cellular Biochem. 21: 95-119.

Lippinocott-Schwartz, J., Yuan, L.C. Bonifacino, J.S. and Klausner, R.D. (1989). "Rapid redistribution of Gplgi proteins into the ER in cells traeted with brefeldin A: evidence for membrane cycling from Gogi to ER". Cell. 56: 801-813.

Liscovitch, M. and L. Cantley (1994). "Lipid second messengers." Cell 77: 329-334.

Louise, C. B. and T. G. Obrig (1991). "Shiga toxin-associated hemolytic-uremic syndrome: combined cytotoxic effects of Shiga toxin, interleukin-1ß, and tumor necrosis factor alpha on human vascular endothelial cells in vitro." Infect Immun 59(11): 4173-4179.

Louise, C. B. and T. G. Obrig (1992). "Shiga toxin-associated hemolytic uremic Syndrome: combined cytotoxic effects of Shiga toxin and lipopolysaccharide (endotoxin) on human vascular endothelial cells in vitro." Inf. Imm. 60(4): 1536-1543.

Lowe, S. W., E. M. Schmitt, S. W. Smith, B. A. Osborne and T. Jacks (1993). "P53 is required for radiation-induced apoptosis in mouse thymocytes." Nature 362: 847-849.

Mahaley, M., M. Urso, R. Whaley and e. al. (1985). "Immunobiology of primary intracranial tumors. Part 10: therapeutic efficacy of interferon in the treatment of recurrent gliomas." J. Neurosurg. 63: 719-725.

Makita, A. (1964). "Biochemistry of organ glycolipids." J Biochem 55(3): 269-276.

Maloney, M. and C. Lingwood (1993). "Interaction of verotoxins with glycosphingolipids." TIGG 5(21): 23-31.

Maloney, M. D. and C. A. Lingwood (1994). "CD19 has a potential CD77 (globotriaosyl ceramide)-binding site with sequence similarity to verotoxin B-subunits: Implications of molecular mimicry for B cell adhesion and enterohemorrhagic Escherichia coli pathogenesis." J Exp Med 180: 191-201.

Mandon, EC., Ehses, I., Rother, J., van Echten, G., Sandhoff, K. (1992). Subcellular localization and membrane topology of serine palmitoyltransferase, 3-Dehydrosphingosine reductase, and sphinganine N-Acytltransferase in Mouse liver. I. Biol. Chem. 267: 11144-11148.

Mangeney, M., C. A. Lingwood, B. Caillou, S. Taga, T. Tursz and J. Wiels (1993). "Apoptosis induced in Burkitt's lymphoma cells via Gb3/CD77, a glycolipid antigen." Cancer Res 53: 5314-5319.

Mangeney, M., Y. Richard, D. Coulaud, T. Tursz and J. Wiels (1991). "CD77: an antigen of germinal center B cells entering apoptosis." Eur. J. Immunol. 21(5): 1131-1140.

Mannori, G., O. Cecconi, G. Mugnai and S. Ruggieri (1990). "Role of glycolipids in the metastatic process: Characteristics neutral glycolipids in clones with different metastatic potentials isolated from a murine fibrosarcoma cell line." Int J Cancer 45: 984-988.

Marques, L. R. M., J. S. M. Peiris, S. J. Cryz and A. D. O'Brien (1987). "Escherichia coli strains isolated from pigs with edema disease produce a variant of Shiga-like toxin II." FEMS Microbiol. Lett. 44: 33-38.

Martinez, O., A. Schmidt, J. Salamero, B. Hoflack, M. Roa and B. Goud ((1994)). "The small GTP-binding protein rab6 functions in intra-Golgi transport." J Cell Biol 127: 1575-1588.

McCormack, B., D. Miller, G. Budzilovich, G. Voorhees and J. Ransohoff (1992). "Treatment and survival of low-grade astrocytomas in adults." Neurosurg 31: 636-642.

Mehl, E. and H. Jatzkewitz (1964). " Eine Cerebrosidsulfatase aus Schweineniere." Hoppe-Seyler's Z. Physiol. Chem. 339: 260-276.

Meredith, J. E., B. Fazeli and M. A. Schwartz (1993). "The extracellular matrix as a cell survival factor." Mol Biol Cell 4: 953-961.

Merlo, G. R., F. Basolo, L. Fiore, L. Duboc and N. Hynes (1995). "P53-dependent and P53-independent activation of apoptosis in mammary epitehlial cells reveals survival function of EGF and insulin." J Cell Biol 128: 1185-1196.

Michael, A., C. Barry, A.,, K. Behnke and N. Eastman (1990). "Activation of programmed cell death (apoptosis) by cisplatin, other anticancer drugs, toxins and hyperthermia." Biochem Pharmacology. 40: 2353-2362.

Middlebrook, J. L. and R. B. Dorland (1984). "Bacterial toxins: Cellular mechanisms of action." Microbiol Review 48(3): 199-221.

Miguel, A., M. D. Valverde, F. V. Sepulveda, D. R. Gill, S. C. Hyde and C. F. Higgins (1992). "Volume-regulated chloride channels associated with human multi-drug-resistance P-glycoprotein." Nature 355: 380-383.

Mikkelsen, T., Cairncross, J., Cavenee, W., (1991). Genetics of the malignant progression of astrocytomas. J. Cell Biochem. 46: 3-8.

Miller, M., M. Park and J. Hanover (1991). "Nuclear pore complex: structure functionand regulation." Physiol. Rev. 71: 909-949.

Misumi, Y., Miki, A., Takatsuki, A. Tamura, J. Ikeharak, Y. (1986). Novel blocked by brefeldin A of intracelluar transport of secretory proteins in culture rat hepatocytes. J. Biol. Chem. 261: 11398-11403.

Morimoto, S., B. Martin, Y. Kishimoto and J. O'Brien (1988). Biochem. Biophys. Res. Commun. 156: 403-410.

Morimoto, S., B. Martin, Y. Yamamoto, K. Kretz, J. O'Brien and Y. Kishimoto (1989). Proc. Natl. Acad. Sci. USA 86: 3389-3393.

Morison, I. M., D. M. Becroft, T. Taniguchi, C. G. Woods and A. S. Reeve (1996). " Somatic overgrowth associated with overexpression of insulin-like growth factor II." Nature Med. 2: 311-316.

Morton, D. L., M. H. Ravindranath and R. F. Irie (1994). "Tumor gangliosides as target for active specific immunotherapy of melanoma in man ."Prog Brain Res 101: 251-275.

Muller, W., D. Afra and R. Schroder (1977). "Supratentorial recurrences of gliomas: morphological studies with relation to time intervals in gliomas." Acta Neurochir 37: 75-91.

Myers, D., X. Jun, K. Waddick, C. Forsyth, L. Chelstrom, R. Gunther, N. Tumer, J. Bolen and F. Uckun (1995). "Membrane-associated CD19-LYN complex is an endogenous p53-independent and Bcl-2-independent regulator of apoptosis in human B-lineage lymphoma cells." Proc Natl Acad Sci 92: 9575-9579.

Myher, J. J., A. Kuksis and S. Pind (1989). "Molecular species of glycerolipids and sphingomyelins of human erythrocytes: improved method of analysis." Lipids 24(5): 396-407.

Nagahama, M., L. Orci, M. Ravazzola, M. Amherdt, L. Lacomis, P. Tempst, J. E. Rothman and T. H. Sollner (1996). "A v-SNARE implicated in intra-Golgi transport." J Cell Biol 133: 507-516.

Naiki, M. and D. M. Marcus (1974). "Human erythrocyte P and P^k blood group antigens: Indentification as glycosphingolipids." Biochem Biophys Res Comm 60(3): 1105-1111.

Nakakuma, H., M. Ari, T. Kawaguchi, K. Horikawa, M. Hidaka, K. Sakamoto, M. Iwamori, Y. Nagai and K. Takatsuki (1989). "Monoclonal antibody to galactosylceramide: discrimination of structural difference in the ceramide moiety." FEBS Lett 258(2): 230-232.

Neill, M. A., J. Agosti and H. Rosen (1985). "Hemorrhagic colitis with Escherichia coli 0157:H7 preceding adult hemolytic uremic syndrome." Arch Intern Med 145: 2215-2217.

Neurogenetics and the molecular biology of human brain tumors. Thapar, K.; Fukuyama, K.; Rutka, J. 69-97.

Newland, J. W., N. A. Strockbine and R. J. Neill (1987). "Cloning of genes for production of Escherichia coli Shiga-like toxin type II." Infect Immun 55(11): 2675-2680.

Nicoletti, I., G. Migliorati, M. Pagliacci, F. Grignani and C. Riccardi (1991). "A rapid and simple method for measuring thymocyte apoptosis by pripidium iodide staining and flow cytometry." J Immunol Meth 139: 271-279.

Norbury, C. J. and P. Nurse (1989). "Control of higher eukaryote cell cycle by p34 homologues." Biochim. Biophys. Acta 989: 85-95.

Nudelman E. Singhal A. Paronetto F. (1992).Expression of sialylated Lewis(x) antigen in chronic and neoplastic liver. Arch. Pathol.& Lab. Medi. 116:643-8.

Nyholm, P.-G. and I. Pascher (1993). "Steric presentation and recognition of the saccharide chains of glycolipids at the cell surface: Favoured conformations of the saccharide-lipid linkage calculated using molecular mechanics(MM3)." Int J Biol Macromol 15: 43-51.

Nyholm, P.-G., G. Magnusson and C. Lingwood (1996). "Two distinct binding sites for globotriaosyl ceramide on verotoxins: molecular modelling and confirmation by analogue studies and a new glycolipid receptor for all verotoxins." Chem Biol 3: 263-275.

Nyholm, P.-G., J. L. Brunton and C. A. Lingwood (1995). "Modelling of the interaction of verotoxin-1 (VT1) with its glycolipid receptor, globotriaosylceramide (Gb$_3$)." Int J. Biol. Macromol. 17: 199-205.

Nyholm, P.-G. and Pascher, I. (1993). " Steric presentation and recognition of the saccharide chains of glycolipids at the cell surface: favoured conformations of the saccharide-lipid linkage calculated using molecular mechanism (MM3). Int. J. Biol. Macromol. 15: 43-51.

O'Brien, A. D. and G. D. LaVeck (1982). "Immunochemical and cytotoxic activities of Shigella dysenteriae 1 (Shiga) and Shiga-like toxins." Infec Immun 35(3): 1151-1154.

O'Brien, A. D. and G. D. LaVeck (1983). "Purification and characterization of a Shigella-dysenteriae 1-like toxin produced by Escherichia coli." Infect. Immun. 40: 675-683.

O'Brien, A. D. and R. K. Holmes (1987). "Shiga and Shiga-like toxins." Microbiol. Rev. 51(2): 206-220.

O'Brien, A. D., T. A. Lively, M. E. Chen, S. W. Rothman and S. B. Formal (1983). "Escherichia coli 0157:H7 strains associated with hemorrhagic colitis in the

United States produce a Shigella dysenteriae 1 (Shiga)-like cytotoxin." Lancet i: 702.

Obeid, L. M., C. M. Linardic, L. A. Karolak and Y. A. Hannun (1994). Programmed cell death indyced by ceramide. Science 259: 1769-1771.

Obrig, T. G., C. B. Louise, C. A. Lingwood and T. O. Daniel (1994). Shiga toxin-endothelial cell interactions. Recent Advances in Verocytotoxin-Producing Eshcerichia Coli Infections. M. A. Karmali and A. G. Goglio, Elsevier Science B.V.: 317-324.

Obrig, T. G., P. J. D. Vecchio, J. E. Brown, T. P. Moran, B. M. Rowland, T. K. Judge and S. W. Rothman (1988). "Direct Cytotoxic Action of Shiga Toxin on Human Vascular Endothelial Cells." Infect. Immun. 56: 2373-2378.

Obrig, T. G., T. P. Moran and J. E. Brown (1987). "The mode of action of Shiga toxin on peptide elongation of eukaryotic protein synthesis." Biochem J 244: 287-294.

Obrig, T., C. Louise, C. Lingwood, B. Boyd, L. Barley-Maloney and T. Daniel (1993). "Endothelial heterogeneity in Shiga toxin receptors and responses." J Biol Chem 268(21): 15484-15488.

Oda, K. Hirso, N. Takami, Y., Misumi, A., Ikehara, Y. (1987). Brefeldin A arrests the intracellular transport of a precursor of complement C3 before its conversion site in rat hepatocytes. FEBS Lett. 214: 135-138.

Ohyama, C., Y. Fukushi, M. Satoh, S. Saitoh, S. Orikasa, E. Nudelman, M. Straud and S.-I. Hakomori (1990). "Changes in glycolipid expression in human testicular tumors." Int J Cancer 45: 1040-1044.

Olsnes, S., B. van Deurs and K. Sandvig (1993). "Protein toxins acting on intracellular targets: cellular uptake and translocation to the cytosol." Med Microbiol Immunol 182: 51-61.

Oltvai, Z. N. and S. J. Korsmeyer (1994). "Checkpoints of dueling dimers foid death wishes." Cell 79: 189-192.

Orci, L., Tagaya, M., Klausner, D., Rothman, J. (1991). Brefeldin A, adrug that blocks secretion, prevents the assembly of non-clathrin-coated buds on Golgi cisternae. Cell. 64: 1183-1195.

Orr, P., Dong, Y., Schroeder, M. and Ogbrrn, M. (1995). P1 blood group antigen expression and epidemic hemolytic uremic syndrom. Pediatr. Nephrol. 9:612-613.

Ozols, R. and R. Young (1994). "Chemotherapy of ovarian cancer." Semin Oncol 11: 251-263.

Palade, G. Intracellular aspects of the processing of protein synthesis. (1975). Science, 189: 347-358.

Pascher, I. and S. Sundell (1977). "Molecular arrangements in sphingolipids. The crystal structure of cerebroside." Chem Phys Lipids 20: 175-191.

Pelham, H. R. B., L. M. Roberts and J. M. Lord (1992). "Toxin entry: How reversible is the secretory pathway?" Trends Cell Biol 2: 183-185.

Pellizzari, A., H. Pang and C. A. Lingwood (1992). "Binding of verocytotoxin 1 to its receptor is influenced by differences in receptor fatty acid content." Biochem 31: 1363-1370.

Perez, R. P. and T. C. Hamilton (1993). Chemotherapy resistance in ovarian cancer. Ovarian Cancer. R. Ozols and R. e. Young: 39-62.

Peter, M. and Herskowitz, I. (1994). "Joining the complex: cyclin-dependent kinase inhibitory proteins and the cell cycle." Cell. 79:181-184.

Petric, M., M. A. Karmali, S. Richardson and R. Cheung (1987). "Purification and biological properties of Escherichia coli verocytotoxin." FEMS Microbiol Letts 41: 63-68.

Pfeffer, S. R. (1994). "Rab GTPases: master regulators of membrane trafficking." Curr Opin Cell Biol 6: 522-526.

Pihlgren, M., Thomas, J., and Marvel, J. (1996). Comparison of two cell death detection methods: In situ nick translation and TUNEL. Biochemica, 3:12-14.

Pines, J. (1995). " Cyclins, CDKs and Cancer." Cancer Biol. 6:63-72.

Plaxe, S. C., L. Deligdisch, P. R. Dottino and C. J. Cohen (1990). "Ovarian intraepithelial neoplasia demonstrated in patients with stage I ovarian carcinoma." Gyneocol Oncol 38: 367-372.

Ponten, J. and E. MacIntyre (1986). "Long-term culture of normal and neoplastic human glia." Pathol Microbiol Scand 81: 791-805.

Pudymaitis, A. and C. A. Lingwood (1992). "Susceptibility to verotoxin as a function of the cell cycle." J Cell Physiol 150: 632-639.

Pulczynski, S., A. M. Boesen and O. M. Jensen (1993). "Antibody-induced modulation and intracellular transport of CD10 and CD19 antigens in human B-cell lines: An immunofluorescence and immunoelectron microscopy study." Blood 81(6): 1549-1557.

Raff, M. (1993). "Social control on cell survival and cell death." Nature (Lond) 356: 397-400.

Ramotar, K., B. Boyd, G. Tyrrell, J. Gariepy, C. A. Lingwood and J. Brunton (1990). "Characterization of Shiga-like toxin 1 B subunit purified from overproducing clones of the SLT-1 B cistron." Biochem. J. 272: 805-811.

Ramu, A., D. Glaubiger, I. T. Magrath and A. Joshi (1983). "Plasma membrane lipid structural order in doxorubicin-sensitive and - resistant P388 cells." Cancer Res 43: 5533-5537.

RasheedBK. and Bringer, S.H. (1991). Genetic alteration in glioma and medulloblastoma. Cancer & Metastasis Rev. 10: 289-299.

Reed, J. (1995). "Regulation of apoptosis by bcl-2 family proteins and its role in cancer and chemoresistance." Current Opinion in Oncol. 7(6): 541-546.

Richardson, S. E., M. A. Karmali, L. E. Becker and C. R. Smith (1988). "The Histopathology of the Hemolytic Uremic Syndrome Associated With Verocytotoxin-Producing Escherichia coli Infections." Hum Pathol 19(9): 1102-1108.

Richardson, S. E., T. A. Rotman, V. Jay, C. R. Smith, L. E. Becker, M. Petric, N. F. Olivieri and M. A. Karmali (1992). "Experimental verocytotoxemia in rabbits." Infect. Immun. 60(10): 4154-4167.

Riley, L. W. (1985). "Hemorrhagic colitis - A "new" disease." Clin. Microbiol. Newsletter 7: 47-49.

Riley, L. W., R. S. Remis, S. D. Helgerson, H. B. McGee, J. G. Wells, B. R. Davis, R. J. Hebert, E. S. Olcott, L. M. Johnson, N. T. Hargrett, P. A. Blake and M. C. Cohen (1983). "Hemorrhagic colitis associated with a rare Escherichia coli serotype." N Engl J Med 308: 681-685.

Riley, R., Kirsh, J., Gallinger, S., Thiessen, J., Damani, M., Hay, K., Polihronis, J., et al., (1992). "Compartmental analysis of radioiodinated monoclonal antibody B72.3 in colon cancer patients." Nucl. Med. Biol. 20: 57-64.

Rivero, J. and S. Adunyah (1996). "Soudium butyrate induces tyrosine phosphorylatio and activatio of MAP kinase (ERK-1) in human K562 cells." Biochem. Biophys. Res. Commun. 224: 796-801.

Robbins, S., N. Quintrell and J. M. Bishop (1995). "Myristoylation and differential palmitoylation of the HCK protein-tyrosine kinases govern their attachmment to membranes and association with calveolae." Mol Cell Biol 15: 3507-3515.

Robinson, L. A., C. Lingwood, R. M. Hurley and D. G. Matsell (1994). The binding and biological effects of E. coli verotoxin (VT-1) on human paediatric glomerular mesangial cells (MC). Recent Advances in Verocytotoxin-Producing Eshcerichia Coli Infections. M. A. Karmali and A. G. Goglio, Elsevier Science B.V.: 361-364.

Rothman, J. E. (1994). "Mechanisms of intracellular protein transport." Nature 372: 55-63.

Rowe, P., E. Orrbine, G. Wells and P. McLaine (1991). "Epidemiology of the hemolytic-uremic syndrome in Canadian children from 1986-1988." J Pediat 119: 218-224.

Ruetz, S. and P. Gros (1994). "Functional expression of P-glycoprotein on secretory vesicles." J Biol Chem 269: 12277-12284.

Ruetz, S. and P. Gros (1994). "Phosphatidylcholine translocase: a physiological role for the mdr2 gene." Cell 77: 1071-1081.

Ruoslahti, E. and J. C. Reed (1994). "Anchorage dependence, integrins and apoptosis." Cell 77: 477-478.

Rutka, J. T., J. R. Giblin, D. Y. Dougherty, H. C. Liu, J. R. McCulloch, C. W. Bell, R. S. Stern, C. B. Wilson and M. L. Rosenblum (1987). "Establishment and characterization of five cell lines derived from human malignant gliomas." Acta Neuropathol (Berl) 75: 92-103.

Rutka, J.T, Apodaca, J., Stern, R., Rosenblum, M. (1988). The extracellular matrix of the central and peripheral nervous system. Structure and function. J. Neuro. Sureg. 69: 155-170.

Rutka, J.T, Dougherty, D.V, Giblin JR., et al, (1987). Growth of a medulloblastoma on normal leptomeningeal cells in culture: Interaction of tumor cells and normal cells. Neurosurgery. 21: 872-878.

Sakai, T., S. Yamashina and S. Ohnishi (1991). "Microtubule-disrupting drugs blocked delivary of endocytosed transferrin to the cytocenter, but did not effect return of transferrin to plasma membrane,." J. Biochem. 109: 528-533.

Sandvig, K., K. Prydz, M. Ryd and B. van Deurs (1991). "Endocytosis and intracellular transport of the glycolipid-binding ligand Shiga toxin in polarized MDCK cells." J Cell Biol 113(3): 553-562.

Sandvig, K., M. Ryd, O. Garred, E. Schweda and P. K. Holm (1994). "Retrograde transport from the Golgi complex to the ER of both Shiga toxin and the nontoxic Shiga B-fragment is regulated by butyric acid and cAMP." J Cell Biol 126(1): 53-64.

Sandvig, K., O. Garred, K. Prydz, J. Kozlov, S. Hansen and B. van Deurs (1992). "Retrograde transport of endocytosed Shiga toxin to the endoplasmic reticulum." Nature 358(6): 510-512.

Sandvig, K., O. Garred, P. K. Holm and B. van Deurs (1993). "Endocytosis and intracellular transport of protein toxins." Biochem Soc Transact 21: 707-711.

Sandvig, K., S. Olnes, J. Brown, O. Peterson and B. van Deurs (1989). "Endocytosis from coated pits of Shiga toxin: A glycolipid-binding protein from Shigella dysenteriae 1." J Cell Biol 108: 1331-1343.

Sanhoff, K and Klein, A. (1994). Intracellular trafficking of glycosphingolipids: role of sphingolipid activator proteins in the topology of endocytosis and lysosomal digestion. FEBS Lett. 346 : 103-107.

Saxena, S. K., A. D. O'Brien and E. J. Ackerman (1989). "Shiga toxin, Shiga-like toxin II variant, and ricin are all single-site RNA N-glycosidases of 28 S RNA when microinjected into Xenopus oocytes." J. Biol. Chem. 264(1): 596-601.

Schmidt, S. L. and J. E. Rothman (1985). "Two classes of binding sites for uncoating protein in clathrin triskelions." J Biol Chem 26: 10050-56.

Schroder, S., F. Schimmoler, B. Singer-Kruger and H. Riezman (1995). "The Golgi-localization of yeast Emp47p depends on its di-lysine motif but is not affected by the ret1-1 mutation in alpha-COP." J Cell Biol 131: 895-912.

Schwartzman, R. A. and J. A. Cidlowski (1993). "Apoptosis: the biochemistry and molecular biology of programmed cell death." Endocrine Rev 14(2): 133-151.

Schwarz, A., E. Rapaport, K. Hirchberg and A. H. Futerman (1995). J Biol Chem 270: 10990-10998.

Schwarzmann, G. and K. Sandhoff (1990). "Metabolism and intracellular transport of glycosphingolipids." Biochem 29(49): 10865- 10871.

Schweizer, A., Fransen, A., Matter, K., Kreis, T., Ginsel, L., and Hauri, H. (1990). Identification of an intermediate compartment involved in protein transport from endoplasmic reticulum to Golgi apparatus. Eur. J. Cell Biol. 53: 185-196.

Schweizer, A., Clausen, H. van Meer, G. Hauri, HP. (1994). Localization of O-glycan initiation, sphingomylin synthesis, and glucosylceramide synthesis in Vero cells with respect to the endoplasmic reticuluGolgi intermediate compartment. J. Biol. Chem. 269: 4035-41.

Scotland, S. M., H. R. Smith and B. Rowe (1985). "Two distinct toxins active on Vero cells from Escherichia coli O157:H7." Lancet ii: 885-886.

Semenza, J. c. H., K.G. Dean, N. Pelham, H.R (1990). "ERD2, a yeast gene required for the receptor-mediated retrieval of luminal ER proteins from the secretory pathway." Cell 61: 1349-1357.

Sexl, V., L. Wagner, M. Wiesholzer, E. Presterl and W. Base (1994). "Treatment of a patient with malignant mesothelioma with interferon-α_2 based on in vitro sensitivity tests." Clin Invest 72: 317-320.

Sidransky, D., Mikkelsen, T., Schwechheimer, K., et, al. (1991). Clonal expression of the P53 mutatant cells is associated with brain tumors Progression. Nature: 355: 846-847.

Silver, P. (1991). "How proteins enter the nuleus." Cell 64: 489-497.

Simon, S., D. Roy and M. Schindler (1994). "Intracellular pH and the control of multi-drug resistance." Proc Natl Acad Sci USA 91: 1128-1132.

Simons, K. and van Meer, G. (1988). Lipid sorting in epithelial cells. Biochem, 27: 6197-6202.

Sixma, T. K., P. E. Stein, W. G. J. Hol and R. J. Read (1993). "Comparison of the B-pentamers of heat-labile enterotoxin and verotoxin-1: Two structures with remarkable similarity and dissimilarity." Biochem 32: 191-198.

Smart, E.J., Ying, Y., Mineo, C., and Anderson, R.G. (1995). A detergent-free method for purifying caveolae membrane from tissue culture cells. Proc. Natl. Acad. Sci. USA, 92: 10104-10108.

Song, W., R. Welti, S. Hafner-Strauss and D. Rintoul (1993). "Synthesis and characterization of N-parinaroooyl analogs of ganglioside GM3 and de-N-acetyl GM3. Interactions with the EGF receptor kinase." Biochem. 32(33): 8602-7.

Sonnino, S., G. Kirschner, R. Ghidoni, D. Aquotti and G. Tettamanti (1985). "Preparation of GM1 ganglioside molecular species having homogeneous fatty acid and long chain base moieties." J Lip Res 26: 248-257.

Stein, P. E., A. Boodhoo, G. J. Tyrrell, J. L. Brunton and R. J. Read (1992). "Crystal structure of the cell-binding B oligomer of verotoxin-1 from E. coli." Nature 355: 748-750.

Stenback, F. (1981). "Benign, borderline and malignant serous cystadenomas of ovary." Pathol Res Pract 172: 58-72.

Stewart, R. J. and J. Boggs (1990). "Dependence of the surface expression of the glycolipid cerebroside sulfate on its lipid environment: comparison of sphingomyelin and phosphatidylcholine." Biochem 29: 3644-3653.

Stewart, R. J. and J. M. Boggs (1993). "Exposure of galactosylceramide to galactose oxidase in liposomes: Dependence on lipid environment and ceramide composition." Biochem 32: 5605-5614.

Stirling, J. W. and P. S. Graff (1995). "Antigen unmasking for immunoelectron microscopy: labeling is improved by treating with sodium ethoxide or sodium metaperiodate, then heating on retriveal medium." J Histochem Cytochem 43(2): 115-123.

Strasberg, P., H. Yeger and I. Warren (1992). "Increased globotriaosylceramide in familial dysautonomia." Lipids 27(12): 978-983.

Strasser, A., A. W. Harris, T. Jacks and S. Cory (1994). "DNA damage can induce apoptosis in proliferating cells via P^{53}-independent mechanisms inhibited by bcl-2." Cell 79(2): 189-192.

Strockbine, N. A., L. R. M. Marques, J. W. Newland, H. W. Smith, R. K. Holmes and A. D. O'Brien (1986). "Two Toxin-Converting Phages from Escherichia coli 0157:H7 Strain 933 Encode Antigenically Distinct Toxins with Similar Biologic Activities." Infect. Immun. 53(1): 135-140.

Strockbine, N. A., M. P. Jackson, L. M. Sung, R. K. Holmes and A. D. O'Brien (1988). "Cloning and sequencing of the genes for Shiga toxin from Shigella dysenteriae type 1." J Bacteriol 170(3): 1116-1122.

Strous, GJ., van Kerkhof, P. van Meer, G. Rijnboutt, S. (1993). Differential effects of brefeldine A on transport of secretort and lysosomal proteins. J. Biol. Chem. 268: 2341-7.

Stults, C. L. M., C. H. Sweeley and B. A. Macher (1989). "Glycosphingolipids; structure biological source and properties." Methods Enzymol. 179: 167-214.

Subramaniam, V. N., F. Peter, R. Philp, S. H. Wong and W. Hong (1996). "GS28, a 28-kilodalton Golgi SNARE that participates in ER-Golgi transport." Science 272: 1161-1163.

Sugita, M., M. Williams, J. Dulaney and H. Moser (1972). "Ceramidase and Ceramide Synthesis in Human Kidney and Cerebellum—Description of a New Alkaline Ceramidase." Biochem Biophys Acta: 125-131.

Suzuki, K. (1994). Trends Glycosci. Glycotech. 6: 215-227.

Sweely, CC., and Siddiqui, B. (1977). In the glycoconjugate. Horowitz, M.and Pigman, W. New Youk: Acadamic Press. 1: 459-540.

Symington, F. W., I. D. Bernstein and S.-I. Hakomori (1984). "Monoclonal antibody specific for lactosylceramide." J Biol Chem 259(9): 6008-6012.

Tamiya-Koizumi, K., K. Koizumi, H. Ishihara and K. Kojima (1985). "Lipid composition of the plasma membrane isolated from hyperplastic nodules of rat liver." J Biochem 97: 773-779.

Tartakoff, AM., and Vassalli, P. (1983). Lectin-binding sites as markers of Golgi subcompatment: ptoximal-to-distal maturation of oligosaccharides. J. Cell Biol. 97: 1243-1248.

Taylor, C. M. (1995). "Verocytotoxin-producing Escherichia coli and the Haemolytic uraemic syndrome." J Infect 30: 189-192.

Taylor, C. M., D. V. Milford, P. E. Rose, T. C. F. Roy and B. Rowe (1990). "The expression of blood group P1 in post-enteropathic haemolytic uraemic syndrome." Pediatr. Nephrol. 4: 59-61.

Tesh, V. L., B. Ramegowda and J. E. Samuel (1994). "Purified Shiga-like toxins induce expression of proinflammatory cytokines from murine peritoneal macrophages." Infect Immun 62(11): 5085-5094.

Trinchera, M. and Ghidoni, R. (1990). Subcellular biosynthesis and transport of gangliosides from exogenous lactosylceramide in rat liver. Biochem. J. 266: 363-369.

Tylor, I. (1990). "A rapid single step staining technique for DNA analysis by flow microfluorimetry." J Histochem 28: 1021-1024.

Tyrrell, D., P. James, N. Rao, C. Foxall, S. Abbas, F. Dasgupta, M. Nashed, A. Hasegawa, M. Kiso and D. Asa, et al. (1991). "Structural requirements for the carbohydrate ligand of E-selectin." Proc. Natl. Acad. Sci. USA 88: 10372-10376.

Tyrrell, G. J., K. Ramotar, B. Toye, B. Boyd, C. A. Lingwood and J. L. Brunton (1992). "Alteration of the carbohydrate binding specificity of verotoxins fro Galα1-4Gal to GalNAcß1-3Galα1-4Gal and vice versa by site-directed mutagenesis of the binding subunit." Proc. Natl. Acad. Sci. USA. 89: 524-528.

van der Bijl, P. Lopes-Cardozo, M. van Meer, G. (1996). Sorting of newly synthesized glactosphingolipids to the two surface doamin of epithelial cells. J. Cell Biol. 132: 813-21.

van der Kar, N. C. A. J., L. A. H. Monnens, M. Karmali and V. W. M. van Hinsbergh (1992). "Tumor necrosis factor and interleukin-1 induce expression of the verotoxin receptor globotriaosyl ceramide on human endothelial cells:Implications for the pathogenesis of the Hemolytic Uremic Syndrome." Blood 80: 2755-2764.

van der Kar, N. C. J., T. Kooistra, M. Vermeer, W. Lesslauer, L. A. H. Monnens and V. W. M. van Hinsbergh (1995). "Tumor necrosis factor α induces endothelial galactosyl transferase activity and verocytotoxin receptors. Role of specific tumor necrosis factor receptors and protein kinase C." Blood 85(3): 734-743.

van Genderen, I. and van Meer, G. (1995). "Differential targeting of glucosylceramide and galactosylceramide analogues after synthesis but not during transcytosis in MDCK cells." J. Cell Biol. 131: 645-654.

van Meer, G. (1993). "Transport and sorting of membrane lipids." Curr. Opinion in Cell Biol. 5(4): 661-673.

Vaux, D. (1993). "Towards an understanding of the molecular mechanisms of physiological cell death." Proc Natl Acad Sci 90: 786-789.

Vaux, D. L., G. Haecker and A. Strasser (1994). "An evolutionary perspective on apoptosis." Cell 76: 777-779.

Velazquez, L., M. Fellous, G. R. Stark and S. Pellegrini (1992). "A protein tyrosine kinase in the interferon α/β signaling pathway." Cell 70: 313-322.

Waddell, T., A. Cohen and C. A. Lingwood (1990). "Induction of verotoxin sensitivity in receptor deficient cell lines using the receptor glycolipid globotriosyl ceramide." Proc. Natl. Acad. Sci. USA. 87: 7898-7901.

Waddell, T., S. Head, M. Petric, A. Cohen and C. A. Lingwood (1988). "Globotriosyl ceramide is specifically recognized by the E. coli verocytotoxin 2." Biochem. Biophys. Res. Commun. 152(2): 674-679.

Wang, Y., L. Szekely, I. Okan, G. Klein and K. G. Wiman (1993). "Wild type p^{53}-triggered apoptosis is inhibited by bcl-2 in v-myc induced T-cell lymphoma line." Oncogene 8: 3427-3432.

Warburg, O. (1955). "On the origin of cancer cells." Science 123: 309-314.

Warnock, D.E., Lutz, M.S., Blakburn, W.A., Young, JR.W., Baenziger, J.U (1994). "Transport of newly synthesized glucosylceramide to the plasma membrane by non-Golgi pathway." Proc. Natl. Acad. Sci. USA. 91: 2708-2712.

Wedegaertner, P., P. Wilson and H. Bourne (1995). "Lipid modifications of trimeric G proteins." J Biol Chem 270: 503-506.

Weidner, N., J. P. Semple, W. R. Welch and J. Folkman (1991). N Engl J Med 324: 1-8.

Weidner, N., P. R. Carrol, J. Flax, W. Blumenfeld and J. Folkman (1993). Am J Path 143: 401-409.

Wenger, D. and K. Inui (1984). In the Molecular Basis of Lysosomal Storage Disorders. New York, Acadamic Press.

Wenger, D., M. Sattler and S. Roth (1982). Biochim. Biophys. Acta. 311: 354-357.

Wenk, J., P. W. Andrews, J. Casper, J.-I. Hata, M. F. Pera, A. von Keitz, I. Damjanov and B. A. Fenderson (1994). "Glycolipids of germ cell tumors: extended globo-series glycolipids are a hallmark of human embryonal carcinoma cells." Int J Cancer 58: 108-115.

Westermark, B., J. Ponten and R. Hugosson (1973). "Determinants for the establishment of permanent tissue culture lines from human gliomas." Acta. Pathol. Microbiol. Scand. 81: 791-805.

Wiels, J., E. H. Holmes, N. Cochran, T. Tursz and S.-I. Hakomori (1984). "Enzymatic and organizational difference in expression of a Burkitt lymphoma-associated antigen (globotriaosylceramide) in Burkitt lymphoma and lymphoblastoid cell lines." J Biol Chem 259(23): 14783-14787.

Wiels, J., M. Mangeney, C. Tétaud and T. Tursz (1991). "Sequential shifts in the three major glycosphingolipid series are associated with B cell differentiation." Int Immunol 3(12): 1289-1300.

Wyllie, A. H. (1981). Cell death: a new classification separating apoptosis from necrosis. I. D. Bowen and R. A. Lockshin. London, Chapman and Hall: 9-34.

Yang, E., J. Zha, J. Jockel, L. H. Boise, C. B. Thompson and S. J. Korsmeyer (1995). "Bad, a heterodimeric partner for Bcl-x$_L$ and Bcl-2, displaces Bax and promotes cell death." Cell 80: 285-291.

Yates, A., H. Saqr and J. Van Brocklyn (1995). "Ganglioside modulation of the PDGF receptor. A model for ganglioside functions." J. Neuro-Oncol 24(1): 65-73.

Yoshida, TC., Chen, M., Zhang, M., Wu, HC. (1991). Disruption of the Gogi appararus by brfeldin A inhibits the cytotoxicity of ricin, modecine and pseudemonas toxin. Exp. Cell Res. 192:389-396.

Younes, A., D. W. Kahn, J. M. Besterman, R. Bittman, H.-S. Byun and R. N. Kolesnick (1992). "Ceramide is a competitive inhibitor of diacylglycerol kinase in vitro and in intact human leukemia (HL-60) cells." J Biol Chem 267(2): 842-847.

Young, Jr. W.W., Lutz, M.S. and Blackburn, W.A. (1992). Endogenous "glycosphingolipids move to the cell surface at a rate consistent with bulk flow estimates". J. Biol. Chem. 267: 12011-12015.

Zakian, V. A. (1995). "ATM-related genes: what do they tell us about the functions of the human gene?" Cell 82: 685-687.

Zerial, M. and H. Stenmark (1993). "Rab GTPases in vescular transport." Curr Opin Cell Biol 5: 613-620.

Zheng, M., H. Fank, T. Tsuruoka, T. Tsuji, T. Sasaki and S.-I. Hakomori (1993). "Regulatory role of G$_{M3}$ ganglioside in α5β1 integrin receptor for fibronectin-mediated adhesion of FUA169 cells." J Biol Chem 268(3): 2217-2222.

Zwelling, L. Z. (1985). "DNA topoisomerase II as a target of neoplastic drug therapy." Cancer Metastasis Rev 4: 263-276.

IMAGE EVALUATION
TEST TARGET (QA-3)

150mm

6"
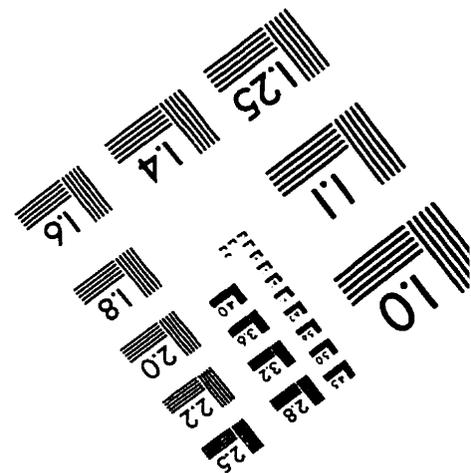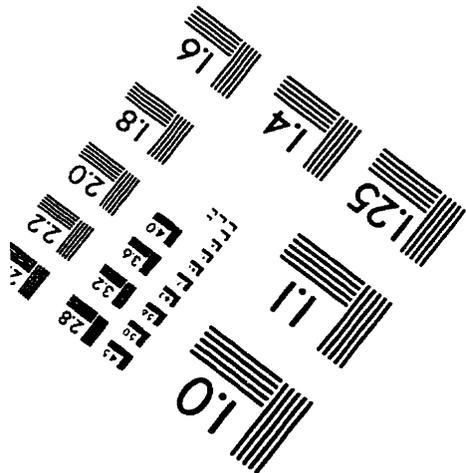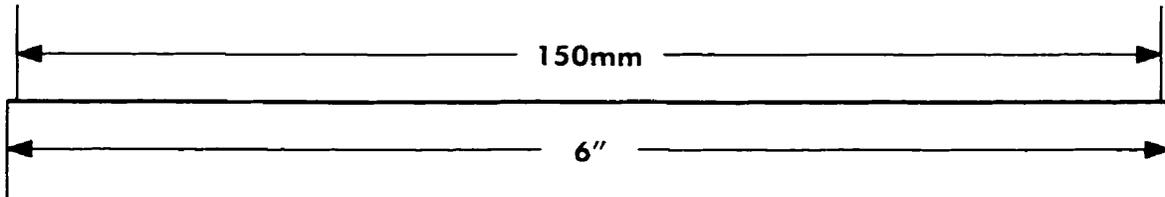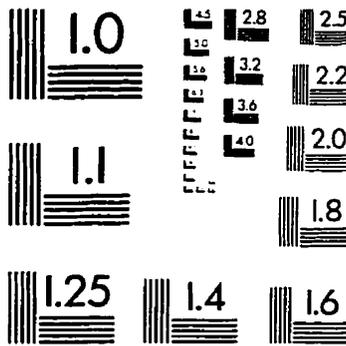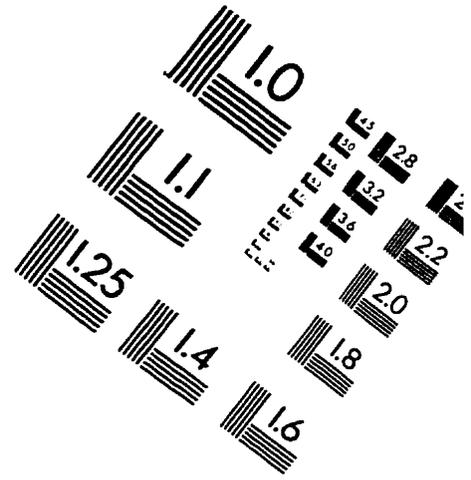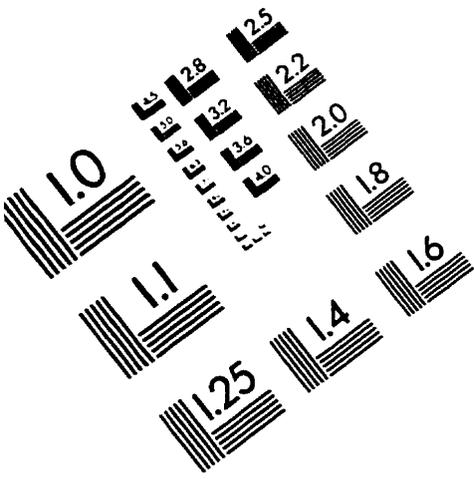
APPLIED IMAGE, Inc
1653 East Main Street
Rochester, NY 14609 USA
Phone: 716/482-0300
Fax: 716/288-5989